TRIAZOLES IN THE MANAGEMENT OF CANDIDA-ASSOCIATED DENTURE STOMATITIS.

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LIST OF PUBLICATIONS AND AWARDS.

Cross, L.J. Bagg, J. & Moseley, H. (1996) Evaluation of an electro-optical device for the measurement of oral mucosal erythema. *Journal of Dental Research*, **75**(2), 1131.

Cross, L.J. Bagg, J. Wray, D. & Aitchison, T. (1996) Clinical and mycological responses to itraconazole and fluconazole in denture stomatitis. *Journal of Dental Research*, **75**(2), 1131.

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' A clinical trial of itraconazole in treatment of denture stomatitis.'

LIST OF ABBREVIATIONS.

AIDS	Acquired Immunodeficiency Syndrome
Anti-DIG-AP	Anti-digoxigenin alkaline phosphatase
CFU/ML	Colony forming units per millilitre
DIG-labelled	Digoxigenin-labelled
DNA	Deoxyribose nucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GES	Guanidine thiocyanate, EDTA, Sarkosyl
HIV	Human Immunodeficiency Virus
kb	Kilobase pairs
K_2HPO_4	Potassium phosphate
LiCl	Lithium chloride
Log_{10}	Logarithm to base 10
µg/L	Micrograms per litre
µl	Microlitre
mg/L	Milligrams per litre
MIC	Minimum Inhibitory Concentration
mmol/L	Millimoles per litre
MOPS	3-[N-Morpholino] Propanesulfonic Acid
NaCl	Sodium chloride
ng/ml	Nanograms per millilitre
n.s.	Not significant
RNA	Ribose nucleic acid
S.D.	Standard deviation
S.D.S.	Sodium dodecyl sulphate
S.E.	Standard error
SSC	Saline sodium citrate
TE	Tris EDTA
TBE	Tris, Boric Acid, EDTA

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Declaration.

I declare that this thesis has been composed by myself and that the work described is my own.

_____

Laura Cross.

SUMMARY.

Denture stomatitis is characterised by erythema and oedema of the palatal mucosa beneath an upper denture. It is common, affecting approximately 50% of denture wearers. The recurrence rate is high and the repeated failure of current methods of treatment of denture stomatitis results in the prescribing of repeated courses of antifungal medication.

The aims of this study were to evaluate the use of itraconazole in the treatment of denture stomatitis and to determine by molecular typing of *C. albicans* species isolated before and after treatment whether there had been a recurrence of infection with the same strain, selection of particular strains or infection with new strains of *C. albicans*. Forty patients with long-standing *Candida*-associated denture stomatitis were enrolled in a single blind clinical trial to compare the new cyclodextrin solution of itraconazole and itraconazole capsules in the management of denture stomatitis. The trial design also encompassed determination of the safety of itraconazole, itraconazole sensitivity of yeasts isolated from the mouths of patients before and after treatment and measurement of itraconazole levels achieved in blood and saliva. Strain differentiation of *C. albicans* isolates was achieved by extracting genomic DNA from selected stored yeast isolates. The DNA was digested with the restriction enzyme EcoR1 then hybridised with the *C. albicans* -specific 27A probe.

A pilot study was conducted to evaluate the use of an electro-optical instrument, the *Erythema meter*, as a means of objectively measuring erythema of the palatal mucosa. A series of non-clinical and clinical investigations were carried out. *Erythema meter* readings were taken from 40 patients with healthy palatal mucosa, 20 denture wearers and 20 non-denture wearers. Readings were also taken from 20 denture stomatitis patients who were then enrolled in a pilot clinical trial comparing fluconazole and itraconazole capsules in the management of denture stomatitis.

As a result of the preliminary investigations the *Erythema meter* was found to be a sensitive and reproducible means of measuring palatal erythema. It was quick and easy to use and was well tolerated by patients. Fluconazole and

itraconazole capsules were found to have similar efficacy in the management of denture stomatitis.

In the main clinical trial patients were randomly assigned to take either the cyclodextrin solution of itraconazole or itraconazole capsules at a dose of 100mg twice daily for 15 days. Of the 40 patients enrolled in the clinical trial, two patients from each treatment group failed to complete the course of medication. Results from the remaining patients indicated that both the cyclodextrin solution of itraconazole and itraconazole capsules showed similar efficacy in the management of denture stomatitis, in terms of reduction in erythema and mycological response. However, a high incidence of gastro-intestinal side-effects was reported by patients in the cyclodextrin group suggesting that, since the response to treatment was similar with both preparations, the itraconazole capsules would be preferred to the cyclodextrin solution of itraconazole for the treatment of denture stomatitis. The multifactorial aetiology of denture stomatitis was highlighted by the results of the trial leading to the conclusions that maintenance of good denture hygiene, the provision of new dentures and the cessation of smoking are all important factors in ensuring a long-term resolution of the condition. Denture stomatitis patients should also be routinely screened for underlying haematinic deficiencies and diabetes mellitus.

The molecular typing of *C. albicans* revealed that the strain of *C. albicans* present six months after treatment commenced is likely to be the same as the strain isolated at baseline in any given patient. This implies that recurrence of denture stomatitis is due to re-colonisation by the original infecting strain, rather than re-infection with a different strain. A wide variety of genotypes of *C. albicans* were isolated in this study, suggesting that no one particular virulent strain of *C. albicans* is associated with denture stomatitis.

CHAPTER ONE

INTRODUCTION AND REVIEW OF THE LITERATURE

1.1. GENERAL INTRODUCTION.

Candida-associated denture stomatitis is one of several clinical types of candidal infection affecting the oral cavity (Table 1.1). In many cases of oral candidosis there is an underlying predisposing factor, which may be local or systemic (Samaranayake, 1990). Management of these infections requires treatment of the underlying factor in addition to that of the infection itself. Many of the antifungal drugs used in the past have been toxic agents which could only be prescribed for topical application. These included nystatin and amphotericin B (Budtz-Jörgensen, 1990b). In recent years the new triazole antifungals, notably fluconazole and itraconazole have become available (Odds, 1993a; Karyotakis and Anaissie, 1994). These drugs can be prescribed for systemic use and compliance is far better. They have been used widely and successfully for managing oral candidosis in immunocompromised patients, for example those with HIV infection (Just-Nübling *et al.*, 1991; Blatchford, 1990) or bone marrow transplant recipients (Goodman *et al.*, 1992). However, their role in the management of oral candidosis in immunocompetent hosts has not been fully researched.

In this thesis, the role of fluconazole and itraconazole in the overall management of patients suffering from *Candida*-associated denture stomatitis has been examined.

Table 1.1.

Classification of oral candidosis.

Pseudomembranous

Erythematous

Hyperplastic

Mucocutaneous

Candida-associated lesions:

Denture stomatitis

Angular cheilitis

Median rhomboid glossitis

(after Samaranayake,1990; Scully *et al.*, 1995)

1.2. THE BIOLOGY OF CANDIDA SPECIES.

1.2.1. Taxonomy.

Fungi are eukaryotes and have a membrane surrounding their nucleus. Their cells are much larger than bacteria and their molecular processes closely resemble those of plants and animals. However, unlike mammalian cells, fungi possess a rigid cell wall surrounding their plasma membrane. Fungi that exist predominantly in the form of independent single cells are usually called yeasts. Although most fungi are capable of sexual reproduction, some species are not known to mate sexually and are called *deuteromycetes* or *hyphomycetes* (previously *fungi imperfecti*) (Odds, 1996). The genus *Candida* falls into this class. It comprises more than 150 species (Odds, 1988). *C. albicans*, *C. tropicalis* and *C. glabrata* are the species most frequently isolated from medical specimens. The other pathogenic *Candida* species are *C. parapsilosis*, *C. stellatoidea*, *C. guillermondii*, *C. krusei* and *C. pseudotropicalis*. *C. stellatoidea* is differentiated from *C. albicans* by its inability to assimilate sucrose. The ability to form pseudohyphae is a distinguishing feature of *Candida* species within the class of Deuteromycetes. *C. glabrata* is the only *Candida* species incapable of this (Shepherd, 1990).

1.2.2. Fungal Structure and Growth.

Typically, fungi grow as either ovoid yeast cells or as thin filamentous hyphal elements. A mass of hyphal filaments is called a mycelium. Yeasts reproduce by

budding. Different morphological forms of *C. albicans,* including budding yeast cells, pseudohyphae, true hyphae and chlamydospores, are exhibited under different environmental conditions. For example, yeast cell growth is favoured at temperatures below 33°C. Bud formation occurs at the polar region of these ovoid cells distal to the birth scar. At higher temperatures and neutral pH mycelial growth is favoured and the conversion of a yeast cell to a hyphal cell occurs via a germ tube. The germ tube develops into a true hyphal cell when the first septum is formed 1-2μm from the mother cell. Pseudohyphae are long branching filaments of elongated yeast cells formed by polar budding and constricted at the site of emergence with a septum. *Candida* produce pseudohyphae in both *in vitro* and *in vivo* conditions (Shepherd, 1990). Round, thick walled chlamydospores develop from hyphae under certain conditions. Chlamydospore formation is a property peculiar to *C. albicans* and to very rare isolates of *C. tropicalis* and *C. stellatoidea* (Silverman *et al.*, 1990). Their function is uncertain.

The various components of the cell wall of *C. albicans* play an important role in the pathogenesis and serodiagnosis of the organism (Odds, 1988). Transmission electron microscopy studies have shown the wall to be composed of at least five distinct layers, made up of mannoproteins, β glucans and chitin. These layers alter during germ tube formation and growth. Mannoprotein forms the outermost layer but is also distributed throughout the wall. This forms the major antigenic component of the wall and two serotypes of *C. albicans* can be distinguished. In addition to the outermost mannoprotein layer, there is a fibrillar layer contained within a fuzzy coat. This layer is believed to be important in the overall virulence of *C. albicans* by affecting adherence and phagocytosis. β-

glucans form the major structural elements of the cell wall. Chitin is located primarily in the bud scars in *C. albicans*, but there is also a layer close to the plasma membrane and chitin is also distributed all over the cell surface. Mycelial cells contain three times more chitin than yeast cells. Receptors on the *C. albicans* cell surface which bind complement fragments consequently enhance the pathogenicity of the organism, because the phagocytic uptake of the yeast by human polymorphonuclear leucocytes is impaired (Shepherd, 1990). Other pathogenic *Candida* species have layered cell wall structures and compositions resembling that of *C. albicans* (Odds, 1988).

The majority of yeasts will grow on simple media containing a source of carbon (glucose) and nitrogen (either ammonium or nitrate). Biotin is an additional growth factor required for *C. albicans*. *Candida* species can grow over the temperature range 20-40°C and within the pH range 2-8 (Shepherd, 1990).

1.2.3. Oral Carriage of *Candida* species.

The oral prevalence of *C. albicans* and other *Candida* species in healthy adults ranges from 3% to 47% (Samaranayake *et al.*, 1986a). However, these figures are dependent on isolation techniques and time of sampling (Cumming *et al.*, 1990; Olsen and Stenderup, 1990). *C. albicans* is the most dominant species, followed by *C. tropicalis*, *C. glabrata*, *C. parapsilosis* and *C. krusei* (Stenderup, 1990). Although *C. albicans* mainly colonises the dorsum of the tongue, the fitting surface of maxillary dentures in the edentulous and the gingival crevice in the

dentate are likely to be the primary reservoirs for oral yeasts (Cumming *et al.*, 1990).

Candida carriage is more common in females than males (Scully *et al.*, 1995). A significant increase in the prevalence of oral yeasts has been found in the warmer summer months (Gergely and Uri, 1966). *Candida* carriage is also subject to diurnal variation, counts being highest in the morning. When dentures, which tend to act as a reservoir for *Candida*, are worn overnight, the early morning *Candida* count is high whereas if dentures are not worn at night, the early morning *Candida* count will be the lowest (Scully *et al.*, 1995).

Several other factors have been reported to influence the establishment of *Candida* species in the oral environment (Arendorf and Walker, 1980; Samaranayake and MacFarlane, 1981b; Oliver and Shillitoe, 1984). For example, acidic saliva or a reduction in salivary flow, nutritional factors such as iron, folate and Vitamin B12 deficiencies, smoking and alterations to the normal oral flora by antibiotic therapy for example, may lead to increases in the carriage of *C. albicans* (Samaranayake, 1990). Non-secretion of blood group antigens in saliva and people of blood group O are more likely to be carriers (Mason *et al.*, 1988). Hospitalisation is also associated with an increase in carrier rates (Cumming *et al.*, 1990).

1.2.4. Culture Methods.

Pathogenic fungi can grow well on many of the media used for the isolation of bacteria from clinical specimens. However, they may grow slowly and without

distinguishing features and so media which specifically promote the growth of fungi have been developed (Mackenzie, 1996). The most commonly used medium for primary isolation of pathogenic *Candida* species is Sabouraud's dextrose agar or malt extract agar. Positive cultures will show cream-coloured, smooth or rough, convex colonies after 48 hours incubation at 37°C under aerobic conditions. *C. albicans* grows very poorly under anaerobic conditions, appearing as characteristic star-shaped colonies. Although coliforms can sometimes grow on Sabouraud's agar, the growth of many commensal oral bacteria is suppressed by the pH of this medium (pH 6). Bacterial growth can be further suppressed by the addition of antibiotics such as chloramphenicol, penicillin, streptomycin and ciprofloxacin (Silverman *et al.*, 1990). Whilst Sabouraud's agar permits the isolation of several different genera, the colonies are very similar in appearance and their subsequent investigation requires considerable investigative time in the laboratory (Beighton *et al.*, 1995).

Several media which allow differential isolation of yeast species through visual differences in colouration have been developed (Nickerson, 1953;1954; Pagano *et al.*, 1958; Costa and Branco, 1964; Odds and Bernaerts, 1994). For example, Nickerson's medium (1953; 1954) relies on the reduction of complex bismuth salts to give light- and dark- coloured colonies. However, it does not adequately differentiate yeast species from each other or from bacteria (Schnell, 1982).

C. albicans isolates give pale-coloured colonies and other yeast species appear with varying degrees of pink, red and mauve colouration on Pagano-Levin medium. This is the result of the reduction of triphenyltetrazolium chloride

incorporated into Sabouraud's agar (Pagano *et al.*, 1958). Pagano-Levin has been found to be as sensitive as Sabouraud's agar in detecting yeast isolates but was superior to the latter in detecting multiple yeast species within a single sample (Yamane and Saitoh, 1985; Samaranayake *et al.*, 1987). In practice it has been found to yield a high rate of false-positive and false-negative results when used to differentiate species (Sinski *et al.*, 1975). Furthermore, high concentrations of tetrazolium chloride may inhibit *C. albicans* and *C. glabrata*, and so it has been suggested that both Pagano-Levin and Sabouraud's agar be used for primary isolation and enumeration of yeasts (Silverman *et al.*, 1990).

A phosphomolybdate agar on which *C. albicans* appears green and other species give white or blue colonies was devised by Costa and Branco (1964). Bump and Kuntz (1968) confirmed the efficacy of this medium.

CHROMagar® *Candida* is a chromogenic medium for presumptive identification of *C. albicans*, *C. krusei* and *C. tropicalis* and the differentiation of these species from other yeasts on the basis of strongly contrasted colony colours produced by reactions of species-specific enzymes with a proprietary chromogenic mix (Odds and Bernaerts, 1994). Studies have indicated that the recovery of different species on Sabouraud's dextrose agar and CHROMagar® *Candida* plates is not significantly different (Odds and Bernaerts, 1994). *C. albicans* is the only species to give a green colour when grown on this medium and *C. tropicalis* gives dark grey colonies with a brown-purple halo formation in the agar (Odds and Bernaerts, 1994; Freydière and Gille, 1994; Beighton *et al.*, 1995). However, *C. glabrata* was found to vary in colour from purple to pale pink which may lead to confusion with *C. parapsilosis* (Beighton *et al.*, 1995). This difficulty with the

correct identification of *C. glabrata* was also noted by Houang *et al.* (1997) who used CHROMagar® *Candida* to identify yeasts from a series of genital specimens. These workers found *C. glabrata* colonies to vary in colour from white or yellow through to different hues of pink or brown, although the most common were glossy pink-purple colonies with a paler edge. In contrast, Pfaller *et al.* (1996) reported satisfactory identification of *C. glabrata* by CHROMagar® *Candida* when they found nine out of ten stock isolates of this species to show dark pink colonies with pale edges. CHROMagar® *Candida* is capable of detecting mixed cultures (Pfaller *et al.*, 1996) and retards the growth of most bacteria (Odds and Bernaert, 1994) but cannot be used satisfactorily if stored at 4°C for more than 14 days (Beighton *et al.*, 1995). Isolates grow well when subcultured from CHROMagar® to Sabouraud's agar, indicating that viability is not lost by growth in the presence of the chromophore contained in this medium (Odds and Bernaert, 1994).

1.2.5. Identification Methods.

The production of hyphal outgrowths (germ tubes) from blastospores when incubated at 37°C in serum for 2-3 hours is a rapid method of identifying *C. albicans* (Odds, 1988). The formation of germ tubes is influenced by a wide range of factors such as temperature, inoculum size, composition of the medium and the strain of *Candida* (Mackenzie, 1962). Although other media have been suggested, serum is the medium of choice for germ tube formation (Silverman *et al.*, 1990). Rarely, germ tube tests can give inaccurate results (Odds, 1988). For example, there have been reports of *C. albicans* isolates not forming germ tubes whilst

some *C. tropicalis* and *C. stellatoidea* isolates do (Silverman *et al.*, 1990). Additional physiological tests for proper speciation should be carried out on any isolates with atypical morphology which form germ tubes in serum (Odds, 1988).

Chlamydospore formation is a property peculiar to *C. albicans* and to very rare isolates of *C. tropicalis* and *C. stellatoidea*. Although a variety of media has been used to induce chlamydospore formation in *C. albicans*, the most popular is corn meal Tween 80 agar. After 1-2 days at room temperature chlamydospore growth could be observed by direct microscopy in regions with low oxygen tension (Silverman *et al.*, 1990).

Final and certain identification of a yeast isolate to species level requires a knowledge of its physiological properties (Odds, 1988). Hence, carbon and nitrogen yeast assimilation tests are used to assess the ability of a particular yeast isolate to assimilate a specific carbohydrate as its sole carbon source. Auxanography is one of the main methods by which assimilation properties may be determined. Sugar solutions, contained in wells cut in a gel or impregnated onto paper discs, diffuse into the surrounding gel which contains the necessary nutrients, with the exception of carbon. If the well or disc contains a sugar which the yeast species can assimilate a circular zone of growth around the wells or disc will be observed. Alternatively, another method involves observation of the growth of *Candida* in tubes containing a liquid basal medium (yeast nitrogen base) to which different carbon sources are added. This method has higher sensitivity and specificity than auxanography and modifications of it are used in rapid commercial systems for the identification of *Candida* species. These methods are also used in tests for assimilation of nitrogen sources, but the basal

media must be free of nitrogen and include glucose as a carbon source. Carbohydrate fermentation is a less sensitive and less dependable test for differentiating *Candida* species than the carbohydrate assimilation test (Silverman *et al.*, 1990).

Commercial systems for yeast identification, offering a high accuracy of identification in comparison to traditional methods, have enhanced the speed and ease of yeast identification in routine clinical laboratories (Odds, 1988). Most of these commercial kits rely mainly on physiological tests but identifications based on physiological evidence alone cannot be assumed to be correct and morphology testing should remain an integral part of any identification process (Odds, 1988).

The API 20C yeast identification system (bioMerieux, Marcy l'Etoile, France) is faster and provides more reliable results than the classic identification methods (Buesching *et al.*, 1979; Land *et al.*, 1979). It is the most popular and widely used of the commercial kits (Odds, 1988). However, as the API 20C system is time-consuming to set up and interpretation of the results may be difficult, the Auxacolour system (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) has been suggested as a quicker, simpler alternative for the routine identification of germ tube negative isolates (Davey *et al.*, 1995). Other systems recommended as alternatives to API 20C include ATB 32C (bioMerieux, Spain) and AMS-YBC (Vitek System, bioMerieux, Spain) (Guttierez *et al.*, 1994).

Candida species have been differentiated according to their heat-sensitive antigenic components or by the use of agglutination tests with species-specific

antisera to distinguish *C. krusei*, *C. guillermondii* and *C. pseudotropicalis* (Silverman *et al.*, 1990).

1.2.6. Typing Methods.

C. albicans strains can be grouped, according to the antigenic characteristics of their cell walls, into one of two serotypes, A and B, by simple slide agglutination. Although commercial versions of this test have been developed, it offers poor discrimination and has been surpassed by other simple and superior typing methods (Silverman *et al.*, 1990).

Biotyping enables *C. albicans* strains to be typed according to their different biochemical characteristics (Silverman *et al.*, 1990). The API ZYM system has been shown to be both a rapid and reproducible method of biotyping. The API 20 C carbon assimilation profile has also been used for the successful biotyping of *C. albicans* strains. However, a highly discriminatory system has been developed using a combination biotyping system comprising the API ZYM, API 20C system and a plate test for boric acid (Silverman *et al.*, 1990).

Other typing methods include resistogram typing, immunoblotting, colony morphotyping and electrophoretic karyotyping (Silverman *et al.*, 1990).

It has been suggested that DNA typing is one of the methods with the greatest potential for discrimination (Merz,1990). DNA fingerprinting was first applied to yeasts by Scherer and Stevens (1987) who examined 17 isolates of *C. albicans*, 10 *C. tropicalis*, 7 *C. parapsilosis*, 1 *C. krusei* and 1 *C.*

pseudotropicalis. Methods of typing *C. albicans* isolated from infected patients are useful in determining whether a new episode of the disease is caused by reinfection or relapse, whether commensally carried strains are the source of infection and whether there are particular strains adapted to particular body niches (Magee *et al.*, 1992). Mathaba *et al.* (1995) determined the genotypic relationships of individual *C. albicans* isolates by hybridisation of a *C. albicans*-specific moderately repetitive sequence, 27A, to *Eco*RI-digested *C. albicans* chromosomal DNA. They concluded that denture stomatitis was due to the overgrowth of commensal strains in the oral cavities of patients and that no one particular virulent strain of *C. albicans* is associated with denture stomatitis. Furthermore, in five of the 18 patients studied, *C. albicans* isolates were obtained from a secondary infection which occurred after antifungal therapy. It was concluded that this was due either to the re-emergence of the original infecting strain or the emergence of a new strain from an exogenous source, or both. The type of antifungal therapy used in this study was not specified.

1.3. DENTURE STOMATITIS.

1.3.1. Historical aspects.

One of the first to describe the condition now recognised as denture stomatitis was McKenzie in 1918. He noted the red and swollen mucous membrane of patients who wore a plate on the roof of the mouth. He also considered that this was a form of infection which was very common among denture wearers where the utmost cleanliness was not observed.

The term 'denture sore mouth' was first used by Cahn in 1936, who was also probably the first to consider that the condition was associated with a yeast infection. Cawson (1963) suggested that 'denture sore mouth' was a misnomer as the degree of soreness complained of by patients was variable and the severity of symptoms was not related to the degree of inflammation. In 1965 Cawson re-iterated that despite the inflammatory changes, soreness is usually absent and because of this suggested 'denture stomatitis' as a better alternative. Lehner (1965) went one stage further and introduced the term chronic atrophic candidiasis. This he described as being a more clinicopathologically precise term, taking its place among the other varieties of oral candidiasis but also, he argued, having the advantage of including candidal angular cheilitis with or without the palatal involvement. However, Holmstrup and Axéll (1990) proposed that the value of using the term 'atrophic' to describe red areas was limited, as redness may be caused not only by reduced epithelial thickness (ie atrophy) but also by increased vascularity (ie. erythema). Nairn (1975) acknowledged the various

synonyms describing inflammation beneath a denture and suggested denture-related candidiasis as an appropriate term. When *Candida* species are involved, the more common terms '*Candida*-associated denture stomatitis' (Budtz-Jörgensen *et al.*, 1988), 'denture-induced candidosis' or 'chronic erythematous candidosis' (Samaranayake and Yaacob, 1990) are used. A further variation was introduced by Walker *et al.* (1981) when they referred to 'denture -induced stomatitis' in their study to evaluate two agents in the treatment of this condition. Other terms of historical interest include 'chronic denture palatitis' (Pryor 1936), 'stomatitis venenata' (Fisher 1956), 'rubber sore mouth' (Lyon and Chick, 1957), 'denture sore mouth syndrome' and 'stomatitis prothetica' or 'stomatopathia prothetica' (Nater *et al.*, 1978).

The concept that denture stomatitis is multifactorial in aetiology was becoming apparent even from the earliest papers (Cahn,1936; Bartels,1937; Lyon and Chick, 1957). Early treatment regimens involved leaving dentures out at night and soaking them in a mild hypochlorite solution (Cahn, 1936). The use of therapeutic agents such as Gentian violet (Cahn,1936) and sodium-caprylate jelly (Lyon and Chick, 1957) has also been recorded.

1.3.2. Classification and Clinical Features.

1.3.2.1. Classification.

Classification of denture stomatitis has generally been based on the clinical appearance of the inflamed mucosa beneath maxillary dentures. The most widely

used classification is that of Newton (1962) who described three forms of the disease:

1) pinpoint hyperaemic foci

2) diffuse hyperaemia of the denture-supporting tissues

3) papillary hyperplasia.

The same changes were described in a slightly different way by Budtz-Jörgensen and Bertram (1970a):

1) simple localised inflammation

2) simple diffuse (generalised) inflammation

3) granular inflammation (papillary hyperplasia).

In a one year follow-up study, Bergendal (1982) concentrated only on the diffuse and papillary varieties of denture stomatitis respectively, referring to these as:

1) atrophic denture stomatitis

2) hyperplastic denture stomatitis.

1.3.2.2. Clinical Features.

Denture stomatitis (Fig.1.1) is characterised by chronic erythema and oedema of part or all of the palatal mucosa that comes into contact with a denture (Budtz-

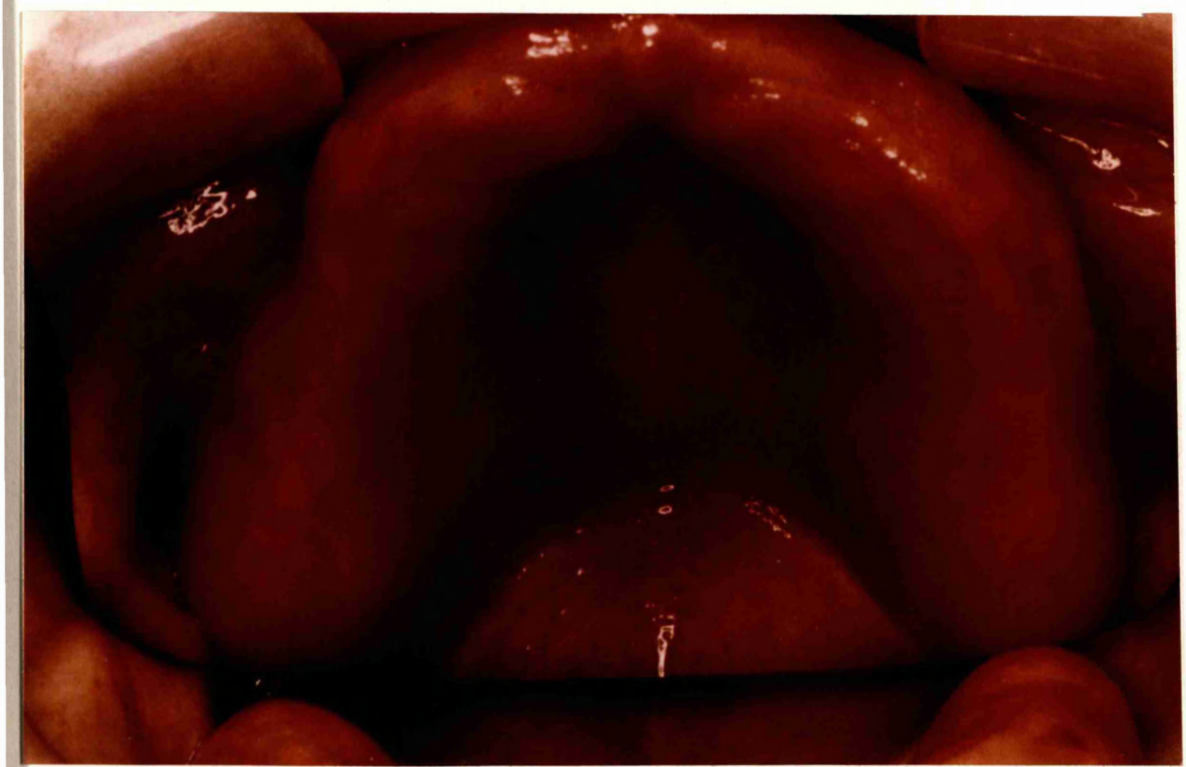


Figure 1.1. Denture stomatitis.

Jörgensen, 1990a). Most authors agree that denture stomatitis affects only the palatal mucosa beneath an upper denture (Budtz- Jörgensen and Bertram, 1970a; Nairn, 1975) although some state that the condition does, on rare occasions, occur beneath the lower denture (Newton, 1962; Ritchie *et al.*, 1969). Neill (1965) observed inflammation of the lower denture-bearing mucosa beneath partial lower dentures only and stated that it was very rare to see the tissues beneath a complete lower denture affected by this condition.

Arendorf and Walker (1987) found that the condition is usually symptomless although severe aching and burning pain has been noted in patients with reduced salivary secretion, for example those with Sjögren's syndrome or advanced cancer (Pollack *et al.*, 1964) . Other oral complaints that have been related to denture stomatitis include mucosal bleeding and swelling, halitosis or an unpleasant taste or dryness in the mouth. It has been estimated that between 28% and 70% of patients with denture stomatitis have oral complaints (Ritchie *et al.*,1969; Budtz-Jörgensen and Bertram, 1970a; Kotilainen, 1972; Budtz-Jörgensen, 1974; Olsen, 1975a; Dorey *et al.*, 1985).

Denture stomatitis has a frequent association with angular cheilitis (Fig.1.2) which is present in 33-82.6% of patients with denture stomatitis (Chick, 1962; Cawson 1965; Mäkilä, 1969; Ritchie *et al.*, 1969; Davenport, 1970; Russotto, 1980; Bergendal and Isacsson, 1983). Although *Candida*-associated denture stomatitis has been proposed as an important aetiological factor of angular cheilitis (Cawson,1963; Nairn, 1975; Rose, 1968), the condition is also found in dentate, non denture-wearing patients (Öhman *et al.*, 1986). It is generally accepted that angular cheilitis has a multifactorial aetiology (Davenport,



Figure 1.2. **Angular cheilitis.**

1970) which includes nutritional deficiencies, such as iron deficiency anaemia (Rose, 1968; Mäkilä, 1969; Burton and Thomson, 1972; Murphy and Bissada, 1979), vitamin B12 deficiency (Mäkilä, 1969) or folic acid deficiency (Rose, 1971). In a study by Öhman *et al.* (1986), the aetiological role of lower serum iron and transferrin could not be confirmed. Shuttleworth and Gibbs (1960) and Cawson (1963) emphasised the aetiological importance of microbial infection, particularly with *C. albicans,* which is frequently present in angular cheilitis lesions. MacFarlane and Helnarska (1976) and Dahlén *et al.* (1982) pointed out the association of organisms other than yeasts, namely *S. aureus* and *β haemolytic streptococci*. The roles of *S. aureus* and *C. albicans* in angular cheilitis are not clear, although Cawson (1963) and Nairn (1975) found that lesions heal rapidly after antifungal therapy. Furthermore, they suggested that topical application of antifungals to the angles of the mouth is unnecessary if denture stomatitis is adequately treated. Prior to this, Lyon and Chick (1957) had already stated that angular cheilitis resolved when the associated denture sore mouth was successfully treated. The association of angular cheilitis with poorly designed or faulty dentures, in particular a loss of the vertical dimension, has been reported by some workers (Ritchie and Fletcher, 1973). However, other studies (Cawson, 1963; Turrell, 1968; Öhman *et al.*, 1986) suggest that neither loss of the vertical dimension nor the condition of the dentures seem to be significant factors in the aetiology of angular cheilitis.

Other pathology occasionally associated with denture stomatitis includes median rhomboid glossitis (Arendorf and Walker, 1984), pseudomembranous candidosis and chronic hyperplastic candidosis (Bergendal and Isacsson, 1983).

1.3.3. Epidemiology.

1.3.3.1. Prevalence.

The prevalence of denture stomatitis depends on the population examined and the diagnostic criteria used (MacEntee, 1985). As a result, the prevalence of denture stomatitis has been reported to vary from 9% to 97% (Budtz- Jörgensen *et al.*, 1975; Manderson and Ettinger, 1975; Smith and Sheiham, 1979; MacEntee, 1985).

In studies using randomised samples, chosen to be representative of the elderly population as a whole (Love *et al.*,1967; Budtz-Jörgensen *et al.*, 1975; Smith and Sheiham,1979; Mikkonen *et al.*, 1984; Ambjörnsen, 1985; Hand and Whitehill, 1986), the prevalence of denture stomatitis ranges from 16.3% to 65%.

Studies in the U.K. by Hoad-Reddick *et al.*(1987) and Tobias (1988) found the prevalence of denture stomatitis to be relatively lower, with values of 7.3% and 9% respectively. In these studies, the samples comprised the institutionalised elderly and those living at home (Hoad-Reddick *et al.*, 1987) or elderly living in sheltered accommodation (Tobias, 1988). In an earlier study, Smith and Sheiham (1979) found denture stomatitis in 20.5% of the elderly population. Numerous studies have been carried out on those living in institutions such as nursing homes and hospitals (Manderson and Ettinger, 1975; Palmquist *et al.*, 1984; Vigild, 1987; Schou *et al.*, 1987; Kaplan and Moskona, 1990; Pietrokovski *et al.*, 1990; Cumming *et al.*, 1990; Jorge *et al.*, 1991; Wilkieson *et al.*, 1991). From these studies it appears that the prevalence of denture stomatitis in the institutionalised elderly ranges from 19% (Pietrokovski *et al.*, 1990) to

54% (Cumming *et al.*, 1990). Lastly, in studies involving dental hospital patients, Nyquist (1952) reported the prevalence of denture stomatitis as 27% and Budtz-Jörgensen (1972) found the condition present in 75% of female and 55% of male dental hospital patients.

1.3.3.2. Age Distribution.

Epidemiological studies into denture stomatitis have produced variable results when relating the prevalence of the condition to age. Kaplan and Moskona (1990) found denture stomatitis to be increasingly common with increasing age. However, Love *et al.*(1967), Mikkonen *et al.* (1984), Palmquist *et al.* (1984) and Vigild (1987) all recorded that denture stomatitis occurs less frequently with increasing age. Furthermore, some studies have reported that the prevalence of denture stomatitis is unrelated to age (Ambjörnsen, 1985; Cumming *et al.*, 1990; Jorge *et al.*, 1991).

1.3.3.3. Sex Distribution.

Most investigators have concluded that denture stomatitis is more common in females (Nyquist, 1952; Budtz-Jörgensen, 1972; Mikkonen *et al.*, 1984; Jorge *et al.*, 1991) although some studies have found no difference in prevalence between males and females (Wilkieson *et al.*, 1991; Ambjörnsen, 1985; Palmquist *et al.*, 1984; Vigild, 1987). No studies have found denture stomatitis to be more prevalent in males. The high proportion of women to men sufferers is most likely

because of the higher incidence of edentulism among women and because of the tendency for women to seek dental treatment more often than men (Dorey *et al.*, 1985).

1.3.3.4. Colonisation Patterns.

Yeasts are most commonly isolated from the midline of the dorsum of the tongue in healthy dentate carriers (Arendorf and Walker, 1980). On the insertion of a denture, however, the density of candidal colonisation in all intraoral sites increases, by comparison with dentate individuals (Arendorf and Walker, 1979). Using various sampling techniques, it has been found that the denture fitting surface and the underlying mucosa are more densely colonised by yeasts in denture stomatitis patients than in denture wearers with healthy palatal mucosa (Budtz-Jörgensen and Bertram, 1970a; Davenport, 1970; Budtz-Jörgensen, 1976; Arendorf and Walker, 1979; Bergendal *et al.*, 1979; Axéll *et al.*, 1985) and higher numbers of yeasts are isolated from the fitting surface of dentures than from the palatal mucosa (Budtz-Jörgensen, 1990). Furthermore, impressions of the maxilla taken in Sabouraud's medium revealed that inflamed sites of the oral mucosa had greater numbers of yeast colonies than other areas (Budtz-Jörgensen and Bertram, 1970a).

1.3.4. Aetiology and Predisposing Factors.

1.3.4.1. Denture Trauma.

It is generally accepted that Newton's (1962) Type I denture stomatitis is caused by trauma alone (Budtz-Jörgensen and Bertram, 1970a). Other forms of denture stomatitis are considered to have a multifactorial aetiology (Budtz-Jörgensen, 1974), of which trauma from ill-fitting dentures is just one aspect. The factors generally considered to be of particular significance in the aetiology of denture stomatitis are summarised in Table 1.2.

Several workers (Nyquist, 1952; Newton, 1962; Bergman *et al.*, 1964; Turrell, 1966; Bastiaan, 1976) have suggested that denture trauma, resulting from problems such as instability, an unbalanced occlusion or ill-fitting dentures, plays an important role in the aetiology of denture stomatitis. Nyquist (1952) examined 1090 complete denture wearers and demonstrated that the incidence of denture stomatitis increased in direct proportion to an increase in the number of traumatic denture factors.

However, Cawson (1963) drew attention to what he felt was a high incidence of denture stomatitis (24% of 1090 denture wearers) in Nyquist's study population and the fact that he had not considered the lower denture-bearing area. Cawson (1963) postulated that denture trauma was not a causative factor in denture stomatitis but a predisposing factor which made the tissues more prone to infection. Incidentally, although the lower denture-bearing area was included in Cawson's 1963 study, no inflammatory changes were noted here.

Nyquist also reported a significant resolution of denture stomatitis following adjustment of the dentures, although other workers (Cawson, 1963; Bergman *et al.*, 1964; Bastiaan, 1976) found that the provision of new dentures with satisfactory stablity and occlusion did not always result in the resolution of denture stomatitis. Bastiaan (1976) carried out a similar study to Nyquist (1952) and found that denture trauma was more prevalent in denture stomatitis patients than in healthy denture wearers.

Table 1.2. **Factors of significance in the aetiology of denture stomatitis:**

- trauma from an ill-fitting denture, including continuous denture wearing
- poor denture hygiene
- candidal infection
- systemic factors
- dietary factors
- allergy to denture base materials

(after Arendorf and Walker, 1987; Lombardi and Budtz-Jörgensen, 1993)

Aware that the exact role of trauma as an aetiological factor in denture stomatitis was still unclear, Bastiaan (1976) considered some of the other factors implicated. As a result he found patients with denture stomatitis to have poorer denture hygiene and a higher incidence of candidal colonisation compared with controls. He also found that a greater number of the denture stomatitis patients wore their dentures continuously. This finding was in contrast to Neill (1965) who, like Nyquist (1952), found no significant difference in the incidence of denture stomatitis amongst those who wore their dentures continuously and those who left them out at night. Several other studies have failed to show any relationship between denture stomatitis and denture wearing habits (Budtz-Jörgensen and Bertram, 1970a; Bergman *et al.*, 1971; Wilkieson *et al.*, 1991).

Bergendal (1982) acknowledges the conflicting evidence as to whether the wearing of dentures at night contributes to the development of denture stomatitis (Budtz-Jörgensen and Bertram, 1970a; Bergman *et al.*, 1971; Arendorf and Walker, 1979; Ettinger, 1975). However, from his one year follow-up study of denture stomatitis, he concludes that the wearing of dentures at night does significantly increase the development of the condition because of the prolonged exposure of the mucosa to the denture plaque.

Most recent authors consider continuous denture wearing to be a factor in the aetiology of denture stomatitis (Arendorf and Walker, 1987). For example, Oksala (1990) found that continuous denture wearing provoked denture stomatitis by enhancing local mucosal trauma, whilst removal of dentures leads to resolution of the infection (Turrell, 1966). Williamson (1972) demonstrated that

patients who wore their dentures at night had much higher early morning *Candida* counts in saliva than those who left their dentures out at night.

Miner (1973) suggested that denture trauma may be the result of micro-roughness on the surface of the denture rather than macroscopic defects. In 1981, Harrison reviewed the use of temporary soft linings as a means of protecting the denture-bearing mucosa from mechanical trauma. More recently, Lombardi and Budtz-Jörgensen (1993) suggested that one aspect of treatment of denture stomatitis should include the smoothing of rough areas on the fitting surface of dentures or the relining of dentures with a soft tissue conditioner.

Budtz-Jörgensen and Bertram (1970a) and Bastiaan (1976) not only assessed the dentures but also the ability of the alveolar ridges to offer good retention and stability for dentures. Both studies found that alveolar mucosa which provides poor support for dentures, for example, a displaceable ridge, was more likely to have an associated denture stomatitis.

There have been several other suggestions as to why the presence of a denture in the mouth is a prerequisite for the development of denture stomatitis (Budtz-Jörgensen, 1990a). For example, Newton (1962) compared denture stomatitis to the 'sweat retention syndrome'. He suggested that occlusion of the palatal minor salivary gland duct orifices by a denture, resulting in lateral spread of saliva into the tissues, may be responsible for the clinical features of denture stomatitis. Ritchie *et al.* (1969) disputed this theory, observing that denture stomatitis did not start at the back, as described by Newton (1962), but seemed to start anteriorly and then spread posteriorly and onto the ridge. Ritchie *et al.*

(1969), like Cawson (1963), also recognised that denture trauma may predispose the tissues to denture stomatitis by reducing their resistance to infection.

This idea was taken further by Riber and Kaaber (1976; 1978) who found that the barrier properties of oral mucosa are affected by denture wearing and that permeability is considerably increased when the mucosa becomes inflamed.

This concept of denture trauma as a predisposing factor to candidal infection was further enhanced when Samaranayake *et al.* (1980) showed that the adhesion of *Candida* to acrylic strips was enhanced by coating the strips with serum and reduced by coating them with mixed saliva. As a result, they suggested that denture trauma may result in an inflammatory exudate which would enhance the adhesion and colonisation of *Candida* to the denture fitting surface.

Other workers (Nater *et al.*, 1978; Bergendal, 1982) have found no difference between the denture trauma present in patients with denture stomatitis and that seen in patients with healthy palatal mucosa. Also, some studies (Vigild, 1987; Jorge *et al.*, 1991) have been unable to demonstrate any significant correlation between trauma from dentures and denture stomatitis.

1.3.4.2. Denture Cleanliness.

Fisher (1956) was one of the earliest to propose that poor denture hygiene might predispose to denture stomatitis. Although Nyquist (1953) reported no such relationship, several workers since then (Love *et al.*, 1967; McKendrick, 1968; Budtz-Jörgensen and Bertram, 1970a, Bergendal,1982) have found a relationship

between lack of denture cleanliness and denture stomatitis. Furthermore, Van Reenen (1973) suggested that bacteria may play a causative role in denture stomatitis and Abelson (1981) suggested that denture plaque was probably of more significance in the aetiology of denture stomatitis than ill-fitting or traumatic dentures.

Tarbet (1982) reported a quantitative approach to assessing the relationship among denture plaque levels, *Candida* species counts and mucosal irritation scores. He found a significant correlation between plaque levels and erythema scores but also found a significant correlation between *Candida* counts and erythema scores. As a result, it was concluded that plaque and yeasts operate as two independent factors which influence mucosal erythema.

Budtz-Jörgensen *et al.* (1983a) analysed one week old denture plaque from semi-quantitative cultures. They found that the rate of plaque formation was ten times higher in stomatitis patients than in controls with clinically healthy palatal mucosae. However, it was not clear whether the difference in microbial counts between patients and controls was the cause or the effect of the disease. The authors considered the possibility that inflammatory exudate of the palatal mucosa may augment the rate of plaque growth on the dentures.

Light and electron microscopic examinations, both in patients with denture stomatitis and in those with healthy mucosa, have shown that denture plaque is composed mainly of bacteria (Theilade and Budtz-Jörgensen, 1980). The plaque is attached to the acrylic surface by a pellicle which appears to be different from the acquired pellicle which forms on enamel (Edgerton and Levine, 1992). In patients with denture stomatitis the pellicle was found to contain serum products,

degradation products and *C. albicans* cell components that were not detected in pellicle from healthy patients.

Theilade and Budtz-Jörgensen (1980) found no substantive difference between the denture plaque of patients with denture stomatitis and those with healthy palatal mucosa.

Budtz-Jörgensen *et al.* (1981) found a predominance of cocci and rods when they studied the microbiology of denture plaque in a group of denture wearers with healthy palatal mucosae, using pieces of sand-blasted self-adhesive tape stuck to the fitting surface of maxillary dentures. Radford and Radford (1993) also found a predominance of coccoid- and rod-shaped bacteria, yet only a small number of yeast cells, when using a scanning electron microscope to examine denture plaque from denture stomatitis patients. This finding compares well with microbial culture studies (Theilade and Budtz-Jörgensen, 1988; Harding *et al.*, 1991) which found yeasts to comprise only a very small proportion of the total cultivable flora in patients with denture stomatitis. However, an earlier scanning electron microscope study (Catalan *et al.*, 1987) and a transmission electron microscope based study, looking at denture stomatitis patients only (Frank and Steuer, 1985), both reported regularly finding yeast cells in denture stomatitis patients. Frank and Steuer (1985) did note a difference between thick plaque, which was composed predominantly of bacteria, and thin plaque deposits, which had a predominance of yeasts.

Some studies (Frank and Steuer, 1985; Walter and Frank, 1985) have shown that the intercellular spaces of the palatal epithelium harbour bacteria and

yeasts and that renewal of denture plaque occurs by proliferation of organisms from this site (Walter *et al.*, 1986).

Frank and Steuer (1985) often observed micro-organisms between the yeast layer and the pellicle and suggested that bacteria may be important in the attachment of yeasts to various surfaces in the mouth. Furthermore, using an agglutination assay, Bagg and Silverwood (1986) found that some common oral bacteria will bind to *C. albicans*, hence possibly acting as a bridge in the attachment of *Candida* to the oral mucosa.

Although bacteria are generally far more numerous in denture plaque than yeasts (Theilade and Budtz-Jörgensen, 1980; Budtz-Jörgensen *et al.*, 1983a; Theilade and Budtz-Jörgensen, 1988) the role of bacteria in the aetiology of denture stomatitis has received little attention.

Microbiological studies indicate that it is the quantity rather than the composition of denture plaque that determines the development of denture stomatitis (Budtz-Jörgensen and Theilade, 1983; Budtz-Jörgensen *et al.*, 1983a; Theilade *et al.*, 1983; Theilade and Budtz-Jörgensen, 1988). Epidemiological studies (Palmquist,1984; Vigild, 1987; Schou *et al.*, 1987) have shown that denture stomatitis patients have significantly greater quantities of denture plaque than those with healthy palatal mucosa. It has also been noted that when plaque accumulation is prevented, mucosal health improves (Lombardi and Budtz-Jörgensen, 1993).

However, qualitative studies by other workers (Koopmans *et al.*, 1988) have found differences between the bacterial flora of patients with denture

stomatitis and healthy controls. For example, there were fewer cocci in the bacterial flora of the former group but higher percentages of obligate anaerobic bacteria in both denture and palatal plaque. Invasion of the epithelial and sub-epithelial tissues by bacteria has been demonstrated (Van Reenen, 1973). However, smears from the palate and dentures of denture stomatitis patients showed leucocytes in association with yeasts more frequently than with bacteria (Budtz-Jörgensen *et al.*, 1975), suggesting that yeasts rather than bacteria are responsible for eliciting the inflammatory response.

1.3.4.3. Candidal Infection.

Yeast infection is assumed to be a major aetiological factor in denture stomatitis, mainly because of the fact that denture stomatitis usually resolves following antifungal therapy (Cawson, 1963; Budtz-Jörgensen, 1974, Quinn, 1985).

High salivary yeast counts are much more common in complete denture wearers than in dentate subjects (Parvinen, 1984). Yeasts are demonstrable in 78-100% of patients with denture stomatitis (Olsen, 1974). However, in microbiological studies of patients with denture stomatitis, yeast counts usually constituted less than 1% of the total microbial counts, although there was a tenfold increase of the yeast counts in denture plaque obtained from denture stomatitis patients compared with healthy controls (Budtz-Jörgensen and Theilade, 1983; Budtz-Jörgensen *et al.*, 1983a).

Cahn (1936) recognised the opportunistic nature of yeast organisms. He suggested that alterations to the environment, as a result of wearing a denture,

could be sufficient to change a harmless yeast into a pathogen. Cahn's proposal that *C. albicans* was responsible for denture stomatitis was backed up by Bartels (1937) who confirmed the presence of these organisms by culturing swabs from Cahn's patients.

Subsequent investigators have been able to demonstrate that yeasts can be cultured far more frequently from the mouths of patients with denture stomatitis than from those with healthy palatal mucosa (Lyon and Chick, 1957; Cawson, 1963; Turrell, 1966; Budtz-Jörgensen and Bertram, 1970a; Bergendal *et al.*, 1979; Palmquist *et al.*, 1984; Cumming *et al.*, 1990; Nanetti *et al.*, 1993) and also that yeasts could be more often isolated from dentures than from mucosal surfaces (Wilkieson *et al.*, 1991; Bergendal *et al.*, 1979; Palmquist *et al.*, 1984). The occurrence in smears of candidal hyphae, believed to be an indication of infection (Winner, 1969), has also been shown to be more frequent in populations with denture stomatitis than those without (Arendorf and Walker, 1987).

In a random sample of the elderly Danish population, Budtz-Jörgensen *et al.* (1975) showed that yeasts could be isolated from the mouths of 93% of those with denture stomatitis. However, among denture wearers with healthy palatal mucosa, they found a yeast carriage rate of 86%.

Sampling techniques throughout the published studies on denture stomatitis have been as diverse as the results produced (Cumming *et al.*, 1990). Methods of quantifying yeasts include impression cultures (Bahn *et al.*, 1962), imprint cultures (Davenport, 1970), saliva cultures (Oliver and Shillitoe, 1984) and oral rinse cultures (Samaranayake *et al.*, 1986a).

Recovery rates of yeasts depend on the technique used. For example, swabbing of mucosal surfaces has been reported to produce inferior recovery rates in comparison to culture of salivary samples (Lilienthal, 1950). Arendorf and Walker (1979) compared different techniques for detecting and quantifying yeasts in dentate individuals and in denture wearers, both with healthy palatal mucosa and with denture stomatitis. As a result, they suggested that imprint cultures were more sensitive than other techniques for detecting candidal carriers. Furthermore, they suggested that quantitative imprint cultures may be useful in differentiating between carrier and disease states. Wilkieson *et al.* (1991) have challenged this suggestion.

Samaranayake *et al.*(1986a), in a study comparing the imprint culture with the concentrated oral rinse technique, recommended the latter as the most sensitive method when screening for the oral carriage of yeasts, coliforms or *S. aureus*.

No difference has been found between the yeast flora isolated from patients with denture stomatitis and that of controls (Turrell, 1966; Budtz-Jörgensen *et al.,* 1975; Budtz-Jörgensen *et al.,* 1983a). *C. albicans* is the most frequently isolated species in both health and disease (Cawson, 1963; Budtz-Jörgensen and Bertram, 1970a; Davenport, 1970; Olsen, 1974; Budtz-Jörgensen *et al.,* 1975; Bergendal *et al.,* 1979; Arendorf and Walker, 1979; Budtz-Jörgensen *et al.,*1983a; Tamamoto *et al.,*1986; Shimizu *et al.,*1987; Schou *et al.,* 1987).

Wilkieson *et al.* (1991) agreed with the majority of workers when they found *C. albicans* to be the organism most frequently isolated in their study group as a whole. However, they also found *C. glabrata* to be the predominant species in patients with denture stomatitis.

In contrast to the majority, Cumming *et al.* (1990) found *C. glabrata* to be the commonest species isolated in their study population.

C. albicans appears to be isolated in pure culture more often than in combination with other species (Budtz-Jörgensen and Bertram, 1970a; Olsen, 1974; Wilkieson *et al.*, 1991). However, unless specific culture media are used it is difficult to identify mixed growth (Samaranayake *et al.*, 1987).

Budtz-Jörgensen (1971a) suggested that some candidal species, such as *C. albicans* or *C. tropicalis*, might be more pathogenic than others. The virulence of candidal species is thought to be dependent on a number of factors (Ruechel, 1990). One of the most important of these factors is adherence and *C. albicans* has been shown to adhere more readily than other species to both mucosal and acrylic surfaces (Critchley and Douglas, 1985).

McCourtie and Douglas (1984) compared the adherence to acrylic and buccal epithelial cells of strains of *C. albicans* isolated from active infections and from asymptomatic carriers. Growth in media containing high concentrations of sucrose and galactose increased the adherence of infective strains many times, whilst only small increases in carrier strains were observed.

It has been shown that a number of different strains of *C. albicans* are involved in denture stomatitis (McCreight *et al.*,1984; Watkinson *et al.*,1985).

These studies failed to produce conclusive evidence that a particular strain was involved in the aetiology.

However, Shakir *et al.* (1983) found *C. albicans* serotype A to cause palatal candidosis more frequently in rats than serotype B. This confirmed earlier clinical indications of the greater role played by serotype A in denture stomatitis (Martin and Lamb,1982). There is also experimental evidence from animal and *in vitro* models that certain strains of *C. albicans* are more virulent than others (Holbrook *et al.*,1983; McCourtie and Douglas, 1984).

A number of studies have indicated that the pathogenesis of denture stomatitis is associated with the presence of *C. albicans* in its hyphal form (Cawson, 1965; Budtz-Jörgensen and Bertram, 1970a; Davenport, 1970). Evidence from an experimental model in monkeys appears to support this view (Budtz-Jörgensen, 1971b). Furthermore, using light microscopy, Allison and Douglas (1973) demonstrated microcolonies of *Candida* with hyphae projecting from the fitting surface of temporary soft lining materials, from patients with denture stomatitis.

Conversely, abundant hyphae have often been demonstrated in denture wearers with healthy mucosa (Budtz-Jörgensen *et al.*, 1975; Olsen and Birkeland, 1977; Arendorf and Walker, 1979) and blastospores have occasionally been found to be the only morphological form present in patients with denture stomatitis (Olsen and Birkeland, 1977; Arendorf and Walker, 1979). Shepherd (1985) produced evidence that either yeast or mycelial forms of *Candida* are

capable of producing disease. Rebora *et al.* (1973) found mycelial forms to be rare in experimental infection of human skin.

Holbrook *et al.* (1983), in experimental oral infection of mice, showed that a virulent strain of *C. albicans* produced few, short pseudohyphae whilst an attenuated strain produced abundant, long pseudohyphae.

It has been suggested that the morphological form of *C. albicans* depends on the local environmental conditions, such as the pH (Budtz-Jörgensen, 1971b;1990a). Denture plaque in patients with denture stomatitis is more acidic than in healthy controls (Olsen and Birkeland, 1977; Samaranayake *et al.*, 1983), particularly following sucrose rinses (Olsen and Birkeland, 1975;1977). No relationship could be demonstrated between the pH of resting denture plaque and the occurrence of hyphae (Olsen and Birkeland, 1975;1977).

Palatal candidosis has been induced by the inoculation of *C. albicans* beneath acrylic plates in monkeys (Budtz-Jörgensen, 1971b;1973;1975; Olsen and Haanaes, 1977) and Wistar rats (Olsen and Bondevik, 1978; Shakir *et al.*,1981;1986a). As gnotobiotic rats were not used, it is not clear whether the denture stomatitis was due to the *Candida* species alone or whether bacteria were involved (Arendorf and Walker, 1987).

An erythematous response, with a histological appearance similar to that of denture stomatitis in man, was produced in monkeys (Budtz-Jörgensen, 1971b). However, this resolved spontaneously after two to three weeks and could only be sustained by repeated inoculation and use of topical tetracycline. In one

animal wearing a plate, but with no candidal inoculum beneath it, localised erythema occurred around the orifices of the palatal minor salivary glands.

The two studies using rat models (Olsen and Bondevik, 1978; Shakir *et al.*, 1981) divided the animals into four experimental groups:

- untreated animals
- animals wearing a plate with no candidal inoculum
- animals wearing a plate with candidal inoculum
- inoculated animals not wearing a plate.

Olsen and Bondevik (1978) reported the most severe inflammation in the plate plus inoculum group. Sporadic inflammation was seen in the other experimental animals but *C. albicans* was isolated from many of the animals prior to the study which complicates the interpretation of the results.

In the study by Shakir *et al.* (1981) no yeasts were isolated from the rats prior to the start of the study. Consequently, they found that inflammation occurred only in the animals wearing a plate with *C. albicans* serotype A inoculated beneath it. This began as localised inflammation and spread to involve the whole of the area covered by the plate. The inflammatory response resolved when the plates were removed but *Candida* organisms persisted in the mouths of the rats (Shakir *et al.*, 1981;1986a). When the plates were subsequently re-inserted without a further inoculum, the inflammation returned (Shakir *et al.*, 1986a). This was interpreted as indicating that the plate caused conversion of the *C. albicans* to its more pathogenic form (hyphae).

The studies by Shakir *et al.* (1981;1986a) demonstrated hyphal invasion of the mucosa. Other studies have not demonstrated this invasion (Budtz-Jörgensen, 1971b; Olsen and Bondevik, 1978).

A few studies have been unable to show a relationship between candidal growth and denture stomatitis (Nater *et al.*, 1978; Bergendal and Isacsson, 1983). Nater *et al.* (1978) used an unusual method of isolating *Candida* species which may have accounted for their findings. An unspecified culture medium was cast in the form of the upper denture and then applied to the palatal mucosa. Bergendal and Isacsson (1983) also used an indirect method.

Monaco and Pickett (1981) denied the involvement of *Candida* species in the hyperplastic form of denture stomatitis, basing their conclusion on the absence of hyphal invasion alone.

Evidence in favour of *C. albicans* as a primary aetiological factor in denture stomatitis is seen in the response of the condition to antifungal agents (Quinn, 1985). However, nystatin and chlorhexidine have been shown to inhibit bacterial populations as well as yeasts (Budtz-Jörgensen and Löe, 1972; Van Reenen, 1973), and penicillin, with a known ability to enhance, directly or indirectly, fungal growth (Seelig, 1966), has been used successfully to treat denture stomatitis (Van Reenen, 1973). As a result, there is difficulty in distinguishing the role of yeasts and bacteria in denture plaque (Arendorf and Walker, 1987).

1.3.4.4. Systemic Factors.

Systemic diseases frequently reported in association with denture stomatitis include diabetes mellitus, immunodeficiency states, anaemia, hypoparathyroidism, nephritis, mercury intoxication, bullous pemphigoid, kidney and urinary tract disease (Budtz- Jörgensen and Bertram, 1970a; Budtz-Jörgensen, 1978; Nater *et al.*, 1978; Bergendal, 1982).

Some studies have shown that candidal carriage is more prevalent among diabetic patients compared to non-diabetic individuals (Tapper-Jones *et al.*, 1981; Lamey *et al.*, 1988). In an earlier study, Peters *et al.* (1966) could find no increase in the prevalence of candidal carriage, nor in the density of growth of *C. albicans*, in diabetic patients compared with controls.

In relation to their ability or inability to secete blood group antigens, Lamey *et al.* (1988) found no significant difference in the prevalence of oral candidal carriage and infection between diabetic patients and non-diabetics.

Knight and Fletcher (1971) showed that saliva from diabetics can promote the growth of *C. albicans* and this finding was correlated with the salivary glucose level.

Odds *et al.* (1978) claimed that candidal carriage rate among diabetics was dependent upon glycaemic control. However, such a relationship has been denied by more recent investigators (Tapper-Jones *et al.*, 1981; Fisher *et al.*, 1987; Lamey *et al.*, 1988; Aly *et al.*, 1991).

Some workers have found that diabetic denture wearers have increased yeast counts when compared to diabetic dentate patients (Tapper-Jones *et al.*, 1981; Fisher *et al.*, 1987). Despite this, Phelan and Levin (1986) demonstrated no significant increase in the prevalence of denture stomatitis in those with either diabetes mellitus or elevated plasma glucose levels.

It is generally accepted that the use of broad spectrum antibiotics and, to a lesser extent, corticosteroids may predispose to the development of oral candidosis (Odds, 1988; Samaranayake, 1990). Knight and Fletcher (1971) produced evidence that antibiotics promote the growth of *C. albicans* by inhibiting bacteria and thus removing the competition for salivary glucose. McKendrick *et al.* (1967) found no relationship between the administration of tetracycline, used in the treatment of chronic bronchitis, and the prevalence or relative numbers of oral *Candida*. Furthermore, McKendrick (1968) could find no significant relationship between tetracycline administration and the occurrence of denture stomatitis. One study (Budtz-Jörgensen, 1975) has produced experimental evidence using an animal model to suggest that corticosteroids may exacerbate denture stomatitis.

The anamnestic data indicating the possible association between the presence of kidney and urinary tract diseases and denture stomatitis (Bergendal, 1982), suggested that repeated and prolonged treatment with antibiotics and sulphonamides may be a predisposing factor.

The carriage rate of *Candida* species has been reported to be elevated in patients taking psychopharmacological drugs (Budtz-Jörgensen, 1990a). Lucas (1993) found that *Candida* species and *S. aureus* were isolated more frequently in

complete maxillary denture wearers undergoing treatment with psychotropic drugs. Palatal inflammation was also present more frequently in these patients than in controls. However, other factors predisposing to denture stomatitis such as a poor standard of denture hygiene, cigarette smoking and high sugar consumption were also more commonly seen in the psychiatric patients.

1.3.4.5. Dietary Factors.

There is evidence that nutritional factors such as deficiencies of iron, folic acid and vitamins may increase the susceptibility of individuals to denture stomatitis (Budtz-Jörgensen, 1990a). In a study of a large group of denture wearers, Ettinger (1973) found the dietary intake of one third of those investigated to be both quantitatively and qualitatively unsatisfactory. Furthermore, Field *et al.* (1995) point out that since oral changes appear to precede many of the systemic indicators of nutritional deficiency, at least in the case of Vitamin B_{12}, early recognition of these oral changes may lead to early diagnosis and the institution of therapy which may prevent more serious sequelae.

Cawson (1963) was the first to describe an apparent relationship between iron deficiency and oral candidosis. Rose (1968) also reported the frequent existence of iron deficiency in denture stomatitis patients. However, Jenkins *et al.* (1977) were unable to find any differences in iron, folic acid and vitamin B_{12} deficiency between patients with denture stomatitis and controls.

Samaranayake and MacFarlane (1981a), in a retrospective study, found cases of iron, folate and vitamin B_{12} deficiency in association with denture

stomatitis. Correction of the deficiencies had little or no effect on the incidence of denture stomatitis.

Joynson *et al.* (1972) and Fletcher *et al.* (1975) demonstrated that iron deficiency appears to exert its effects through impairments in lymphocyte function, although Fletcher *et al.* (1975) found impairments in lymphocyte function in iron deficient patients both with and without oral lesions. Once iron levels were returned to normal, cell-mediated immunity to *C. albicans* was restored (Joynson *et al.*, 1972).

Rennie *et al.* (1984) suggested that iron deficiency may produce an impairment of iron-dependent enzyme systems thereby affecting the metabolism and the kinetics of the rapidly dividing oral epithelial cells. As a result the epithelial surface may be more conducive to the adhesion, growth and invasion of *Candida* (Rennie *et al.*, 1982).

Saliva from iron-deficient patients has been shown to support the growth of *C. albicans* in vitro more readily than did saliva from controls (Fletcher *et al.* 1975).

Jennings and MacDonald (1990) found that denture stomatitis developed in patients with haematological abnormalities when candidal colonisation was less dense than in patients with normal haematological values.

There is evidence to suggest that a high intake of sugars in the diet is associated with oral candidosis (Shuttleworth and Gibbs, 1960; Olsen and Birkeland, 1976; Samaranayake and MacFarlane, 1981a, b). Olsen and Birkeland (1976) found that sucrose rinses could not only aggravate existing denture

stomatitits but also initiate denture stomatitits in denture wearers with previously healthy oral mucosa.

In vitro studies have suggested that glucose promotes the growth of *Candida* species in saliva (Knight and Fletcher, 1971; Samaranayake *et al.*, 1986b). The rapid decline in pH associated with this growth may be involved in the pathogenesis of oral candidosis (Samaranayake *et al.*, 1986b). For example, reduced pH levels may potentiate candidal virulence by enhancing its growth, multiplication and adherence to host tissues whilst activating the acidic proteases and phospholipases of the yeast. Furthermore, the direct cytotoxic effect of the acidic metabolites may exacerbate the host inflammatory response (Samaranayake and MacFarlane, 1985).

Other studies (Samaranayake *et al.*,1980; McCourtie and Douglas, 1984) have shown that sugar substrate appears to enhance the ability of *C. albicans* to adhere to both acrylic and to buccal epithelial cells.

1.3.4.6. Xerostomia.

The protective role of saliva against candidal infection, through its mechanical washing effect and antifungal properties, is widely recognised (Samaranayake,1990).

C. albicans may be found in significantly greater numbers in the mouths of patients with Sjögren's Syndrome compared to healthy individuals (MacFarlane and Mason, 1974). Suppressed salivary flow in monkeys caused aggravation of

inflammation produced by inoculating *C. albicans* beneath acrylic palatal plates (Olsen and Hanaaes, 1977). Knight and Fletcher (1971) demonstrated the inhibition of the growth of *C. albicans* by unsterilised human saliva whilst Samaranayake *et al.* (1980) found that pre-coating strips of acrylic with mixed human saliva reduced the adhesion of *C. albicans*.

However, although xerostomia may increase the severity of the symptoms associated with denture stomatitis, there is no direct evidence that drug-induced xerostomia predisposes to yeast infection in denture wearers (Budtz-Jörgensen, 1990a).

1.3.4.7. Tobacco Smoking.

Arendorf and Walker (1980) claimed that smoking increased the rate of oral candidal carriage. Conversely, however, Oliver and Shillitoe (1984) claimed that smoking had no effect on the oral candidal carriage rate.

Two epidemiological studies have indicated that tobacco smoking is associated with a significantly increased prevalence of denture stomatitis (Hand and Whitehill, 1986; Cumming *et al.*, 1990).

Arendorf *et al.* (1983) found tobacco smoking and denture wearing to be important factors in the aetiology of candidal leukoplakia. Approximately one third of patients with this condition also had denture stomatitis.

1.3.4.8. Allergic Reactions to Denture Base Materials.

Early studies (Rattner, 1936; Bradford, 1948) recorded allergic and primary irritant reactions to denture base materials as possible aetiological factors in denture stomatitis. However, Fisher (1956) showed that what had originally been regarded as a positive patch test to the patients' dentures was actually due to a non-specific traumatic event.

Neill (1965) and Turrell (1966) suggested that the allergic reaction may be attributed to substances absorbed by the denture during wear. A report by Danielewicz (1971) appears to support this view, however, the effects of plaque and its components have not been taken into account (Arendorf and Walker, 1987).

Nater *et al.* (1978) carried out patch tests on the palatal mucosa and skin of the back of patients with denture stomatitis, using denture wearers with healthy palatal mucosa as controls. Although several constituents of denture base materials were used as allergens, no significant differences between the two groups were found in the patch test results.

Occasional reports of a true allergic reaction have occurred (Giunta *et al.*, 1979; Walker, 1982). However, it is now generally accepted that true allergy to denture base materials or its constituents is rare (Arendorf and Walker, 1987).

1.3.5. Pathogenesis and Host Resistance.

1.3.5.1. Histopathology and Tissue Invasion.

Biopsy specimens from the palatal mucosa of denture stomatitis patients have been examined by several investigators (Budtz-Jörgensen, 1970; Wictorin, 1975; Bergendal and Isacsson, 1983; Morimoto *et al.*, 1987). Their findings have been quite consistent and the main features are summarised in Table 1.3.

The histological appearance of the granular type of denture stomatitis is similar to that of the generalised simple form, except for the presence of papillary projections in the former (Budtz-Jörgensen, 1970; Bergendal and Isacsson, 1983; Morimoto *et al.*, 1987). These projections consisted of either markedly hyperplastic epithelium with a narrow core of connective tissue or hyperplastic connective tissue with a covering epithelium of almost normal thickness (Bergendal and Isacsson, 1983). The crypts between the papillae harboured microbial plaque and débris.

Budtz- Jörgensen (1970) could find no qualitative differences in the histopathological appearance between denture stomatitis caused by trauma and that caused by candidal infection. However, pronounced intraepithelial leucocyte infiltration and the occurrence of leucocytes in smears were much more pronounced in the *Candida*-associated lesions. It was also shown that these histological changes could be reversed by appropriate treatment. The persistence of subepithelial inflammation in biopsies taken from the granular form of denture stomatitis could be explained by the difficulty of removing the microbial plaque and débris from the deep epithelial crypts.

Table 1.3.

Main histopathological features of denture stomatitis.

- Absence of keratin layer or parakeratosis.
- Epithelial hyperplasia with deeply penetrating rete pegs, alternating with areas of epithelial atrophy.
- Intracellular and intercellular oedema within the epithelium.
- Leucocyte infiltration.
- Chronic inflammation of the lamina propria, usually extending into the epithelial layers.
- No evidence of hyphae or blastospores invading the epithelium.

(after Budtz-Jörgensen, 1970; Wictorin *et al.*, 1975; Bergendal and Isacsson, 1983; Morimoto *et al.*, 1987).

The inflammatory cell infiltrate in denture stomatitis has been found to be similar to that seen in chronic marginal periodontitis and chronic apical periodontitis. It consists mainly of T lymphocytes, plasma cells and macrophages (Johannessen *et al.*, 1986).

Ostlund (1958) found a moderate increase in the number of mitoses seen in denture stomatitis biopsies compared to normal mucosa. In contrast, Van Mens *et al.* (1975) found a significantly lower mitotic index in the palatal epithelium of denture stomatitis patients compared to denture wearers with healthy palatal mucosa. Although palatal epithelial thickness and the number of mitotic figures was initially reduced when palatal candidosis was experimentally induced in rats (Shakir *et al.*, 1986b), both epithelial thickness and mitotic activity later showed a sharp increase.

In humans, epithelial thickness alternates with areas of extreme atrophy (Ostlund, 1958; Budtz-Jörgensen, 1970; Wictorin *et al.*, 1975; Bergendal and Isacsson, 1983; Morimoto *et al.*, 1987).

Tissue invasion by yeasts in oral candidosis is usually limited to the superficial keratotic or parakeratotic layers (Jepsen and Winther, 1965; Cawson, 1966). Several studies have demonstrated that *Candida* species seldom invade the tissues in denture stomatitis and that relatively few yeast cells are revealed in mucosal smears (Cawson, 1966; Budtz-Jörgensen, 1970; Davenport, 1970; Bergendal and Isacsson, 1983). Budtz-Jörgensen (1990c) has suggested that the keratotic epithelium with deeply penetrating rete pegs and alternating areas of atrophy (Budtz-Jörgensen, 1970; Bergendal and Isacsson, 1983) may be a poor substrate for the keratophilic yeast cells. He also points out that because

pseudohyphae are abundant in smears from the mucosal surface and fitting denture surface, this indicates that yeast growth is limited to host detritus and the microbial plaque between the mucosa and the denture. Furthermore, in experimentally induced denture stomatitis in monkeys and rats, intraepithelial invasion by hyphae was an inconsistent finding even when yeast proliferation was enhanced by treatment with broad-spectrum antibiotics (Budtz-Jörgensen, 1971b; Olsen and Hanaaes, 1977; Shakir *et al.*, 1981).

1.3.5.2. Hypersensitivity.

Eglitis *et al.* (1981) suggested that the inflammatory response seen in Newton's Type III denture stomatitis (Newton,1962) arises from a local disturbance of the host-parasite relationship which enables an endogenous yeast to cause disease. Also concentrating on hyperplastic denture stomatitis, Johannessen *et al.* (1986) used various immunological techniques to demonstrate the presence of IgG, IgA, IgM, complement factors, T-lymphocytes and macrophages in the inflammatory cell infiltrate. They concluded that denture stomatitis is a complex inflammatory lesion with elements of both humoral and cell-mediated immune responses. The immunofluorescent technique used in this study failed to demonstrate *Candida* antigens in the epithelium. However, Johannessen *et al.* (1986) agreed with the suggestion of Budtz-Jörgensen *et al.* (1983a) that it is likely that these antigens are present in the tissues and play an important role in the initiation and maintenance of the disease. The extent to which bacterial or candidal antigens contribute to the inflammation has not yet been ascertained (Budtz-Jörgensen, 1990a).

Evidence that a delayed type hypersensitivity reaction against *C. albicans* contributes to the inflammatory reaction in denture stomatitis has been provided by Budtz-Jörgensen (1971b). In this study, re-inoculation of *C. albicans* beneath an acrylic plate in monkeys produced a recurrence of inflammation, with histological evidence of a delayed hypersensitivity reaction to the yeast. In the same experimental model, the cellular response was studied directly by observing *in vitro* leucocyte migration and was seen to reach its peak at the same time as the inflammation resolved (Budtz-Jörgensen, 1973; 1975). When this immune response was suppressed by immunosuppressive drugs, thrush-like lesions developed and hyphal invasion of the superficial epithelial layers occurred (Budtz-Jörgensen, 1973; 1975). This inflammation persisted until after the immunosuppressant had been discontinued. The cellular immune response was suppressed by the drug and did not occur until the drug was stopped. The humoral response in these animals was early and strong but failed to clear the infection (Budtz-Jörgensen, 1973). A similar picture was observed in monkeys treated with corticosteroids, which also suppressed the cell-mediated immune response (Budtz-Jörgensen (1975).

It is known that cellular immunity is important in combatting systemic fungal infections, whereas humoral immunity may be less important (Folb and Trounce, 1970).

1.3.5.3. Virulence Factors.

There is no single virulence factor of *Candida* species which is predominantly responsible for the establishment of candidosis (Ruechel, 1990).

Dobias (1964) suggested that *C. albicans* may produce an endotoxin. However, other workers (Chattaway *et al.*, 1971; Cutler *et al.*, 1972) have found insufficient levels of endotoxin *in vivo* to produce toxic effects. Odds (1988) stated that it was unlikely that an opportunistic pathogen would produce a potently bioactive toxin and remain opportunistic.

Secretory lipases of *C. albicans* and, to a lesser extent, other *Candida* species have been studied as possible virulence factors (Ruechel, 1990). Pugh and Cawson (1977) have shown histochemically that phospholipases are concentrated at the tips of fungal hyphae and localised to where invasion is occurring in chick embryonic membranes, although their role is still to be determined. Samaranayake *et al.* (1984a) showed that phospholipase production by *C. albicans* was limited to acidic growth conditions and was inversely related to the concentration of glucose or galactose in the media.

Price and Cawson (1977) differentiated the lipolytic activity of *C. albicans* into phospolipase A and lysophospholipase. Although these enzyme activities were found in most *C. albicans* strains, Samaranayake *et al.* (1984b) failed to demonstrate phospholipase activity in organisms known to be less virulent than *C. albicans*, such as *C. glabrata*, *C. tropicalis* and *C. parapsilosis*. Furthermore, Williamson *et al.* (1986) found significant differences between the phospholipase activity of different clinical isolates of *C. albicans*.

Evidence linking lipolytic activity with virulence of fungi has been provided by Barrett-Bee *et al.* (1985) and Ibrahim *et al.* (1995).

Extracellular proteinases which can degrade the salivary proteins have been implicated in the pathogenicity of *C. albicans* (Borg and Ruechel, 1988). The ability of acid proteinases to cleave IgA1, IgA2 and secretory IgA has been demonstrated (Budtz-Jörgensen, 1990c). The low pH necessary for activation of these acidic enzymes is found in denture plaque, particularly after sugar intake (Olsen and Birkeland, 1976). Furthermore, Theilade and Budtz-Jörgensen (1988) found that this cleavage of salivary antibacterial immunoglobulins by the various *Candida* proteinases may enhance the pathogenic potential of the bacterial components of denture plaque.

The variable ability of different *Candida* species to secrete extracellular proteinases largely reflects the virulence of these opportunistic organisms (Ruechel, 1990). For example, virtually all isolates of the more virulent species, *C. albicans* and *C. tropicalis* secrete proteinases (MacDonald, 1984). In contrast, the extracellular proteolytic activity of *C. parapsilosis* is expressed under *in vitro* conditions only (Ruechel *et al.*,1986) and Reinholdt *et al.* (1987) found *C. glabrata* to have only weak proteolytic actvity.

In a study relating the germ tube formation and proteinase activity of *C. albicans* to the virulence of this organism for mice, Okamoto *et al.* (1993) found that clinical isolates from patients with denture stomatitis were lethal for mice, regardless of their proteinase activity. However, the overall results from this study led these workers to conclude that the virulence of *C. albicans* is partly due to proteinase activity.

Evidence against proteinase as a virulence factor has also been reported (King *et al.*, 1980; MacDonald and Odds, 1983; Cutler, 1991).

Candida species adhere to a variety of surfaces, including acrylic (Samaranayake *et al.*, 1980; Samaranayake and MacFarlane, 1980; Minagi *et al.*, 1986). Some investigators have suggested a relationship between the adherence of *C. albicans* to acrylic and epithelial surfaces and its ability to colonise and cause disease (Kennedy *et al.*, 1988; Soll, 1992). Hyphal forms of the organism tend to adhere more strongly than yeast forms (Samaranayake and MacFarlane, 1982; Tronchin *et al.*,1988). Adherence also varies with environmental conditions, for example, it is promoted by sugars (Minagi *et al.*, 1985; Rotrosen *et al.*, 1986). Other factors which contribute to adherence are listed in Table 1.4.

Table 1.4. **Factors affecting adhesion of yeasts.**

• Yeast cell attributes:	medium/cultivation, phenotype, germ tubes/hyphae, extracellular polymeric material, floccular/fibrillar surface layers, mannan, chitin, hydrophobicity, cellular lipids.
• Host cell attributes:	cell source, mucosal size and viability, fibronectin, fibrin, sex hormones, yeast carriers versus patients with overt candidosis.
• Environmental factors:	cations, pH, sugars, saliva, humoral antibody and serum, antibacterial drugs, bacteria, lectins.

(after Olsen, 1990; Scully *et al.*, 1995).

C. albicans frequently exhibits variant colonial forms when grown *in vitro* (Shepherd, 1990). Studies have linked switching *in vitro* with virulence *in vivo* (Soll, 1988). Mechanisms to explain the relationship of colony switching to pathogenicity of *C. albicans* are not yet known (Nelson *et al.*, 1991). However, the pathogenic mechanisms which may be potentiated by switching include enhanced adhesion of different phenotypes to each other and to epithelial cells (Kennedy *et al.*, 1988), avoidance of elimination by neutrophils (Kolotila and Diamond, 1990) or enhanced potential for *C. albicans* cells to adapt rapidly to new microniches (Soll *et al.*, 1991). Furthermore, switching may enhance tissue penetration, secretion of enzymes such as proteinases and phospholipases and the capacity to escape antifungal treatment (Shepherd, 1990).

1.3.5.4. Host Defences.

Scully *et al.* (1995) divided host oral defences against *Candidal* infection into immune and non-immune factors. As non-immune factors they included iron, lysozyme, salivary histidine-rich polypeptides, lactoferrin, lactoperoxidase and salivary glycoproteins.

Immune factors, such as phagocytosis and cell-mediated and humoral immunity, provide a second line of defence against candidal infection (Budtz-Jörgensen, 1990a). *C. albicans* is primarily controlled by phagocytosis (Ashman and Papadimitriou, 1990; 1992). Conditions where candidal phagocytosis and/or killing by polymorphonuclear leucocytes or monocytes is depressed are often associated with serious candidal infections (Odds, 1988). Similarly, infections with

Candida species are consistently seen where there is depressed cell-mediated imune function (Scully *et al.*, 1995).

Although patients with denture stomatitis do not have any serious cell-mediated immune defects, they may be deficient in migration-inhibition factor and may have overactive suppressor T cells or other T lymphocyte or phagocyte defects (Iacopino and Wathen, 1992).

Secretory IgA is the major humoral factor which defends against yeast organisms by aggregation or by prevention of adherence (Challacombe, 1990).

1.3.6. Diagnosis of Denture Stomatitis.

1.3.6.1. Clinical Findings.

The characteristic presenting signs of denture stomatitis are chronic erythema and oedema of the mucosa that contacts the fitting surface of the denture (Budtz-Jörgensen, 1990a). Lesions are often free of symptoms and the presenting complaint is commonly associated with angular cheilitis (Odds, 1988).

There are no reliable clinical criteria which can distinguish between a *Candida*-associated-, bacterial induced-, or trauma-induced- denture stomatitis and an allergic reaction to the denture base material (Budtz-Jörgensen, 1990a; b; c). Therefore, Budtz-Jörgensen (1990a) stated that in order to establish a diagnosis it is necessary to carry out a mycological examination.

1.3.6.2. Microbiological Investigations.

Scully *et al.* (1995) have suggested that the detection of yeasts in a clinical specimen should start with direct microscopic examination of smears of the lesion. Although valuable in many situations, smears have been shown to be less sensitive than culture techniques (Silverman *et al.*, 1990).

A sterile cotton-tipped swab rubbed over the lesional tissue or over all surfaces irrespective of clinical signs, and subsequently cultured in the laboratory, is a useful method to detect the presence or absence of *Candida* spp.. However, this will not provide a quantitative estimate (Silverman *et al.*, 1990). Odds (1982) found that *C. albicans* can remain viable for at least 24 hours on a moist swab.

Discrimination between candidal carriage and frank oral candidosis can be made using the imprint culture technique (Arendorf and Walker, 1979). Sterile plastic foam pads dipped in Sabouraud's broth are placed directly onto the clinical surface and then directly onto a suitable culture medium. According to Arendorf and Walker (1979), colony counts in excess of 30CFU cm^{-2} of mucosa in the dentate and 49CFU cm^{-2} in denture wearers may suggest a candidal infection.

The concentrated oral rinse technique was first described by McKendrick *et al.* (1967) and later modified by Samaranayake *et al.* (1986a). This method is more simple to perform than the imprint culture technique, especially if multiple sites are involved. It also removes the risk of inhalation of the sterile foam pads (Silverman *et al.*, 1990). Samaranayake *et al.* (1986a) found that although the imprint culture technique and the concentrated oral rinse method had comparable sensitivity, the latter was superior in quantifying yeast, coliform and *S. aureus*.

The impression culture technique (Bahn *et al.*, 1962), although useful in quantifying the relative distribution of yeasts on various oral surfaces, is purely a research tool (Silverman *et al.*, 1990).

No single method of detecting oral *Candida* species is sensitive enough (Silverman *et al.* 1990) and the choice of method should depend on whether a semi-quantitative or a quantitative estimate of candidal carriage is required.

1.3.7. Management of Denture Stomatitis.

1.3.7.1. Denture Hygiene Advice.

Denture hygiene is an important factor in the treatment of denture stomatitis (Thomas and Webb, 1995). Several surveys have revealed that a large number of people do not know how to clean their dentures satisfactorily, and this has been attributed to a lack of advice or to failure to follow the advice given (Neill, 1968; Polyzois, 1983; Jagger and Harrison, 1995).

Odds (1988) states that denture stomatitis may be managed by antifungal therapy in combination with strict denture disinfection and advice to patients not to wear their dentures at night. Furthermore, as dentures act as a reservoir of infection, the risk of relapse following antifungal therapy is reduced if patients maintain meticulous oral and denture hygiene and continue to remove their dentures at night (Budtz-Jörgensen, 1990a).

Walker *et al.* (1981) found that both the yeast concentration on the denture-fitting surface and palatal inflammation were as effectively reduced with

mechanical removal of plaque alone, as with a combination of plaque removal, both mechanically and using a proprietary peroxide denture cleaner, and amphotericin B.

The importance of good oral hygiene was further supported when Berge *et al.* (1987) found that professional plaque control resulted in an improvement of the mucosal state, whilst withdrawal lead to recurrence of the denture stomatitis (Lombardi and Budtz-Jörgensen, 1993).

It has been suggested that mechanical methods of denture cleansing, such as scrubbing dentures after meals and massaging the denture-bearing mucosa with a soft toothbrush, should be introduced to denture wearers with and without denture stomatitis (Lombardi and Budtz-Jörgensen, 1993). However, some studies have shown no relationship between the frequency of brushing and the amount of denture plaque (Schou *et al.*, 1987) nor the frequency of denture brushing and the prevalence of denture stomatitis (Nyquist, 1953; Mikkonen *et al.*, 1984).

In addition to the mechanical removal of plaque from the denture fitting surface and mucosa, numerous solutions, pastes and powders are available for the chemical cleansing of dentures (Jagger and Harrison, 1995). The most common of these consist of alkaline hypochlorite or alkaline peroxide (Abelson, 1985). Acids (Lambert and Kolstad, 1986) and enzymes (Budtz-Jörgensen, 1977) have also been used with some success.

In their review of the treatment of denture stomatitis, Lombardi and Budtz-Jörgensen (1993) stated that alkaline peroxides had no significant effect on

either denture plaque accumulation or palatal inflammation, whereas hypochlorites removed plaque efficiently but had the disadvantage of having a harmful effect on certain denture base materials, such as soft lining materials and metals.

Lambert and Kolstad (1986) and Iacopino and Wathen (1992) found that *C. albicans* was eliminated from dentures using a denture soak containing benzoic acid. Granata and Staffanou (1991) found that this compound showed no harmful effects on soft as well as hard denture lining materials, a further advantage.

The use of enzymes which may disrupt the plaque matrix is based on the assumption that denture plaque is similar to dental plaque, consisting of mutan, dextran and salivary glycoprotein (Budtz-Jörgensen and Kelstrup, 1977; Connor *et al.*, 1977; Budtz-Jörgensen *et al.*, 1983b). Enzymes have been shown to be effective at removing established plaque from dentures (Budtz-Jörgensen and Kelstrup, 1977; Connor *et al.*, 1977; Odma, 1992) but also help to reduce the formation of denture plaque (Budtz-Jörgensen *et al.*, 1983b).

Antibacterial substances used in the treatment of denture stomatitis include chlorhexidine and Listerine antiseptic (Lombardi and Budtz-Jörgensen, 1993).

Chlorhexidine has been used as a denture disinfectant and as a topical medicament (Budtz-Jörgensen and Löe, 1972; Olsen,1975a; 1975b; 1975c; Budtz-Jörgensen and Knudsen, 1978; Lal *et al.*, 1992). It has been shown to reduce both the clinical signs of denture stomatitis (Budtz-Jörgensen and Löe, 1972; Olsen,1975b; Lal *et al.*, 1992) and yeast colonisation of the palate and denture surface (Budtz-Jörgensen and Löe, 1972; Olsen,1975a; Lal *et al.*, 1992). However, once chlorhexidine therapy was terminated, the inflammation recurred

and this was followed by increased yeast colonisation (Budtz-Jörgensen and Löe, 1972; Olsen,1975a; b; Lal *et al.*, 1992). Prolonged use of a 0.2% chlorhexidine solution as an overnight denture soaking agent prevented both clinical and mycological relapse (Olsen,1975c).

Brushing dentures with a 1% chlorhexidine gel proved more effective in preventing plaque formation than a commercial alkaline peroxide soaking agent (Steradent). However, the use of chlorhexidine gel was no more effective than brushing with a placebo gel (Budtz-Jörgensen and Knudsen, 1978).

Olsen (1975a; b) found a combination of chlorhexidine and amphotericin B to be effective in the treatment of denture stomatitis, although a pronounced 14 day relapse rate in all study groups was also reported. *In vitro* testing has indicated that combining nystatin and chlorhexidine reduces the anti-candidal effect of both agents, possibly as a result of salt formation (McCourtie *et al.*, 1986; Barkvoll and Attramandal, 1989).

McCourtie *et al.* (1986) suggested that chlorhexidine may exert a beneficial effect by reducing the adhesion of *Candida* species to acrylic resin.

Listerine antiseptic has been found to be effective against microorganisms isolated from the dentures of hospitalised patients (DePaola *et al.*,1984). Studies by the same group of workers also found Listerine to be as effective as nystatin in reducing inflammation and yeast colonisation in denture stomatitis patients (DePaola *et al.*, 1986) and as effective as a commercial soft denture relining product (Coe Comfort) at reducing inflammation (DePaola *et al.*, 1990). Plaque

scores were not reduced in either of these studies, neither were patients assessed after the cessation of treatment to determine the incidence of relapse.

Although there have been few reports concerning the effects of denture treatment solutions on denture base materials (Aldana *et al.*, 1994), most of these have indicated that disinfecting solutions have deleterious effects on acrylic resins (Burns *et al.*, 1976) and soft linings (Davenport *et al.*;1986; Harrison *et al.*, 1989).

Aldana *et al.* (1994) suggested that light-activated resins may be the materials of choice for denture stomatitis patients, as they demonstrated less overall degradation from the candidal treatment modalities investigated in their study.

Staining of the denture base material is a frequent complication when chlorhexidine is used as a denture disinfectant (Olsen, 1975b). Furthermore, little is known about the possible effects of chlorhexidine on the denture-bearing mucosa (Lombardi and Budtz-Jörgensen, 1993).

Jagger and Harrison (1995) recommended alkaline hypochlorite as the most effective denture cleansing agent. They also suggested that it was suitable for use when dentures were lined with temporary soft lining materials. Furthermore, they advised that the problem of corrosion of metal based dentures by alkaline hypochlorite can be overcome by reducing the soaking time from twenty minutes to ten, followed by thorough rinsing and soaking in water.

Alternatively, Stafford *et al.* (1986) found a significant reduction in *Candida* colonisation, without any significant dimensional changes to the dentures, if they were allowed to dry in air for 8 hours overnight.

Sterilization of dentures by microwaves has been recommended (Rohrer and Bulard, 1985). Thomas and Webb (1995) found that dimensional changes in dentures were almost negligible after 6 minutes microwaving at 350 watts. However, they stated that investigations were continuing as regards the effect that this level of radiation has on microorganisms.

1.3.7.2. Prosthodontic Treatment.

The removal of denture trauma, by providing new, well-fitting dentures with good occlusion, has been advocated by some workers as a successful method of treating denture stomatitis (Budtz-Jörgensen and Bertram, 1970a; Davenport, 1970; Bastiaan, 1976; Graham *et al.*, 1991). However, as denture stomatitis has a multifactorial aetiology (Lombardi and Budtz-Jörgensen, 1993), several investigators have found that more successful treatment regimes reflect this (Ritchie *et al.*, 1969; Arikan *et al.*, 1995; Salonen *et al.*, 1996). For example, Ritchie *et al.* (1969) concluded that although denture stomatitis may be resolved by new, well-fitting, stable, non-traumatic dentures, most patients also require treatment with antimycotic drugs. This conclusion was confirmed when Budtz-Jörgensen and Bertram (1970b) found that although prosthodontic treatment alone did result in reduced inflammation in *Candida*-induced denture stomatitis, it did not eliminate hyphal structures. Furthermore, Arikan *et al.* (1995) found a

combination of fluconazole and chlorhexidine to have a greater fungicidal effect and result in greater improvement of palatal inflammation, than either treatment with fluconazole or the provision of new dentures alone. However, the beneficial effects of prosthodontic treatment seen in patients with Newton's Type I (Newton, 1962) denture stomatitis, indicated that the provision of new dentures without antifungal therapy could provide a successful means of treating this form of denture stomatitis (Arikan *et al.*, 1995).

In the case of Newton's Type III denture stomatitis (Newton, 1962), although the renewal of dentures results in healing to some extent (Salonen, 1994), again, the best results are obtained by combining antifungal therapy with the provision of new dentures (Salonen *et al.*, 1996).

The use of tissue conditioning materials (Budtz-Jörgensen and Bertram, 1970b; Olsson and Bergman, 1971; Zakhari and McMurry, 1977) and hard laboratory relines (Olsson and Bergman, 1971) have been shown to reduce the severity of and, in some cases, resolve palatal inflammation among patients with denture stomatitis. Although the internal denture base acrylic which has been penetrated by yeasts is removed prior to placing a soft liner (Iacopino and Wathen, 1992), the lining material itself should be replaced frequently, as these materials do not inhibit candidal growth (Burns *et al.*, 1976). In fact, an increased incidence of denture stomatitis has been reported in association with soft linings (Mäkilä, 1976).

This problem may be reduced by the incorporation of antimycotic agents into soft denture reline materials (Quinn, 1985; Carter *et al.*, 1986). Truhlar *et al.* (1994) acknowledge that more research is required in this area, but advise that the

incorporation of a single dose of antimycotic agent into the reline material of a denture that allows release of the agent over a two week period, provides a promising treatment modality for the management of denture stomatitis.

Budtz-Jörgensen and Kaaber (1986) showed that glazing of the denture fitting surface was effective in reducing plaque formation and palatal erythema in denture stomatitis patients. However, the glazing system tested was not particularly durable.

1.3.7.3. Identification and Correction of Underlying Systemic Factors.

Denture stomatitis rarely occurs without at least one predisposing factor that lowers patient resistance (Iacopino and Wathen, 1992). Estimates of haemaglobin, lymphocyte and white blood cell counts, corrected whole blood folate, vitamin B12 and serum ferritin can all be important (Scully *et al.*, 1995).

It is well known that uncontrolled diabetics are predisposed to oral candidosis (Samaranayake, 1990) and an assessment of the random blood glucose level should be included in investigations. There have been isolated reports of two patients with persistent denture stomatitis (Cawson, 1965) and another with angular cheilitis (Burman *et al.*, 1956) whose lesions resolved after correction of the diabetic state (Samaranayake, 1990).

In the treatment of fungal infection, attention to the underlying cause will avoid the need for prolonged or repeated courses of treatment. However,

intermittent or prolonged topical therapy may be necessary where the underlying cause is unavoidable or incurable (Scully *et al.*, 1995).

1.3.7.4. Antifungal Therapy.

Antifungal therapy may be indicated in cases of *Candida*-associated denture stomatitis (Lombardi and Budtz-Jörgensen, 1993). Until relatively recently, topical nystatin, amphotericin B and miconazole were the mainstay of treatment for oral candidosis (Budtz-Jörgensen, 1990a). Although these drugs have been shown to be effective in producing an initial improvement in denture stomatitis, recurrence rates are high and treatment regimens tend to be prolonged (Cawson, 1963; Nairn, 1975; Bergendal and Isacsson, 1980; Bergendal, 1982; Martin *et al.*, 1986; Lamb and Douglas, 1988). The systemic triazoles, fluconazole and itraconazole, have provided potentially important additional agents for treatment of this infection (Odds, 1993a; Karyotakis and Anaissie, 1994).

Nystatin (Mycostatin) is a specific polyene antifungal drug which has been used in clinical practice since 1950. The polyene mechanism of action involves an effect on the fungal plasma membranes, leading to augmented permeability (Lombardi and Budtz-Jörgensen, 1993). It is effective against candidiasis and, in particular, denture stomatitis (Cawson, 1963). Nystatin is insoluble and toxic when given parenterally and so it is limited to topical use (Scully *et al.*, 1995) as a suspension, ointment or troche (Iacopino and Wathen, 1992). The treatment regimen consists of oral rinses kept in the mouth for five minutes, three or four times a day for two to three weeks (Iacopino and Wathen, 1992; Lombardi and

Budtz-Jörgensen, 1993). Nystatin has a bitter taste which may lead to poor patient compliance and nausea has occasionally been reported (Lombardi and Budtz-Jörgensen, 1993).

Amphotericin B is also a polyene antifungal, discovered a few years after nystatin (Lombardi and Budtz-Jörgensen, 1993). Amphotericin B binds to the membrane sterols of fungal cells, causing impairment of their barrier function and loss of cell constituents (Warnock, 1991). Amphotericin B is not absorbed by the gut, but has been used intravenously for systemic candidosis. However, it does possess adverse side-effects such as nausea and nephrotoxicity (Lombardi and Budtz-Jörgensen, 1993). The use of amphotericin B in oral candidosis is usually limited to topical application (Epstein, 1990; Lal *et al.*, 1992). Amphotericin B has been widely used in the treatment of denture stomatitis (Walker *et al.*, 1981), either in the form of tablets, to be dissolved orally with the denture removed, or as an ointment applied to the fitting surface of the denture for three to four weeks (Lombardi and Budtz-Jörgensen, 1993). Although most of the major fungal pathogens of man are sensitive to amphotericin B (Scully *et al.*, 1995), occasional strains of *C. albicans* which are resistant to amphotericin B are now being reported (Conly *et al.*, 1992). Earlier, Ritchie *et al.* (1969) claimed to have demonstrated resistance of *C. albicans* to both amphotericin B and nystatin. However, Martin *et al.*(1986) tested *C. albicans* isolates from 500 cases of denture stomatitis and found no resistance to nystatin.

A number of studies have investigated the efficacy of nystatin (Cawson, 1963; 1965; Budtz-Jörgensen and Bertram, 1970b; Van Reenen, 1973; Douglas and Walker, 1973; Nairn, 1975; Bergendal and Isacsson,1980; Bergendal, 1982;

Martin *et al.*, 1986; Johnson *et al.*, 1989) and amphotericin B (Ritchie *et al.*, 1969; Nairn, 1975; Olsen, 1975a;b; Budtz-Jörgensen *et al.*, 1983a; Jegnathan *et al.*, 1987) in the treatment of denture stomatitis. Some of these studies demonstrated that patients could be completely cured (Cawson, 1963; Ritchie *et al.*, 1969; Budtz-Jörgensen and Bertram, 1970b; Nairn, 1975) whilst others showed that although clinically the majority of patients had improved, only a small number were completely clinically cured (Cawson, 1963; Budtz-Jörgensen and Bertram, 1970b; Bergendal, 1982; Budtz-Jörgensen *et al.*, 1983a). In a direct comparison, nystatin and amphotericin B appeared to be equally efficacious (Nairn, 1975). High rates of relapse and recurrence following cessation of treatment with these drugs were a common finding (Budtz-Jörgensen and Bertram, 1970b; Nairn, 1975; Olsen, 1975a; Bergendal, 1982; Budtz-Jörgensen *et al.*, 1983a). Possible reasons for this tendency to relapse include re-infection from exogenous sources as well as endogenous sites (Cawson, 1963; 1965; Bergendal and Isacsson, 1980). As mentioned earlier, the denture is an important source of re-infection (Budtz-Jörgensen and Bertram, 1970; Olsen, 1975a).

In most studies, although clinical improvement was usually accompanied by mycological improvement, the density of candidal colonisation quickly returned to pre-treatment levels on withdrawal of the drug (Ritchie *et al.*, 1969; Budtz-Jörgensen and Bertram, 1970b; Olsen, 1975a; Bergendal and Isacsson, 1980; Johnson *et al.*, 1989). In patients followed up one year after treatment of denture stomatitis with either a combination of nystatin and new dentures or new dentures alone, nystatin was found to confer no long-term benefits (Bergendal, 1982).

Antifungal therapy has been shown to improve subjective symptoms (Cawson, 1963; Budtz-Jörgensen and Bertram, 1970b; Olsen, 1975b) and other oral conditions (Cawson 1965; Budtz-Jörgensen and Bertram, 1970; Olsen, 1975b) associated with denture stomatitis. It has also been shown to lead to reversal of the histopathological (Budtz-Jörgensen, 1970) and immunological (Jegnathan *et al.*, 1987) changes seen in the condition. Furthermore, Van Reenen (1973) found that nystatin inhibited the growth of some streptococci isolated from denture stomatitis patients.

Miconazole (Daktarin) is an imidazole-derived antifungal drug (Lombardi and Budtz-Jörgensen, 1993). It is fungistatic through the azoles' property of changing membrane permeability (Scully *et al.*, 1995), but also bacteriostatic for Gram-positive microorganisms, simultaneously blocking steroid and protein synthesis in microbial cell membranes (Lombardi and Budtz-Jörgensen, 1993). In the treatment of denture stomatitis, miconazole is used topically in the form of a 2% gel, placed in contact with the fitting surface of the denture three times a day for 15 days (Lombardi and Budtz-Jörgensen, 1993). Topical miconazole gel, applied in this way, effectively reduced erythema in denture stomatitis patients and also resulted in decreased candidal colonisation of various oral sites (Watson *et al.*, 1982). Furthermore the effectiveness of miconazole against certain bacteria makes it useful for the treatment of angular cheilitis (Watson *et al.*, 1982).

Ketoconazole was the first of the imidazole-derived agents to be developed (Lombardi and Budtz-Jörgensen, 1993) and the first to be shown capable of achieving therapeutic blood levels when given orally (Scully *et al.*, 1995). Systemically administered ketoconazole has been reported to be effective

in the treatment of denture stomatitis in one patient (Johnson and George, 1986). However, serious side-effects, including hepatotoxicity, have restricted its use (Lombardi and Budtz-Jörgensen, 1993; Scully *et al.*, 1995).

Fluconazole is a fluorinated bis-triazole antifungal (Odds, 1996) that acts by inhibiting the cytochrome c-dependent demethylation step in fungal ergosterol production, essential in cell wall formation (Lewis *et al.*, 1991). Fluconazole is well absorbed if taken orally and is widely distributed throughout body fluids, including saliva (Grant and Clissold, 1990). Fluconazole may reduce the adherence of *Candida* species to human buccal epithelial cells (Darwazeh *et al.*, 1991). Fluconazole was found to be efficacious in the treatment of denture stomatitis in an open, non-comparative study (Wragg *et al.*, 1986) and in another placebo-controlled double blind trial (Budtz-Jörgensen *et al.*, 1988). In the trial by Budtz-Jörgensen *et al.* (1988) fluconazole, at a dose of one 50mg capsule daily for 14 days, not only reduced palatal erythema and yeast colonisation, but also proved to be a safe, well-tolerated drug. The clinical and mycological relapse, which occurred in the majority of patients four weeks after treatment started, was attributed to possible reinfection from exogenous sources and to failure to eradicate yeasts from the denture plaque. Patients in this trial were not given denture hygiene advice and the authors acknowledged the importance of meticulous oral and denture hygiene in obtaining a more long-lasting cure of denture stomatitis after antifungal therapy. It was also concluded that a daily dose of fluconazole at 50mg for 2 weeks was not more efficient than topical treatment with antimycotic drugs for a similar treatment period. In contrast, however, Bissell *et al.* (1993) found systemic fluconazole to be as effective as topical amphotericin B in patients who were given denture hygiene advice in addition to

antifungal therapy. In this study, as in others (Budtz-Jörgensen and Bertram, 1970b; Nairn, 1975; Bergendal *et al*, 1979; Bergendal, 1982; Budtz-Jörgensen *et al*., 1983a; Budtz-Jörgensen *et al*., 1988), there was a high rate of clinical relapse and recurrence after therapy in both the amphotericin B and the fluconazole treatment groups.

Itraconazole is an orally active dioxalane tri-azole derivative which differs from fluconazole in several key areas (Odds, 1993a; 1996). It is a lipophilic molecule which achieves high and often persistent levels in skin and deep tissues. It also has a much broader spectrum of activity than fluconazole, including *Aspergillus* species and less common *Candida* species. Itraconazole is available as 50mg and 100mg capsules. The most recent formulation to be described is a new cyclodextrin solution of itraconazole, which combines the effect of topical application to the oral mucosa and the systemic effects after absorption (Blatchford, 1990). Studies of itraconazole treatment so far have concentrated on patients who are greatly predisposed to the infection, for example those with AIDS (Blatchford, 1990). There have been no therapeutic trials of itraconazole among patients with denture stomatitis.

Lamb and Douglas (1988) treated ten patients, previously resistant to antifungal therapy, by means of a miconazole sustained drug-delivery device in the maxillary denture. Half the patients were cured. Those who were not cured had high levels of candidal colonisation prior to treatment and predisposing systemic factors. A single dose of miconazole varnish applied to the fitting surface of dentures has been found to be equally efficacious to miconazole gel (Budtz-Jörgensen and Carlino, 1994). Parvinen *et al*. (1994) also found a miconazole

lacquer to be as effective as gel but suggested that repeated applications of the lacquer would be required to achieve a prolonged effect. Konsberg and Axéll (1994) found a single application of miconazole lacquer to reduce the number of yeasts for a substantial period of time but suggested weekly re-application for most effectiveness. Furthermore, although miconazole lacquer was found to be a viable treatment of *Candida*-associated denture stomatitis in a cohort of Chinese patients (Dias *et al.*, 1997), it was also concluded that to obtain lasting relief it was essential to apply the drug three times at weekly intervals and monitor treatment closely to detect recalcitrant infections requiring additional treatment sessions. Dias *et al.* (1997) found the major advantages of the miconazole lacquer to be its minimal dosage, compared with conventional formulations, and its application by a clinician, devoid of direct patient involvement.

1.4. THE TRIAZOLES.

1.4.1. History of Use.

Fungal infections are a major cause of morbidity and mortality in immunocompromised individuals. For over 30 years amphotericin B has been considered the gold standard for the treatment of life-threatening fungal infections, but severe adverse reactions and treatment failures have obscured its status (Karyotakis and Anaissie, 1994). The introduction of ketoconazole provided an alternative to amphotericin B, but problems of resistance, hepatotoxicity, poor gastric absorption and failure to successfully treat fungal infections in the immunosuppressed have limited its use (Sugar, 1993). The need for safer and more effective systemic antifungals led to the synthesis of the triazoles, fluconazole and itraconazole (Karyotakis and Anaissie, 1994). These agents are currently the most widely used of their class for the management of deep-seated fungal infections (Odds, 1996). Fluconazole is a well tolerated drug with an excellent safety profile (Osterloh, 1992). It has been used to treat superficial and systemic mycoses (Odds, 1993a). Prolonged administration of fluconazole for prophylaxis has raised concerns about the emergence of resistance (Karyotakis and Anaissie, 1994). Itraconazole is safe and effective in the treatment of both superficial and systemic candidoses (Odds, 1993a; 1996). It has a broader spectrum of activity than fluconazole, including *Aspergillus* species and less common *Candida* species (Odds, 1993a).

1.4.2. Mechanism of Action and Spectrum of Activity.

The azoles are fungistatic through their property of changing membrane permeability (Scully *et al.*, 1995). They interfere with cytochrome P-450-dependent enzyme C-14a-demethylase, which is responsible for the conversion of lanosterol to ergosterol, the major component of the fungal cell membrane. The disruption of ergosterol synthesis causes functional and structural changes that render fungal cells more susceptible to osmotic and immune-mediated damage and result in deficient fungal cell adherence (Karyotakis and Anaissie, 1994).

Fluconazole, whilst active against most pathogenic yeasts, has limited activity, *in vitro*, against the moulds, including *Aspergillus* species. However, there is some evidence that higher doses may be effective in clearing aspergillus infections (Hay, 1991). Itraconazole's spectrum of activity both *in vitro* and clinically is very broad (Odds, 1993a). However, neither triazole is active against *C. krusei*, *C. glabrata*, *Fusarium* species, and the zygomycetes. Other *Candida* species, such as *C. parapsilosis*, *C. lusitaniae* and *C. guillermondii*, exhibit strain-dependent susceptibility (Karyotakis and Anaissie, 1994).

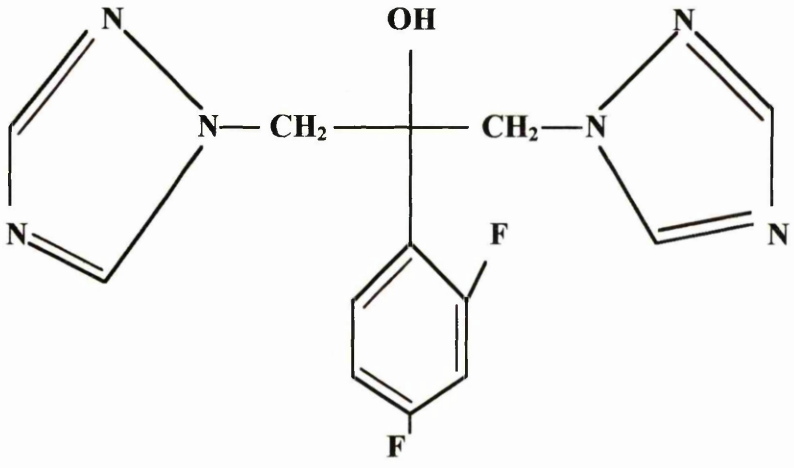
1.4.3. Fluconazole.

1.4.3.1. Chemistry.

Fluconazole is a triazole derivative, the important determinant of activity being the triazole moiety (Fig. 1.3). Fluconazole is unique among clinically available azole derivatives by being highly water-soluble. It is minimally protein-bound in

the circulation (Odds, 1996). As a result it is distributed throughout body water. It is available as a capsule and as an aqueous solution for intravenous administration (Sugar, 1993).

Fig. 1.3. The structure of fluconazole.



1.4.3.2. Pharmacology.

Fluconazole, being very water soluble, has excellent absorption and is widely distributed throughout the body (Hay, 1991). High concentrations appear in CSF (50-100% serum concentrations) and peritoneal fluid (Odds, 1993a). Fluconazole is minimally metabolized and 80% of the administered dose is excreted unchanged in the urine (Karyotakis and Anaissie, 1994). The terminal half-life of fluconazole is approximately 24 hours. Serum concentrations increase sequentially with increasing dose (Hay, 1991) and, without a loading dose, steady state concentrations are reached in about five days. Optimal dosing depends on the indication, with doses ranging from 50mg to 400 mg given once daily (Sugar,

1993). The half-life of fluconazole is markedly increased in patients with severely depressed glomerular filtration rates; hence the dose should be reduced in patients with renal impairment (Karyotakis and Anaissie, 1994).

1.4.3.3. Adverse Effects and Drug Interactions.

Fluconazole is a well tolerated drug (Osterloh, 1992). The main side-effect is gastrointestinal intolerance (Karyotakis and Anaissie, 1994; Odds, 1996). Headaches, dizziness, abdominal pain and pruritis may also occur (Karyotakis and Anaissie, 1994). Transient elevations of serum enzymes related to liver function occur frequently in AIDS patients treated with fluconazole but there are no effects on steroid metabolism related to adrenal or testicular function (Odds, 1996). Rare side-effects reported with fluconazole include anaphylaxis, Stevens-Johnson syndrome and thrombocytopenia (Sugar, 1993).

Fluconazole elevates or prolongs levels of phenytoin, cyclosporin, sulphonylurea and other oral hypoglycaemic agents such as chlorpropamide and tolbutamide (Odds, 1996). Potentiation of warfarin effects on prothrombin time have been noted (Sugar, 1993; Odds, 1996). Levels of ethinyl oestradiol are reduced in patients taking oral contraceptives (Odds, 1996). Rifampin has been reported to cause a modest reduction of fluconazole serum levels (Sugar, 1993; Odds, 1996).

1.4.3.4. Clinical Efficacy.

The efficacy of fluconazole has been demonstrated in superficial fungal infections such as vaginal candidosis (Andersen *et al.*, 1989) and in oropharyngeal candidosis in patients who are HIV positive (Dupont and Drouhet, 1988; DeWit *et al.*, 1989). Oropharyngeal and oesophageal candidosis in the neutropenic patient have also been successfully treated with fluconazole (Sugar, 1993). Doses range from as low as 50mg daily, for superficial fungal infections, up to and exceeding 400mg daily for systemic candidal and cryptococcal infections (Osterloh, 1992). In the treatment of oral candidosis in patients with AIDS, although there is usually a rapid response to therapy there is also a high recurrence rate, usually within 30 days (Sugar, 1993). As a result, either maintenance therapy or intermittent therapy with fluconazole is essential to prevent relapse after cessation of treatment (Lewis *et al.*, 1991). Fluconazole has been successfully used to treat chronic disseminated candidosis and, less convincingly, patients with candidaemia (Sugar, 1993). The efficacy of fluconazole in the treatment of *Candida*-associated denture stomatitis has been reviewed above (see Section 1.3.7.4).

1.4.3.5. Antifungal Prophylaxis.

The low toxicity of fluconazole has led to its use over long time intervals as a prophylactic agent in severely compromised patients, for example bone marrow transplant recipients and those with AIDS (Just-Nübling *et al.*, 1991; Stevens *et al.*, 1991; Goodman *et al.*, 1992). The poor *in vitro* activity of fluconazole against

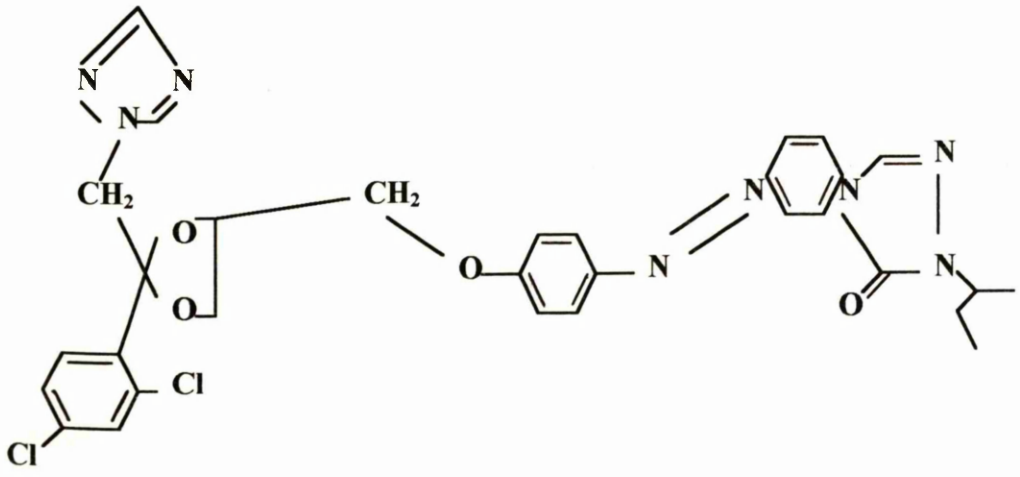
aspergilli is a possible disadvantage in the use of fluconazole as oral antifungal prophylaxis (Hay, 1991). Furthermore, there is concern about the emergence as pathogens of yeasts that are less virulent than *C. albicans* but which are resistant to fluconazole, for example *C. krusei* (Wingard *et al.*, 1991; Goodman *et al.*, 1992).

1.4.4. Itraconazole.

1.4.4.1. Chemistry.

Itraconazole is a triazole derivative (Fig. 1.4). It is insoluble in aqueous media and is highly protein-bound in the bloodstream. Itraconazole is found concentrated in lipid tissues. It is dispensed as a lactose bead-encapsulated capsule and recently as a new liquid formulation using hydroxypropyl-β-cyclodextrin, which offers more reliable absorption and near total bioavailability of the drug (Hostetler *et al.*, 1992). There is no intravenous preparation of itraconazole (Sugar, 1993).

Fig. 1.4. The structure of itraconazole.



1.4.4.2. Pharmacology.

Itraconazole is highly lipophilic and essentially insoluble in aqueous solutions and at neutral pH (Sugar, 1993; Karyotakis and Anaissie, 1994). Conditions of low intragastric acidity interfere with the absorption of itraconazole. As a weak base, it requires low gastric pH and is better absorbed when administered with food (Karyotakis and Anaissie, 1994; Odds, 1996). A low pH also aids in maximising concentration of itraconazole in the blood (Sugar, 1993). Bradford *et al* (1991) have indicated that in cases of apparent failure of itraconazole therapy, measurement of serum concentrations of the drug may be helpful in determining the cause of the lack of response. Oral doses of 100-200mg itraconazole taken daily produce serum concentrations of 0.3mg/ml, one to three hours later (Hay, 1991). However, repeated administration for two weeks results in a peak plasma concentration of around 2mg/ml (steady state) in healthy subjects. Furthermore, a loading dose (600mg/day for several days) may help to attain higher serum levels before the steady state is achieved (Karyotakis and Anaissie, 1994). Itraconazole is highly protein-bound to plasma proteins, and concentrations of itraconazole in body fluids, such as serum or CSF, are low (Sugar, 1993; Odds, 1996). However, when itraconazole is assayed in tissues, concentrations two to five times higher than those in blood are often found (Van Cutsem *et al.*, 1987). As a result, itraconazole appears to reach potential sites of infection in concentrations that should provide an antifungal effect (Sugar, 1993). Itraconazole undergoes extensive metabolism in the liver (Karyotakis and Anaissie, 1994). At least one of the metabolites, hydroxyitraconazole, has an antifungal effect (Sugar, 1993; Odds, 1996). Excretion of itraconazole and its metabolites is principally via bile (Odds, 1996).

1.4.4.3. Adverse Effects and Drug Interactions.

Itraconazole has an excellent safety profile (Odds, 1993a) causing only minor side-effects, most of which are gastrointestinal in nature (Odds, 1993a; 1996). Headaches, dizziness, abdominal pain and pruritis may also occur (Karyotakis and Anaissie, 1994). Hepatotoxicity is not a serious problem with itraconazole (Odds, 1993a), although transient elevations of serum aminotransferases have been reported in some patients and itraconazole usage should be avoided in patients with liver disease (Odds, 1996). Itraconazole may occasionally interfere with steroid metabolism and can cause accumulation of substances with aldosterone-like activity, capable of producing hypokalaemia, pedal oedema and hypertension (Karyotakis and Anaissie, 1994). These effects should not occur at normal dosages of itraconazole (Odds, 1996).

Itraconazole should not be used concomitantly with cyclosporin, digoxin, phenytoin and terfenadine to avoid increasing the serum levels of these drugs. Conversely, serum levels of itraconazole are lowered if it is administered concomitantly with rifampicin, rifabutin, antacids or H_2 blocking agents. The anticoagulant effect of warfarin is prolonged and enhanced by itraconazole (Odds, 1996).

1.4.4.4. Clinical Efficacy.

Itraconazole is an effective agent for the treatment of both superficial and systemic mycoses (Odds, 1993a). Itraconazole's broad spectrum of activity includes less common *Candida* species and *Aspergillus* species (Van Cutsem *et*

al., 1984; Van Cutsem *et al.*, 1987). In a randomised comparative study with intravenous amphotericin B, itraconazole showed comparable activity with the latter in the treatment of fungal infection in neutropenic patients (Van t'Wout *et al.*, 1991). Studies of itraconazole treatment of oral candidosis have concentrated so far on patients who are greatly predisposed to the infection, for example those with AIDS (Blatchford, 1990). Itraconazole gave equally good clinical results when compared to ketoconazole in the treatment of oral and oesophageal candidosis in patients with HIV infection, although relapse rates were identical with both drugs (Smith *et al.*, 1991). The new cyclodextrin solution of itraconazole (see 1.4.4.1.) has been used successfully among a group of AIDS patients with a history of failed treatment for oral candidosis using other azoles (Cartledge *et al.*, 1994).

1.4.4.5. Antifungal Prophylaxis.

A combination of itraconazole and oral amphotericin B, when compared to oral amphotericin B alone, as a prophylactic measure in neutropenic patients with haematologic malignancies, reduced the incidence of *C. albicans* infection but made no difference to the incidence of aspergillosis, overall fungal infections or mortality. (Vreugdenhil *et al.*, 1993). In two retrospective studies in neutropenic patients, prophylactic itraconazole was more effective than nystatin or ketoconazole and a combination of oral itraconazole and nasal amphotericin B effectively reduced the incidence and mortality of invasive aspergillosis compared with historical controls in patients with haematologic malignancies (Karyotakis and Anaissie, 1994).

1.4.5. The Problem of Azole Resistance.

Therapeutically, azole resistance is a substantial problem (Denning, 1995). Fluconazole has been implicated in instances of emergent resistance more often than ketoconazole, and ketoconazole more often than itraconazole (Odds, 1993b). The first reports of fluconazole resistance in the context of AIDS did not emerge until 1990 (Fulton and Philips, 1990). *C. krusei* and *C. glabrata* are frequently cited as emergent colonizing flora during frequent or sustained clinical use of fluconazole in immunocompromised or debilitated patients and in treatment failures of diagnosed disease, both in disseminated infections and in mucosal candidal infections. Resistance to fluconazole in *C. albicans* is less common but does exist (Odds, 1993b). Evidence exists that strains of *C. albicans* are becoming more resistant to fluconazole, because of decreased cell wall permeability, when the drug is given in a low dosage and on a long-term basis to patients with AIDS for prophylaxis of oral candidosis (Dupont, 1995).

To date, there have been only anecdotal reports of itraconazole inducing resistance, but it has been suggested that this may reflect the much wider use of fluconazole (Milne, 1997). Barchiesi *et al.* (1994) have found a significant proportion of fluconazole-resistant *C. albicans* strains to be cross-resistant to itraconazole *in vitro*.

Three possible mechanisms of resistance have been described (Odds, 1993b):

1) reduced permeability of the *C. albicans* membrane to azole antifungals

2) mutation in the target fungal cytochrome P450 enzyme resulting in decreased binding affinity for the azole drugs

3) overproduction of the target cytochrome P450 enzyme.

The relative importance of each, and for which azole, is unknown (Denning, 1995). Stategies for the prevention of azole resistance include restraint in the use of these drugs, the avoidance of antifungal prophylaxis in AIDS patients, except when there are frequent episodes of oesophageal candidosis or for cryptococcal meningitis, and possibly the use of alternative antifungals to fluconazole, such as itraconazole formulated in cyclodextrin (Janssen) or the azole D0870 (Zeneca) (Denning, 1995). However, caution should be exercised if alternative antifungals to fluconazole are to be used in the longer term, as in cases where fluconazole-specific resistance has been demonstrated isolates have also shown a loss of sensitivity to itraconazole, although to a lesser degree (Johnson *et al.*, 1995).

1.5. AIMS.

Candida-associated denture stomatitis is common, affecting approximately 50% of denture wearers. Unfortunately, current treatments result in a high rate of recurrence (Budtz-Jörgensen, 1990). Until now, there have been no therapeutic trials of itraconazole among patients with denture stomatitis, despite the poor patient compliance with topical drugs and the rapid relapse reported following treatment with fluconazole (Budtz-Jörgensen *et al.*, 1988; Bissell *et al.*, 1993). The most recent formulation of itraconazole, the cyclodextrin solution, could prove advantageous in the armamentarium of denture stomatitis management by combining the effect of topical application to the mucosa and the systemic effects after absorption.

The objectives of this study were:

1. To determine by means of a single blind clinical trial the value of cyclodextrin solution of itraconazole at a dose of 100mg twice daily in the treatment of *Candida*-associated denture stomatitis.

The trial design also encompassed:

- Determination of the safety of and tolerance for itraconazole.
- Determination of itraconazole sensitivity of yeasts isolated from the mouths of patients before and after treatment.
- Measurement of itraconazole levels achieved in blood and saliva.

2. To determine, by molecular typing of *C. albicans* species isolated before and after treatment, whether there had been recurrence of infection with the same strain(s), selection of particular strains or infection with new strains of *C.albicans*. This may permit a fuller understanding of the reasons for any relapses following treatment.

CHAPTER TWO

MATERIALS AND METHODS

2.1. PRELIMINARY INVESTIGATIONS.

2.1.1. Evaluation of the Erythema Meter.

Many diseases of the oral mucosa result in inflammation, manifesting clinically as an increase in mucosal erythema. Reduction in erythema, indicative of resolving inflammation, can therefore be used as an objective indicator of successful treatment. Clinical trials of antifungal drugs have commonly used a reduction in erythema as an outcome measure. To date, however, the major difficulty has been the lack of an objective method for assessing erythema of the oral mucosa. Previous studies have relied on a subjective visual assessment (Bergendal and Isaacson, 1980; Bergendal, 1982; Johnson *et al.*, 1989) or have used photographs (Olsen, 1975a; Budtz-Jörgensen, *et al.*, 1988; Lal *et al.*, 1992), but neither of these methods is ideal.

The *Erythema meter* (Fig. 2.1) was developed originally to measure cutaneous erythema induced by ultraviolet radiation (Diffey *et al.*, 1984). The instrument is based on the principle that when white light, between 400nm and 700nm, is reflected off a cutaneous or mucosal surface, haemaglobin in the vasculature selectively absorbs green light but has little effect on red. An 'erythema index', defined as the logarithm of the ratio of red to green reflected light, can be determined. The difference between the 'erythema index' measured before and after treatment is related to reduced vasodilation following a reduction in the degree of inflammation.

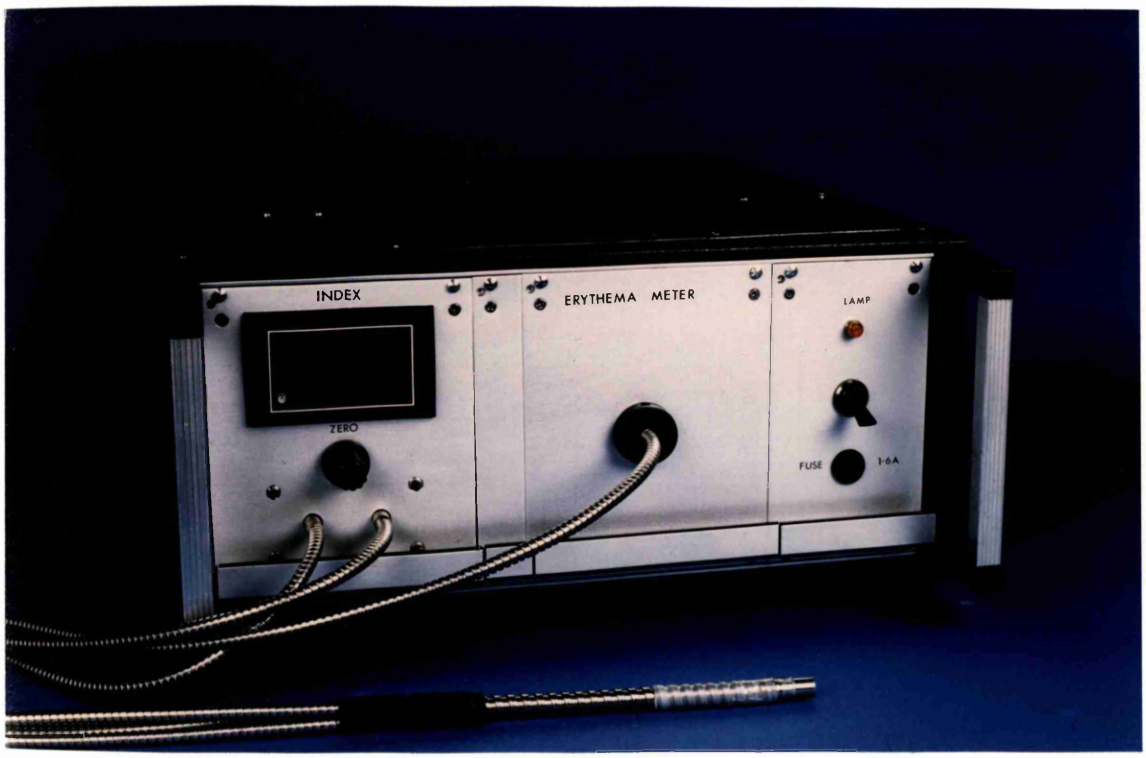


Figure 2.1. **A photograph of the *Erythema Meter* - an electro-optical instrument for measuring oral mucosal erythema.**

The aims of the preliminary investigations were:

1. To evaluate the sensitivity and reproducibility of the *Erythema meter* for quantifying erythema of the palatal mucosa.

2. To determine the usefulness of the *Erythema meter* in a clinical trial situation.

2.1.1.1. Laboratory Study.

A series of non-clinical *Erythema meter* readings were taken from a check-standard used by graphic artists for colour reproduction. This check-standard (Fig.2.2) had a range of colours from pale pink to dark red. To the human eye these colours appeared similar to those of healthy and inflamed mucosa. The colours were composed of varying percentages of magenta, starting at 20% magenta for the palest pink shade and moving up to 100% magenta for the darkest red. The *Erythema meter* was used to record an erythema index for each shade on three separate occasions and the results averaged.

2.1.1.2. Clinical Investigations.

The protocol for the clinical study was approved by the local ethical review committee. All patients gave informed consent prior to participation.

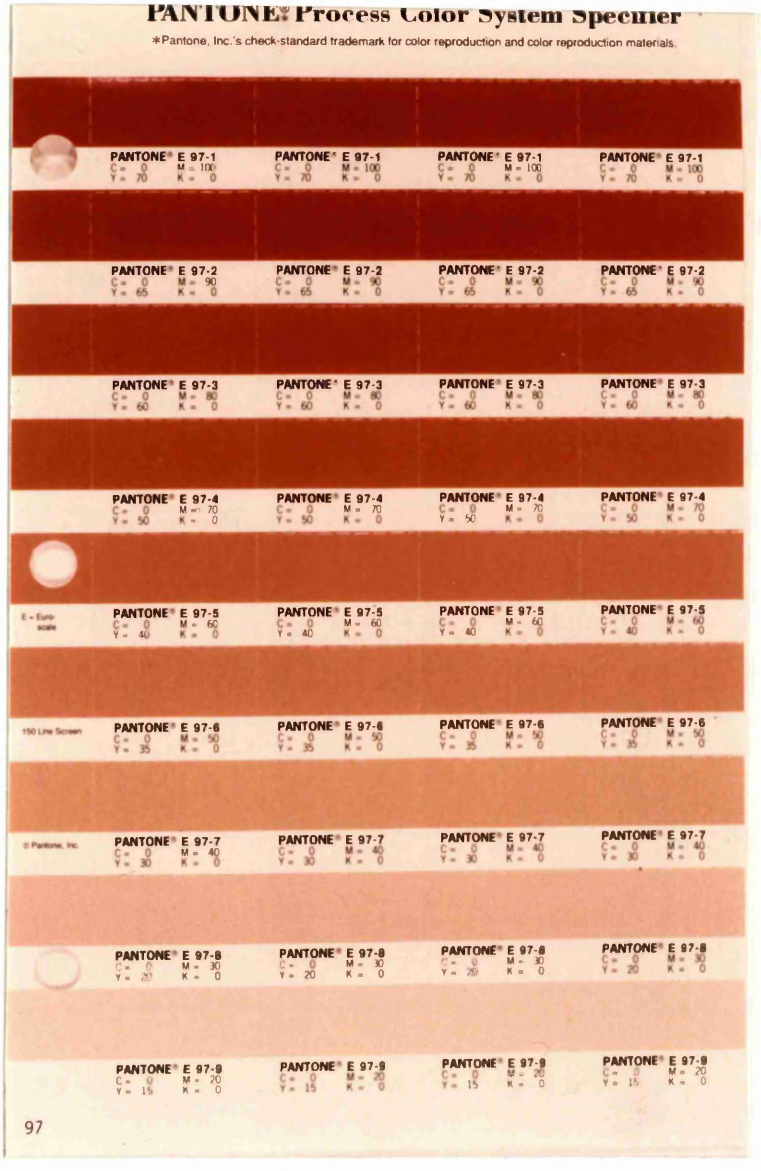


Figure 2.2. A photograph of the check-standard used by graphic artists for colour reproduction.

2.1.1.2.1. Patients.

Two groups of patients were studied. The first comprised 40 individuals with clinically healthy palatal mucosa, 20 of whom were dentate and 20 of whom wore complete dentures. The second group consisted of another 20 complete denture wearers, all of whom had clinical evidence of long-standing denture stomatitis. The complete denture wearers were recruited from the Oral Medicine and Prosthodontic Departments of Glasgow Dental Hospital and School N.H.S.Trust, while the dentate participants were staff and students from Glasgow Dental Hospital and School N.H.S.Trust. Table 2.1 shows the sex and age distribution of the groups.

Table 2.1. **Age and sex distribution for the three study groups.**

		n	Mean age (range)	Male	Female
Healthy palatal mucosa	Non-denture controls	20	29.7(18-53)	9	11
	Denture controls	20	64.7(34-88)	10	10
Denture stomatitis		20	68.8(43-85)	7	13

2.1.1.2.2. Baseline Investigations.

All patients were examined by the author. Following a full medical and dental history, the clinical assessment included an oral examination and both visual and objective assessments of erythema of the palatal mucosa.

The age and fit of dentures and existing denture hygiene practices were recorded for the complete denture wearers. Denture stomatitis, if present ,was classified as localised erythematous (Newton's Type I), diffuse erythematous (Newton's Type II) or hyperplastic granular (Newton's Type III) (Newton, 1962).

Visual assessment of the degree of palatal erythema was recorded, from zero to three, according to Budtz-Jörgensen and Bertram (1970b).

Erythema meter readings were then taken from each side of the palate, repeated three times and averaged, as recommended by Diffey *et al.* (1984). This allowed the derivation of an 'erythema index'. A removable attachment was fitted onto the end of the fibre optic cable to ensure the tip was positioned a standard distance above the mucosal surface. This attachment was heat-sterilised between patients. The sites measured were recorded on a diagram so that the readings could be repeated from exactly the same sites at the next visit.

Two weeks later the visual scores and the *Erythema meter* readings for the 40 patients with healthy palatal mucosa were repeated and recorded, to allow assessment of the reproducibility of the *Erythema meter* scores.

2.1.1.3. Comparison of Fluconazole and Itraconazole in the Management of Denture Stomatitis.

2.1.1.3.1. Patients.

The twenty complete denture wearers with clinical evidence of long-standing denture stomatitis (see Section 2.1.1.2.1.) were enrolled in a clinical trial comparing the efficacy of fluconazole and itraconazole in the management of denture stomatitis.

The following groups of patients were excluded from the trial:

1. Female patients who were pregnant, lactating or of child-bearing age and not using reliable contraception.

2. Patients with impaired renal or hepatic function.

3. Those who had used an antifungal agent within the preceding 10 days.

4. Those taking any drug listed in the British National Formulary (1996) as interacting with the azole group of antifungal drugs.

5. Those with a known sensitivity to the azole group of antifungal drugs.

6. Those intending to donate blood during or three weeks after the study period.

2.1.1.3.2. Baseline Investigations.

The clinical history and investigations carried out were those described in Section 2.1.1.2.2. In addition, the denture fitting surfaces of the denture stomatitis patients were assessed visually and by scraping with a blunt instrument, then allocated a clinical denture cleanliness score of 0 to 4, depending on the amount of plaque visible on the denture fitting surface (Blair *et al.*, 1995). Zero represented no visible plaque and no plaque adherent to a flat plastic instrument on light scraping of the denture fitting surface; 1, no visible plaque but matter was adherent to the instrument on light scraping; 2, deposits of plaque which were just visible without the need to confirm by scraping; 3, deposits of plaque which were clearly visible and 4, gross plaque deposits (Blair *et al.*, 1995).

Venous blood was collected for routine haematological and biochemical investigations including a full blood count, serum ferritin, folate, vitamin B_{12} and a random blood glucose level.

Microbiological samples including imprint cultures (Arendorf and Walker, 1979) from the palate and tongue, an oral rinse (Samaranayake *et al.*, 1986a) and a denture disc specimen (Blair *et al.*, 1995) were collected (see Section 2.2.3.4.1).

2.1.1.3.3. Treatment Protocols and Follow-up.

At baseline, all patients were instructed in denture hygiene measures and given an instruction leaflet containing relevant standardised information. Provision of specific antifungal medication was undertaken on an open basis. Ten consecutive

patients were prescribed fluconazole capsules at a dose of 50mg once daily for 14 days, and a further ten consecutive patients provided with itraconazole capsules at an oral dose of 100mg once daily for 15 days. These dosages were those recommended in the British National Formulary (1996).

All patients were reviewed after 14 days, on completion of their course of antifungal medication, and the clinical and microbiological investigations described at baseline were repeated.

2.1.1.3.4. Microbiological Methods.

2.1.1.3.4.1. Specimen Processing.

Imprint cultures (Samaranayake *et al.*, 1986a) were inoculated sequentially onto plates of Sabouraud's dextrose agar (Life Technologies, Paisley, Scotland) and CHROMagar® *Candida* (Life Technologies, Paisley, Scotland) at the chairside.

After concentration, oral rinse specimens (Samaranayake *et al.*, 1986a) were inoculated by spiral plating onto blood agar (at dilutions of 10^{-2} and 10^{-3}), Sabouraud's dextrose agar and CHROMagar® *Candida*.

Denture disc specimens (Blair *et al.*, 1995) were collected by applying a pre-sterilised filter paper disc (Whatman Grade 1) to a standardised area of the denture fitting surface for 20 seconds. The disc was immediately placed into 9ml sterile phosphate buffered saline (pH 7.4). On arrival at the laboratory the specimen was vortex mixed for 10 seconds and an aliquot tenfold diluted to 10^{-4}.

Each dilution was spiral plated onto duplicate sets of blood agar and incubated for 48 hours at 37°C both aerobically and anaerobically. Neat specimen was inoculated onto Sabouraud's agar and CHROMagar® *Candida*. Logarithmic conversion of the total anaerobic count produced a 'denture cleanliness score' (Blair *et al.*, 1995).

All plates were incubated at 37°C for 48 hours.

2.1.1.3.4.2. Yeast Identification.

C. albicans was identified on the basis of germ tube production. A sucrose assimilation test was performed on germ tube positive yeasts to distinguish from *C. stellatoidea* or *C. tropicalis*. Germ tube negative yeasts were identified by the API 32C test (bio-Merieux S A, Marcy-l'Etoile, France)(see Section 2.2.3.4.2).

2.2. A CLINICAL TRIAL COMPARING THE EFFICACY OF A CYCLODEXTRIN SOLUTION OF ITRACONAZOLE AND ITRACONAZOLE CAPSULES IN THE MANAGEMENT OF *CANDIDA*-ASSOCIATED DENTURE STOMATITIS.

2.2.1. Study Design.

The study was a parallel design, single blind clinical trial which compared the effectiveness of denture hygiene instruction and treatment with the cyclodextrin solution of itraconazole against denture hygiene instruction and treatment with itraconazole capsules in the management of *Candida*-associated denture stomatitis.

The protocol for the clinical trial was approved by the local ethical review committee. All patients gave written informed consent to participate in the study (Appendix I).

2.2.2. Patients.

Patients were recruited from the Prosthodontic and Oral Medicine Departments of Glasgow Dental Hospital and School N.H.S. Trust. Edentulous denture wearers with clinical evidence of denture stomatitis were examined for yeasts by swabbing the palatal mucosa and dorsum of the tongue. Patient details were completed on a

standard proforma (Appendix II). In the laboratory, the swabs were inoculated onto Sabouraud's dextrose agar which was then incubated at 37°C for 48 hours.

Forty patients from whom yeasts were cultured at both sites and who fulfilled the other criteria were enrolled in the clinical trial. Nine were male and thirty-one were female with ages ranging from 29 to 81 years (mean age 62 years). Table 2.2 shows the sex and age distribution of the two groups. Exclusion criteria were identical to those stated in Section 2.1.1.3.1.

Table 2.2. **Age and sex distribution for the two study groups.**

	n	Mean age (range)	Male	Female
Cyclodextrin group	20	62.1(35-79)	6	14
Capsule group	20	65.2(29-81)	3	17

2.2.3. Baseline Investigations.

2.2.3.1. Clinical History.

Clinical details were recorded on a standard proforma (Appendix II). Demographic details were collected, together with a full medical and dental

history. In particular, local factors predisposing to oral candidosis were sought. These included continuous denture wearing, inadequate denture hygiene practices, smoking, use of steroid inhalers and oral dryness.

Similarly, systemic predisposing factors were identified, including relevant drug history, (steroids and other immunosuppressive medication, antibiotics and drugs inducing xerostomia), haematological or endocrine abnormalities and recognised immunodeficiency states.

2.2.3.2. Clinical Examination.

All patients were examined by the author. A complete oral examination was undertaken and the age and fit of dentures recorded. Mucosal changes suggestive of candidosis were noted. Denture stomatitis was classified visually as localised erythematous (Newton's Type I), diffuse erythematous (Newton's Type II) or hyperplastic granular (Newton's Type III) (Newton, 1962).

Palatal erythema was visually assessed and awarded a score from 0 to 3, according to Budtz-Jörgensen and Bertram (1970a). *Erythema meter* readings were then taken from that area of the palate which appeared to be the most inflamed. The reading from this site was repeated three times and averaged, as recommended by Diffey *et al.* (1984). The site measured was recorded on a diagram (Appendix II) so that the readings could be repeated from exactly the same site at subsequent visits.

The denture fitting surfaces were assessed visually and by scraping with a blunt instrument, then allocated a clinical denture cleanliness score of 0 to 4, depending on the amount of plaque visible on the denture fitting surface (Blair *et al.*, 1995).

2.2.3.3. Haematology and Blood Chemistry.

The following investigations were undertaken for each patient at enrolment:

- full blood count
- serum ferritin
- serum folate
- vitamin B12
- random blood glucose
- urea and electrolytes
- liver function tests
- C-reactive protein

2.2.3.4. Microbiological Methods.

2.2.3.4.1. Specimen Collection and Primary Cultures.

- Imprint Cultures.

Imprint cultures (Arendorf and Walker, 1979) were collected from the hard palate, denture fitting surface and tongue. A modified version of the imprint culture technique was employed. Sterile foam pads were placed lightly onto the site to be sampled and then immediately inoculated at the chairside onto plates of Sabouraud's dextrose agar followed by CHROMagar® *Candida*. After incubation at 37°C for 48 hours, yeast isolates were processed as described below.

- Oral Rinse.

An oral rinse (Samaranayake *et al.*, 1986a) was collected from all patients. Each patient was asked to rinse the mouth for one minute with 9ml sterile phosphate buffered saline (pH 7.4) before returning the solution to the container provided. After concentration, by centrifugation at 3200 r.p.m. (Centaur) for 5 minutes (Samaranayake *et al.*, 1986a), the supernatant was poured off and the oral rinse specimens were inoculated by spiral plating onto blood agar (at dilutions of 10^{-2} and 10^{-3}), Sabouraud's dextrose agar, CHROMagar® *Candida* and MacConkey agar. All plates were incubated at 37°C for 48 hours.

Staphylococcus aureus was identified by a commercially available coagulase test (Slidex Staph-Kit, bioMerieux SA, Marcy-l'Etoile, France) and β-haemolytic streptococci were Lancefield grouped (bioMerieux SA, Basingstoke). Coliforms were tested as oxidase positive or negative using a paper strip for the

detection of cytochrome oxidase (PYO-TEST, Medical Wire and Equipment Company (Bath) Limited). Oxidase negative coliforms were identified by the Analytical Profile Index (API 20E, bioMerieux SA, Marcy-l'Etoile, France) and oxidase positive coliforms by API 20NE (bioMerieux SA, Marcy-l'Etoile, France). Yeasts were investigated as described below.

- Denture Disc Specimens.

Denture disc specimens (Blair *et al.*, 1995) were collected from the fitting surface of the maxillary denture. A pre-sterilised filter paper disc (Whatman Grade 1) was applied to a standardised area of the denture fitting surface for 20 seconds. It was immediately placed into 9ml sterile phosphate buffered saline (pH 7.4). On arrival at the laboratory the specimen was vortex mixed for 10 seconds and an aliquot tenfold diluted to 10^{-4}. Each dilution was spiral plated onto duplicate sets of blood agar and incubated for 48 hours at 37°C both aerobically and anaerobically. Neat specimen was inoculated onto selective media for yeasts and coliforms as described earlier.

Logarithmic conversion of the total anaerobic count provided a denture cleanliness score of relevance for patients with denture stomatitis. Isolates of coliforms, *Staphylococcus aureus* and β-haemolytic streptococci were identified as described above. Yeasts were analysed as described below.

2.2.3.4.2. Selection of Yeasts from Primary Plates.

One colony of each morphological type on CHROMagar® *Candida* (Fig. 2.3) was plated for purity, identified and stored at -70°C. The proportion of each type on these plates was also recorded. For each patient, four further colonies were removed from the Sabouraud's plates, selected at random from the specimens available. Each isolate was formally identified and stored at -70°C for further investigation.

2.2.3.4.3. Yeast Identification.

Although yeast species could be differentiated by their morphological appearance on CHROMagar® *Candida*, their identification was always confirmed by appropriate physiological testing.

C. albicans was initially identified by the green colouration of its colonies on CHROMagar® *Candida* and this was confirmed by the production of germ tubes. A small portion of the yeast colony to be investigated was removed with a loop of wire, emulsified in 0.5ml serum in a tube and incubated at 37°C for 2 hours. A drop of this mixture of serum and yeast was then removed, placed on a microscope slide and examined for the presence of germ tubes. If present, the yeast was identified as *C. albicans*. In the absence of germ tubes, the inoculated serum was reincubated for a further 60 minutes. If there was no evidence of germ tube production after this period of time, the isolate was classed as germ tube negative.

A sucrose assimilation test was performed on germ tube positive yeasts to distinguish *C. albicans* from *C. stellatoidea* or *C. tropicalis*. Yeast identification agar was melted and allowed to cool to 45°C. One colony of the yeast to be investigated was emulsified in 5ml distilled water. Five drops of this mixture were then added to the molten agar, which was poured into a petri dish and allowed to set. Five discs, each saturated with a different carbohydrate (sucrose; lactose; trehallose; cellobiose; raffinose) were placed onto the surface of the agar around the periphery of the plate. After incubation at 30°C for 24-48 hours, the plate was examined for growth around the discs to determine the assimilation profile.

Germ tube negative yeasts were identified using the ID 32C test (bioMerieux S A, Marcy-l'Etoile, France).

2.2.3.5. Oral Hygiene.

All participants were instructed on denture hygiene and given an instruction leaflet (Appendix III) containing relevant standardised advice. This included instructions to clean dentures regularly after each meal using a soft brush and soap. Patients were also advised to remove their dentures at night for sleeping and immerse them overnight in luke-warm water containing an alkaline peroxide denture cleanser, such as Milton® or Steradent®.

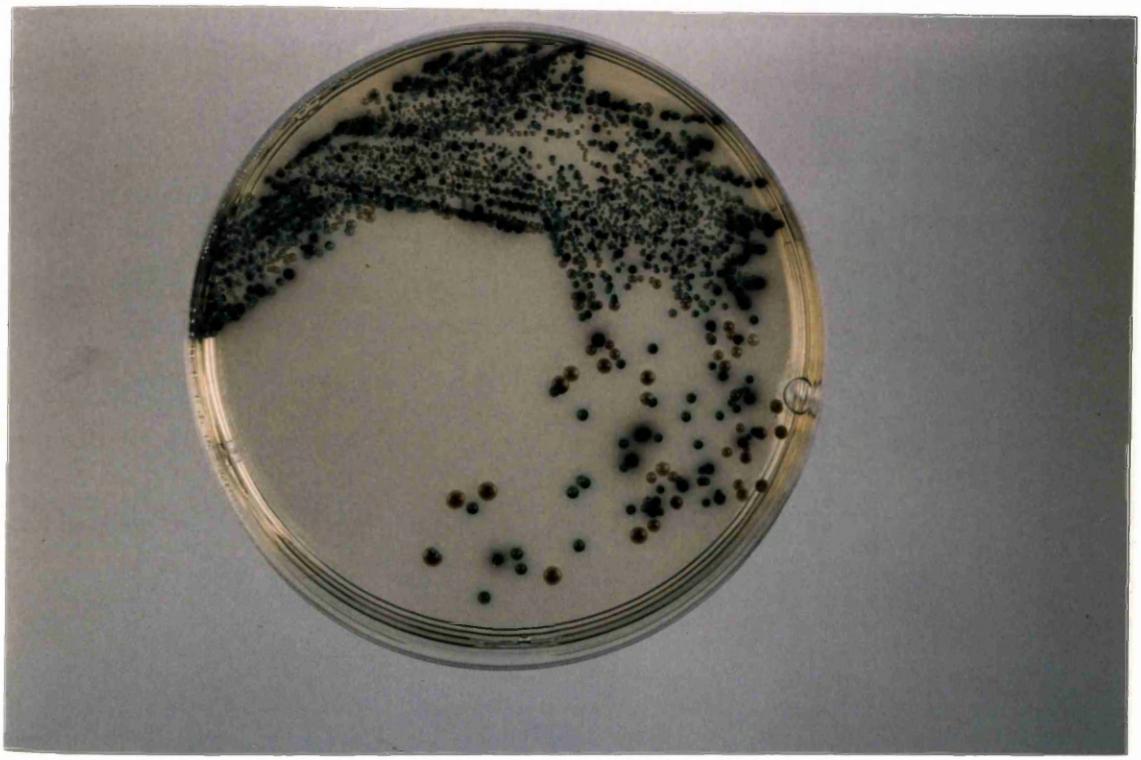


Figure 2.3. **A photograph of a CHROMagar® *Candida* plate showing a mixed growth of *C. albicans* (green colonies) and *C. glabrata* (purple colonies).**

2.2.3.6. Azole Treatment.

The forty patients were randomised to receive either cyclodextrin solution of itraconazole (Sporanox Liquid®, Janssen Pharmaceuticals) at a dose of 100mg twice daily for 15 days or itraconazole capsules (Sporanox®, Janssen Pharmaceuticals) at the same dosage, also for 15 days. These dosages were those recommended in the British National Formulary (1996). As the clinician examining the patients was blinded to the treatment code, the medication was dispensed by nursing staff in the Oral Medicine Department. Patients received a bag containing either itraconazole liquid or capsules along with printed instructions stating the dose to be taken and the appropriate time at which to take it (Appendix III). Itraconazole capsules were to be taken after food and cyclodextrin solution one hour before food. Patients in the cyclodextrin group were asked to remove their dentures and briefly swill the solution around their mouth before swallowing.

2.2.4. Assessment of Response.

Patients were reviewed after 15 days, on completion of their course of antifungal medication. Any patient who had failed to complete the course of medication was then excluded from the trial. Further reviews were undertaken at one and six months after treatment commenced.

At each review visit a full oral examination, including visual and objective assessment of any changes in the appearance of the palatal mucosa was made. An

oral rinse, imprint cultures and denture disc specimen were also collected at each visit. The yeasts isolated were selected, identified, stored at -70°C and analysed as described above (see Section 2.2.3.4).

Blood was taken for a full blood count, liver function tests, urea and electrolytes and C-reactive protein measurement at the end of the 15 day study period. A further blood sample was taken at six months for a full blood count, serum ferritin, serum folate, vitamin B_{12} and a random blood glucose level.

An additional blood sample and at least 1ml of whole saliva were collected at the end of the 14 day study period to enable measurement of itraconazole levels in serum and saliva. Itraconazole concentrations were measured by means of reversed-phase high-performance liquid chromatography (HPLC) with a lower limit of detection of 10μg/L and coefficients of variation of 2.2 - 7.8% over the concentration range of 20-1600μg/L (Warnock *et al.*, 1988). The standard curve was linear from 10 to 10,000μg/L. These measurements were made by the Public Health Laboratory Service Mycology Reference Laboratory in Bristol.

On completion of the antimycotic therapy patients were requested to complete a questionnaire (Appendix IV). This was designed to assess patient compliance and record the incidence of side-effects whilst enabling the clinician to remain blind to the treatment code until after the clinical trial.

2.2.5. Additional Microbiological Methods.

2.2.5.1. Molecular Typing of *Candida albicans* Isolates Before and After Treatment.

2.2.5.1.1. DNA Extraction.

Pairs of isolates from those patients with *C. albicans* present at baseline and six months after treatment commenced,were removed from storage at -70°C and plated onto Sabouraud's agar. The Sabouraud's plates were incubated at 37°C for 48 hours. DNA was extracted using a method similar to one based partly on that originally devised by Scherer and Stevens (1988), as described by Anthony *et al.* (1995). Growth from a Sabouraud's plate was emulsified in 10ml YEPD broth (0.3 % yeast extract, 1 % mycopeptone, 2% dextrose) and incubated overnight at 30°C in an orbital incubator. Cells from 1.5ml of the broth were pelleted in a centrifuge, the supernatant removed, and the cells washed by resuspending in 1000µl 1M sorbitol. The cells were then pelleted in a centrifuge for 3 minutes, when the supernatant was removed and discarded. Cell walls were digested by re-suspending in 1M sorbitol plus 50mM K_2HPO_4 (pH 7.5) and adding to each tube 3µl β-mercaptoethanol and 20µl of freshly prepared 20mg/ml lyticase 20T (Sigma Chemical Company Limited, Dorset, UK) in 1M sorbitol and incubating at 37°C for 90 minutes. The resulting spheroblasts were pelleted by centrifugation (3 minutes), the supernatant removed and lysed by adding 0.5ml GES reagent (30g guanidium thiocyanate dissolved at 65°C in 20ml 0.5mol/l EDTA at pH 8 and then 2.5ml of 10% w/vol sarkosyl added, finally made up to 50ml with distilled water) (Pitcher *et al.*, 1989). After 20 minutes at room temperature, samples were

centrifuged again for 3 minutes and the supernatant was then removed into a new tube, leaving the protein pellet behind to be discarded. The supernatant volume was made up to 200µl with autoclaved distilled water. 100µl phenol and 100µl chloroform were added to the tube. This mixture was shaken well and centrifuged for a further 3 minutes. The top layer was then removed into a new tube and 100µl of 5M potassium acetate and 0.5ml chloroform pentanol (24:1 V:V) was added. The mixture was centrifuged for 5 minutes, the top layer pipetted off into a new tube and the bottom layer discarded. The extracted DNA was precipitated with propan-2-ol, pelleted and resuspended in TE buffer (10mM Tris and 1mM EDTA, pH 7.5). RNA was digested with 1µl of 10mg/ml RNAase A stock solution for 15 minutes at 37°C and the DNA re-precipitated in propan-2-ol, washed with 70% ethanol, then dissolved in 60µl of TE. The presence of DNA was confirmed by running samples on a 1% agarose gel.

2.2.5.1.2. Restriction Enzyme Digestion.

Thirty microlitre aliquots of the DNA extract were restricted with EcoRI according to the manufacturer's instructions (Boehringer Mannheim Biochemica). For example, for digestion of ten DNA samples, 40µl 10 x R3 buffer (Boehringer Mannheim), 8µl 1M spermidine and 30µl autoclaved distilled water were mixed together in an Eppendorf tube. This mixture was then aliquoted into ten new Eppendorf tubes (7.8µl per tube). Thirty microlitres of each DNA sample was added to each tube followed by 2.2µl of the enzyme EcoR1. The mixture was briefly vortex mixed, pulse spun to collect the liquid and placed in a water bath at

37°C. After three hours an additional 1.5µl of EcoR1 was added to each tube to enhance the digestion. The digestion was continued at 37°C overnight. The restriction digests were then run on a 0.8% agarose gel at a constant 30v for 18 hours in TBE buffer (Appendix V).

2.2.5.1.3. Southern Blotting.

Fragments of DNA separated by electrophoresis through an agarose gel were transferred to a nylon membrane (Boehringer Mannheim Biochemica) by Southern blotting. The method used was that of Sambrook *et al.* (1989).

The gel was washed with three different solutions prior to the transfer process. Firstly, the gel was soaked in a pyrex dish containing 250ml 0.25M HCl for 5 minutes, with continuous shaking. The gel was then rinsed briefly in water. Next, the DNA was denatured by soaking the gel twice in 1.5M NaCl / 0.5M NaOH (Appendix V) for 15 minutes each time. Lastly, the DNA fragments were neutralised by soaking in 1.5M NaCL / 0.5M Tris (Appendix V) for 15 minutes. This process was also repeated. Each washing step was carried out in a pyrex dish at room temperature with constant rotational shaking. After each 15 minute stage, the solution was poured off and the gel washed briefly in distilled water before adding the next solution.

The top left hand corner was cut off the gel for orientation purposes. Six pieces of Whatman 3mm chromatography paper were cut, allowing some overlap of the gel. One piece of positively charged nylon membrane (Boehringer

Mannheim Biochemica) was cut to the same dimensions and the top left hand corner was removed. Care was taken to avoid contamination of the membrane. Both the nylon membrane and the Whatman paper were soaked in transfer buffer (20 x SSC - Appendix V), until completely saturated. The restriction digests were capillary blotted onto the nylon membrane overnight (Sambrook *et al.*, 1989). The membrane was placed in an ultraviolet crosslinker for 1 minute, without prior washing, to ensure that the DNA was bound to it. The membrane was then washed briefly in distilled water before being left to airdry, DNA side up, on Whatman paper. It was then sealed in a plastic bag and stored at room temperature until hybridization.

2.2.5.1.4. EcoRI 27A Fragment Purification and Labelling.

The repeated sequence 27A originally described by Scherer and Stevens (1988) was provided, cloned in pUC18, plasmid number 824, by B.B. Magee (University of Minnesota).

Plasmid DNA containing the 27A fragment was restriction digested using EcoR1 and run in a 0.8% agarose gel. The top band containing the 27A was cut out of the gel and placed in a Spin-X column (Costar) on ice at -20°C for 30 minutes. This was then centrifuged for 15 minutes at 13K (Eppendorf centrifuge 5415C) to leave purified 27A DNA at the bottom of the tube. This template DNA was linearized and purified by adding equal volumes (1:1) of phenol and chloroform. After shaking well, the mixture was centrifuged for 3 minutes. The

top layer containing the DNA was transferred to a new tube, 500µl chloroform added, mixed well and spun for a further 3 minutes. The top layer was then pipetted off into a new tube and the bottom layer discarded. The template DNA was re-precipitated with 0.1volume of 3M sodium acetate and 2 volumes of 100% ethanol at -20°C overnight.

The re-precipitated DNA was centrifuged for 3 minutes and the supernatant discarded to leave a small pellet of DNA. The pellet was air-dried for approximately 5 minutes and then resuspended in 40µl of autoclaved distilled water. One microlitre of the suspension was run out in a 0.8% agarose gel to allow estimation of the yield of template DNA prior to labelling.

The template DNA (1µg) was diluted to a total volume of 15µl and denatured by heating for 10 minutes in a boiling water bath and quickly chilling on ice. Hexanucleotide mixture (2µl of10x concentration of hexanucleotide reaction mixture as supplied), 2µl dNTP mixture and 1µl Klenow enzyme were added on ice to the denatured DNA. This was mixed, centrifuged briefly and incubated overnight at 37°C. EDTA (2µl; 0.2M; pH 8.0) was then added to stop the reaction. The labelled DNA was precipitated by adding 2.5µl 4M LiCl and 75µl pre-chilled ethanol and mixing well. It was then left for 30 minutes at 70°C before spinning in a centrifuge for 15 minutes. The supernatant was removed and the pellet washed with 50µl cold 70% ethanol. The DNA pellet was then briefly vacuum dried and dissolved in 50µl TE buffer (Boehringer Mannheim Biochemica, 1993a).

2.2.5.1.5. Prehybridization and Hybridization.

The nylon membrane was placed in a roller tube containing 20ml standard prehybridization solution (Appendix V) and prehybridized at 68°C for 4 hours. The prehybridization solution was then discarded and hybridization solution (Appendix V) containing the DIG-labelled probe was added and the probe allowed to hybridize at 68°C overnight (Boehringer Mannheim Biochemica, 1993b).

Following the overnight hybridization, the membrane was removed from the roller tube and placed in a plastic lunch box. Stringency washes were then carried out. This involved two 5 minute washes in 2 x SSC, 0.1% SDS (Appendix V) at room temperature and two 15 minute washes in 0.1 SSC, 0.1% SDS (Appendix V) at 68°C, under constant agitation on a rotashaker (Boehringer Mannheim Biochemica, 1993b).

2.2.5.1.6. Detection of DIG-Labelled Probe.

The membrane was rinsed briefly (1-5 minutes) in maleic acid buffer (buffer 1 - Appendix V) before incubating for 30 minutes in 100ml blocking solution (buffer 2, 1 x concentration - Appendix V). Anti-DIG-AP conjugate was diluted in buffer 2 to 75mU/ml (1:10000) in blocking solution (1x concentration) and the membrane was incubated for 30 minutes in 20ml of this antibody solution. Unbound antibody was removed by washing twice with 100ml maleic acid buffer (buffer1). Equilibration was carried out for 2-5 minutes in 20ml detection buffer

(buffer 3 - Appendix V). The membrane was then incubated for 5 minutes in 10ml freshly prepared colour substrate solution (Appendix V), sealed in a box in the dark. Colour precipitate started to form within a few minutes and was complete after 12-16 hours. The reaction was stopped by washing the membrane with 50ml distilled water for 5 minutes and the membrane was photographed for documentation (Boehringer Mannheim Biochemica, 1993c).

2.2.5.2. Itraconazole Sensitivity Testing.

The sensitivity to itraconazole of all the *C. albicans* species isolated at baseline was measured by a broth microdilution assay. The buffers and stock solutions required for this method were made up as described in Appendix V.

C. albicans isolates taken from each patient at baseline were removed from storage at -70°C. Each isolate was plated onto Sabouraud's dextrose agar and incubated at 37°C for 48 hours.

Following incubation, five colonies were taken from the purity plate, emulsified in 5ml sterile distilled water and diluted to a concentration of 1 in 10 by adding 0.5 ml of this suspension to 4.5ml RPMI broth (Appendix V). This suspension was used for the yeast counts using a Neubauer haemocytometer.

A multichannel pipette with a setting of 100µl was used to add RPMI broth to columns one and three, through to column 12, of a 96-well flat-bottomed microtitre plate (Alpha Laboratories). Column two of the microtitre plate was left empty. One hundred microlitres of the stock itraconazole solution (32 mg/l -

Appendix V) was added to columns one, two and three. Again using the multichannel pipette, 100µl was transferred from column three to column four and this step was repeated from column four to column five and so on, up to column 11 (medium control). Lastly, 100µl of the yeast suspension was added to all wells except column one (inoculum free control). A control strain (*C.glabrata* NCPF 3931) was set up in row one of every microtitre plate. The control strain was kept refrigerated on Sabouraud's dextrose agar. Single colonies were subcultured every two weeks. The microtitre plate was incubated at 28°C for 48 hours.

An ELISA multiscan plate reader was used to interpret the results. It was set to 450nm and the blank was B1. The MIC was the lowest drug concentration at which the growth of the organism was reduced by 50% of the drug free control well (column 12).

2.3. STATISTICAL ANALYSIS.

The data were analysed using the Minitab Statistical Programme, Release 11 for Windows.

It was decided that a level of significance of $p < 0.05$ would be appropriate. Parametric statistical tests, such as two-sample-t-tests, were judged to be suitable for both within group and between group comparisons. Treatment effects of each formulation of itraconazole and the effect of time were analysed by Repeated Measures Analysis of Variance. Analysis of any changes between baseline and subsequent visits, for variables such as mean *Erythema meter* indices, was by Analysis of Covariance. *Chi*-squared tests were used to compare the mycological response of trial participants who received new dentures with those who did not and to compare the frequency of zero and non-zero saliva itraconazole concentrations in each treatment group.

Other analyses were made on a purely descriptive basis.

CHAPTER THREE

RESULTS

3.1. RESULTS OF PRELIMINARY INVESTIGATIONS.

3.1.1. Evaluation of the Erythema Meter .

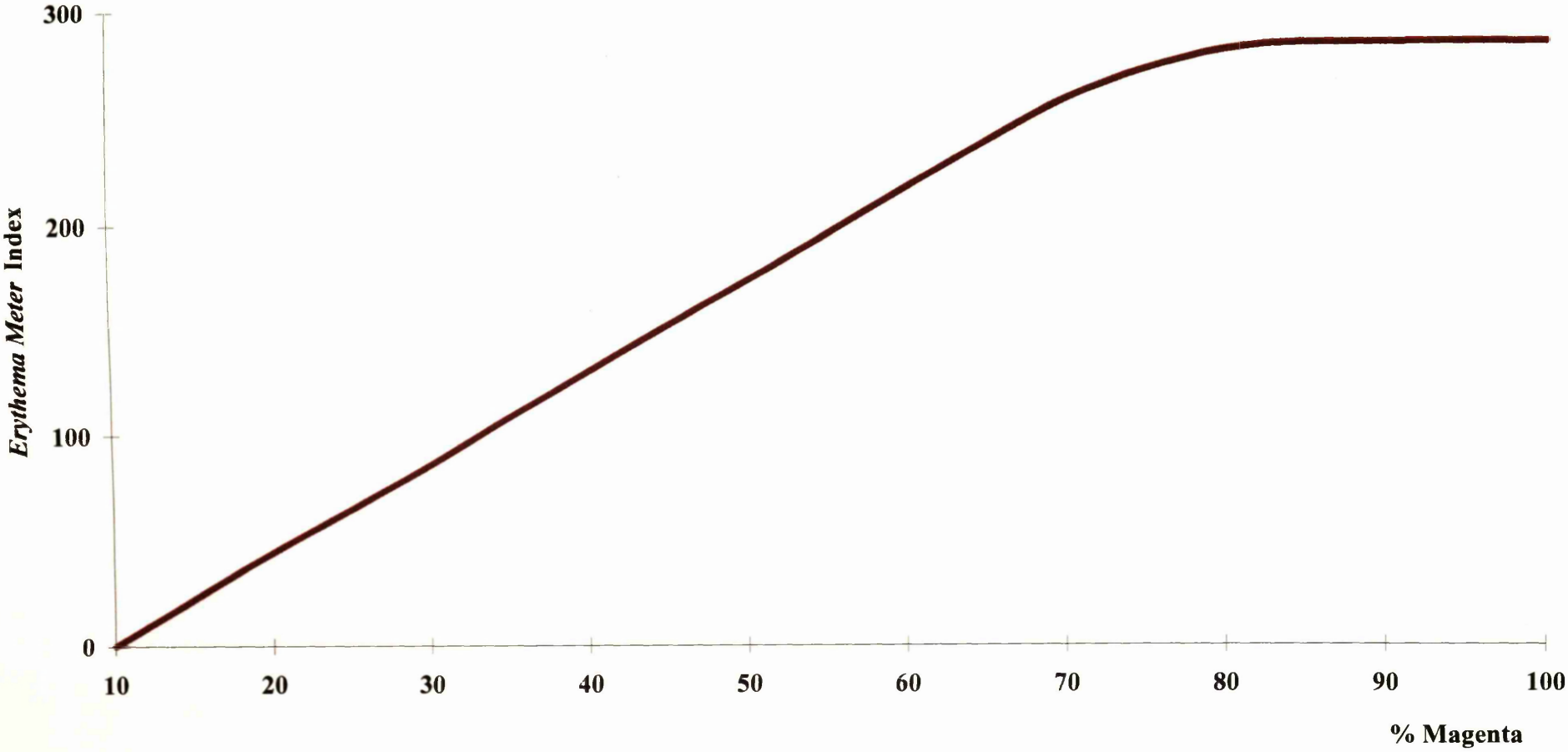
3.1.1.1. Laboratory Study.

Table 3.1 shows the mean erythema indices as they corresponded to the varying percentages of magenta in the colour card standards. These standards gave a range of erythema index readings from 44 for the palest pink strip (20% magenta) to 285 for the darkest red strip (100% magenta). Fig. 3.1 is a graphical representation of the same data and shows that the erythema index increases steadily by about 40-50 units each time the proportion of magenta increases by 10%. This steady proportional rise continues until the *Erythema meter* records an index of 270, after which the graph levels off.

Table 3.1. **Non-clinical erythema indices corresponding to the percentage of magenta in the colour card standards.**

Percentage of magenta	Erythema meter index
20	44
30	86
40	123
50	174
60	210
70	258
80	280
90	281
100	285

Figure 3.1. The mean *Erythema meter* indices corresponding to varying percentages of magenta in the colour card standards.



3.1.1.2. Clinical Study.

The mean age of dentures worn in each of the denture-wearing groups was seven years. In general, maxillary dentures were retentive but ill-fitting. The most common form of denture stomatitis, seen in 14 of the patients, was Newton's Type II. One patient had Newton's Type I and 5 patients had Newton's Type III denture stomatitis. At the initial visit, 11 of 20 denture wearers with healthy palatal mucosa and four of 20 denture stomatitis patients admitted removing their dentures for sleeping.

For the 40 patients with healthy palatal mucosa, the clinical erythema scores ranged from 0 (no inflammation) to 1 (mild inflammation), whereas the scores for the denture stomatitis patients ranged from 2 (moderate inflammation) to 3 (severe inflammation) (Table 3.2).

Table 3.2. **Clinical visual erythema scores for patients with healthy palatal mucosa and those with denture stomatitis.**

Clinical erythema score	Healthy palatal mucosa (n=40)	Denture stomatitis (n=20)
0	35	0
1	5	3
2	0	10
3	0	7

Table 3.3 shows the median erythema index derived from the *Erythema meter* readings for all patients at the initial visit. The 40 patients with healthy mucosa were re-assessed after two weeks. The mean erythema indices of each patient, at the initial and the follow-up visits, are plotted in Fig. 3.2. The points lie on or very close to the line of equality in the majority of cases, having changed very little from one visit to the next. A components of variance analysis allowed the standard variation of the erythema index across patients to be estimated at 8.8, while the standard deviation within patients was 3.2. This gave a reproducibility coefficient of 88%.

Table 3.3. **Median erythema indices and range recorded at initial visit.**

	n	Median erythema index	Range
Healthy non-denture wearers	20	12	2-38
Healthy denture wearers	20	5	0-29
Denture stomatitis patients	20	95	36-117

To compare the objectivity of the *Erythema meter* with the more subjective visual erythema scores, the latter were plotted against the mean erythema index for each patient at that visit (Fig. 3.3). Visual erythema scores of zero (no inflammation) corresponded to low erythema indices of less than 20, whilst scores of 3 (severe inflammation) corresponded to high erythema indices of greater than 100. There was some overlap when comparing clinical erythema scores of 1 (mild inflammation) and 2 (moderate inflammation) with the corresponding erythema index for that patient.

Figure 3.2. A comparison of the mean erythema indices of 40 patients with healthy palatal mucosa at the initial and follow-up visits.

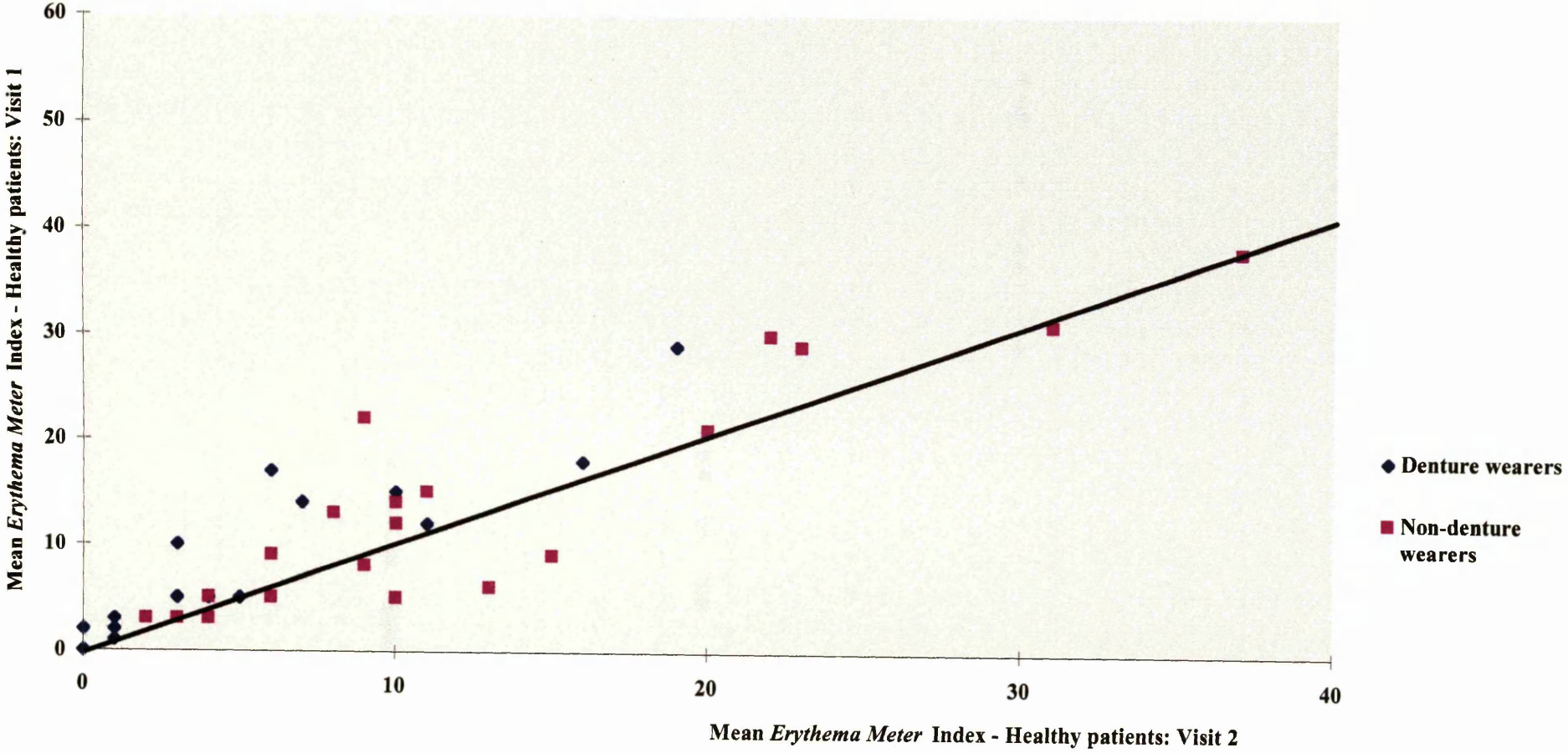
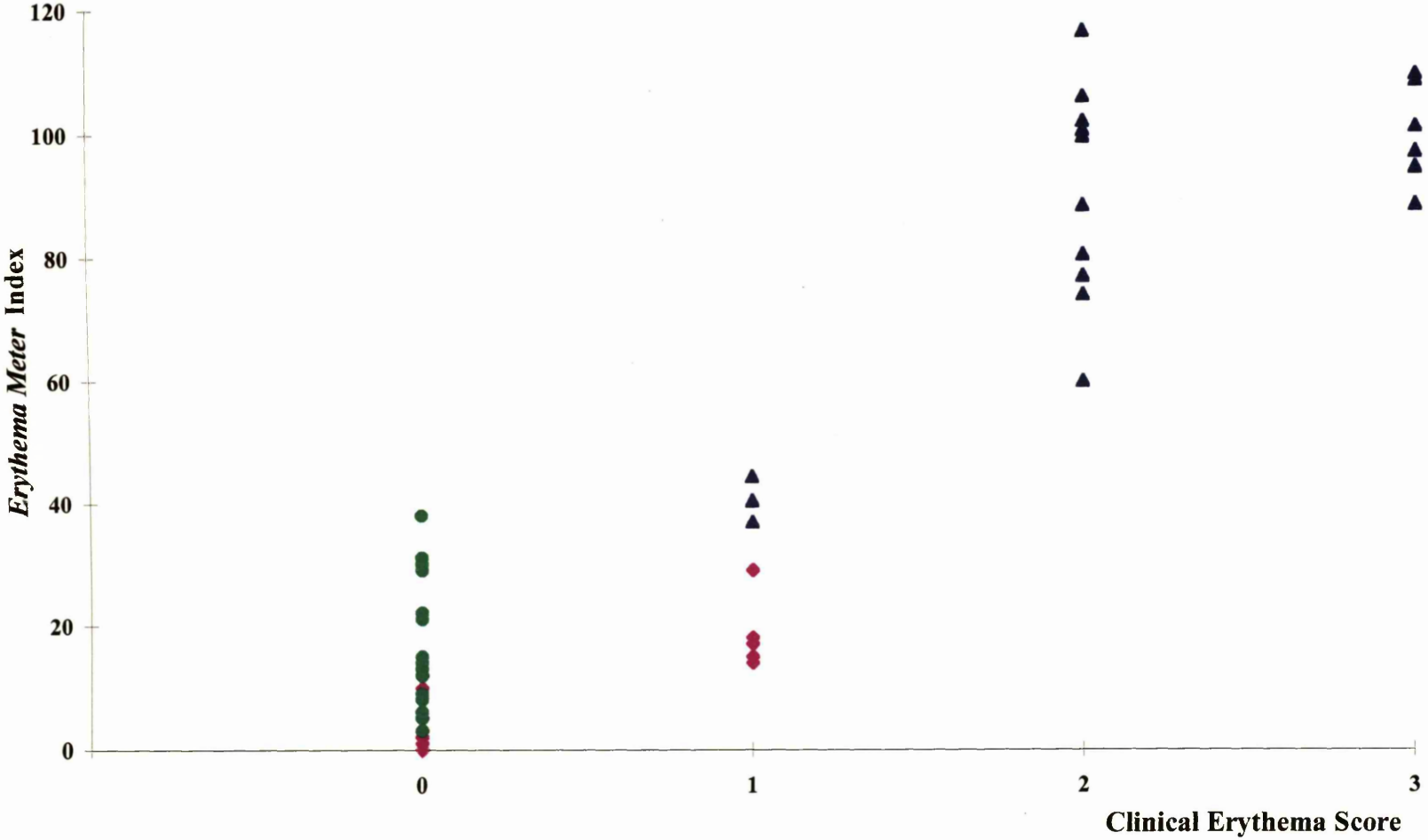


Figure 3.3. A comparison of the objective *Erythema meter* indices recorded at the initial visit with the more subjective visual clinical erythema score. Triangle: denture stomatitis; diamond: healthy denture wearers; circle: healthy non-denture wearers.



3.1.2. A Pilot Study Comparing Fluconazole and Itraconazole in the Treatment of Denture Stomatitis.

3.1.2.1. Clinical Investigations.

Newton's Type II denture stomatitis affected seven patients in both the itraconazole and the fluconazole groups respectively. One patient in the fluconazole group had Newton's Type I denture stomatitis and 5 patients in total had Type III denture stomatitis, three in the itraconazole group and two in the fluconazole group.

The median erythema indices recorded at baseline and on completion of the relevant course of antifungal treatment are given in Table 3.4. There was a reduction in erythema with both fluconazole and itraconazole. While this difference appears greater for the fluconazole group, the median score at the beginning of the study was lower for those on the itraconazole arm, with correspondingly less room for improvement with treatment. *Erythema meter* scores of between zero and 38 have been recorded for those with healthy mucosa (Table 3.3).

Table 3.4. **Median erythema indices before and after antifungal treatment.**

	Before	After	Difference	Range of differences
Fluconazole (n=10)	100	48	52	9-71
Itraconazole (n=10)	74	43	31	7-57

The mean erythema indices for each patient, at the initial and the follow-up visits, have been plotted for those in the fluconazole and itraconazole groups in figure 3.4. In most cases the points plotted lie above the line of equality, indicating a reduction in erythema following the course of antifungal therapy. One patient in the fluconazole group and two in the itraconazole group showed little change in their erythema indices between visits.

At the initial visit, only four of the 20 patients admitted removing their dentures for sleeping. However, all patients responded to the advice given and by visit 2 were removing their dentures at night for sleeping. However, analysis of the clinical visual denture cleanliness scores revealed a variable picture (Figs. 3.5 and 3.6). In the fluconazole group, four patients showed improvement in their denture cleanliness scores, with the scores reducing from one or three to zero in all four cases. The remaining patients had the same amount of plaque present on their denture fitting surface from one visit to the next, except for one patient whose score increased at the review. In the itraconazole group, five patients showed varying degrees of improvement in their denture cleanliness scores, with the remaining five patients showing no change. Results from the objective denture disc method were in agreement with the visual scores and are described in Section 3.1.2.2.

Figure 3.4. The mean *Erythema meter* indices for each patient in the fluconazole and itraconazole groups at the initial and follow-up visits. Points above the line of equality indicate a reduction in erythema and therefore clinical improvement.

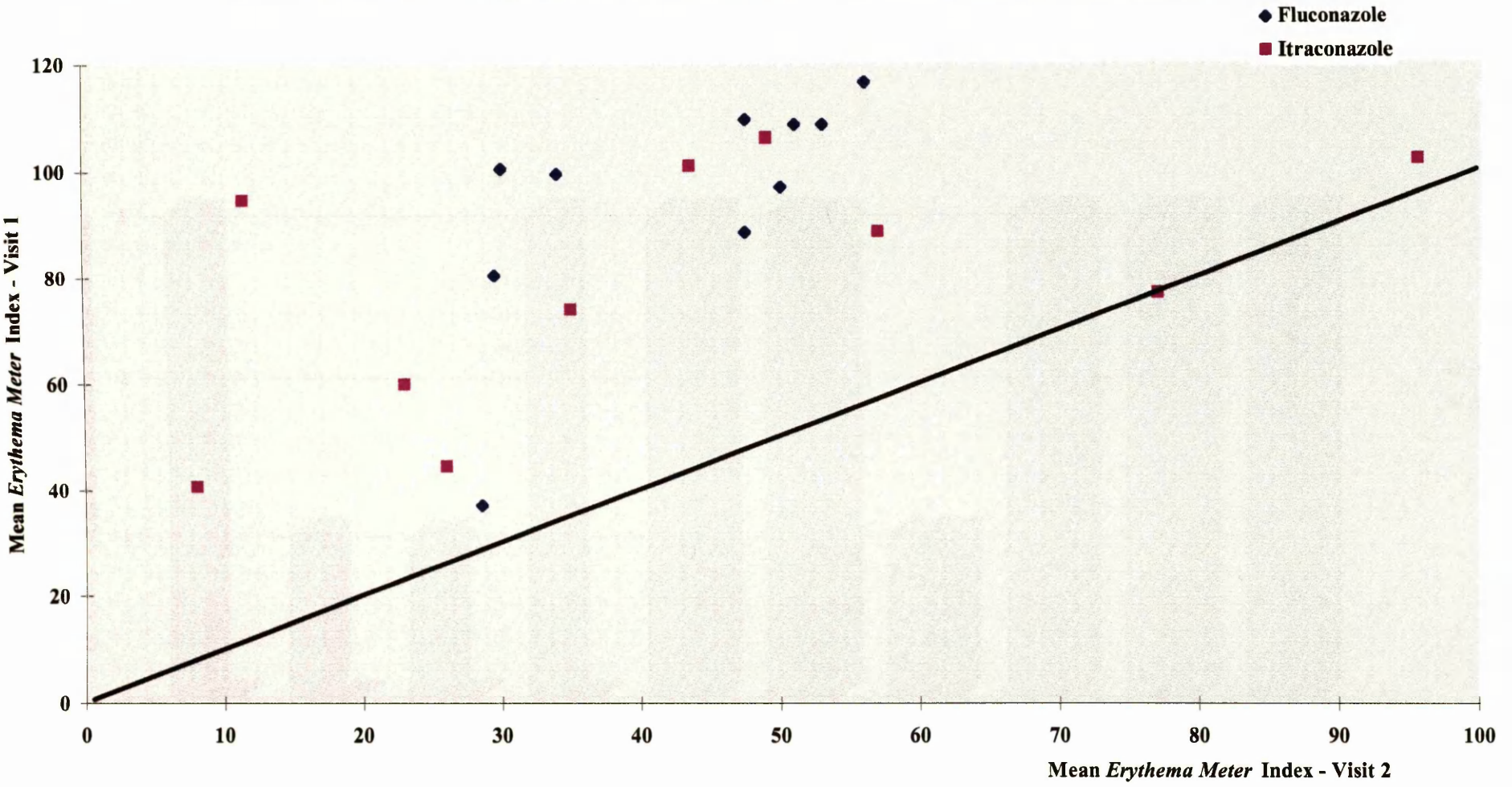


Figure 3.5. Clinical visual denture cleanliness scores for patients in the fluconazole group before and after treatment.

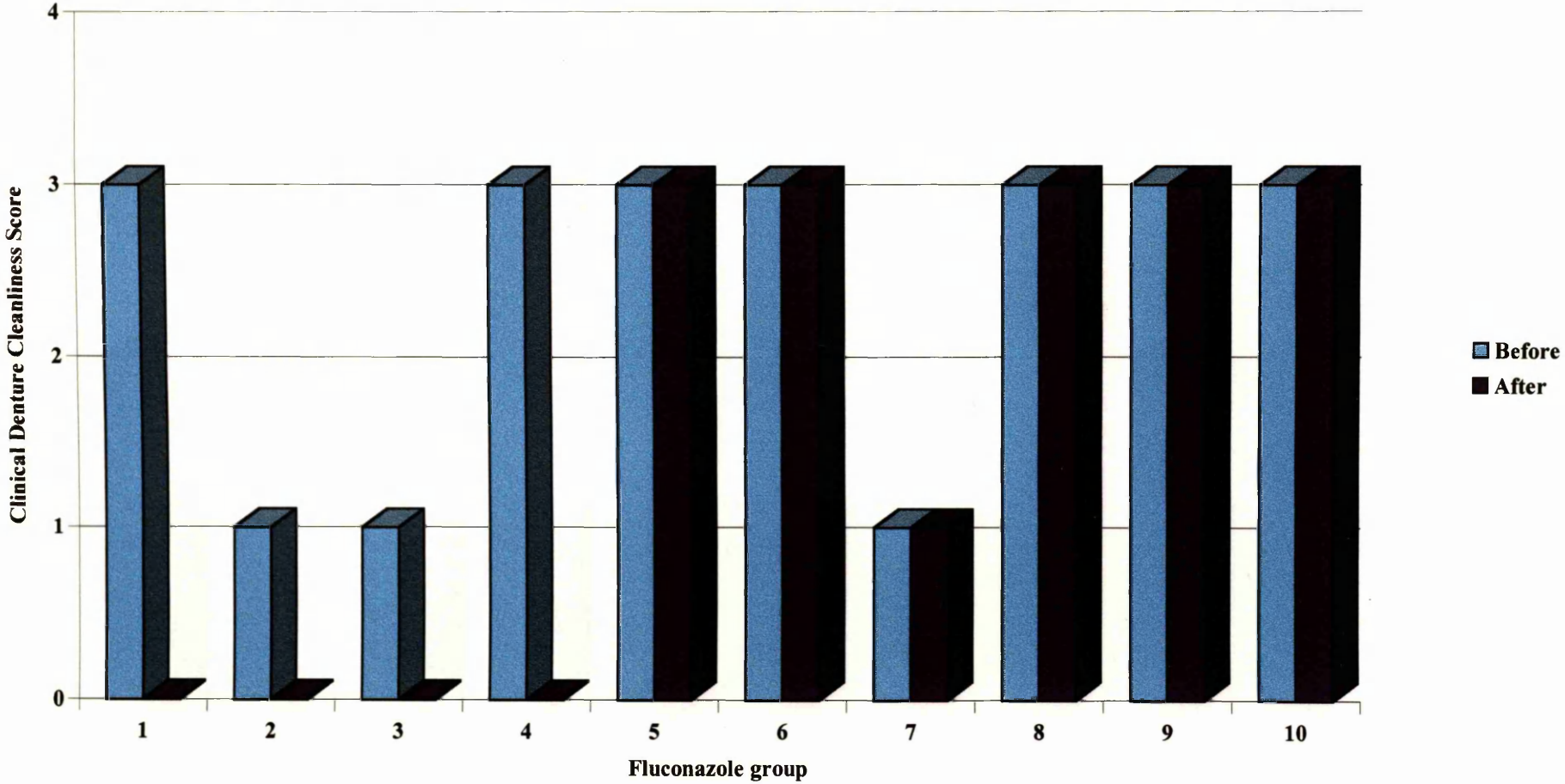
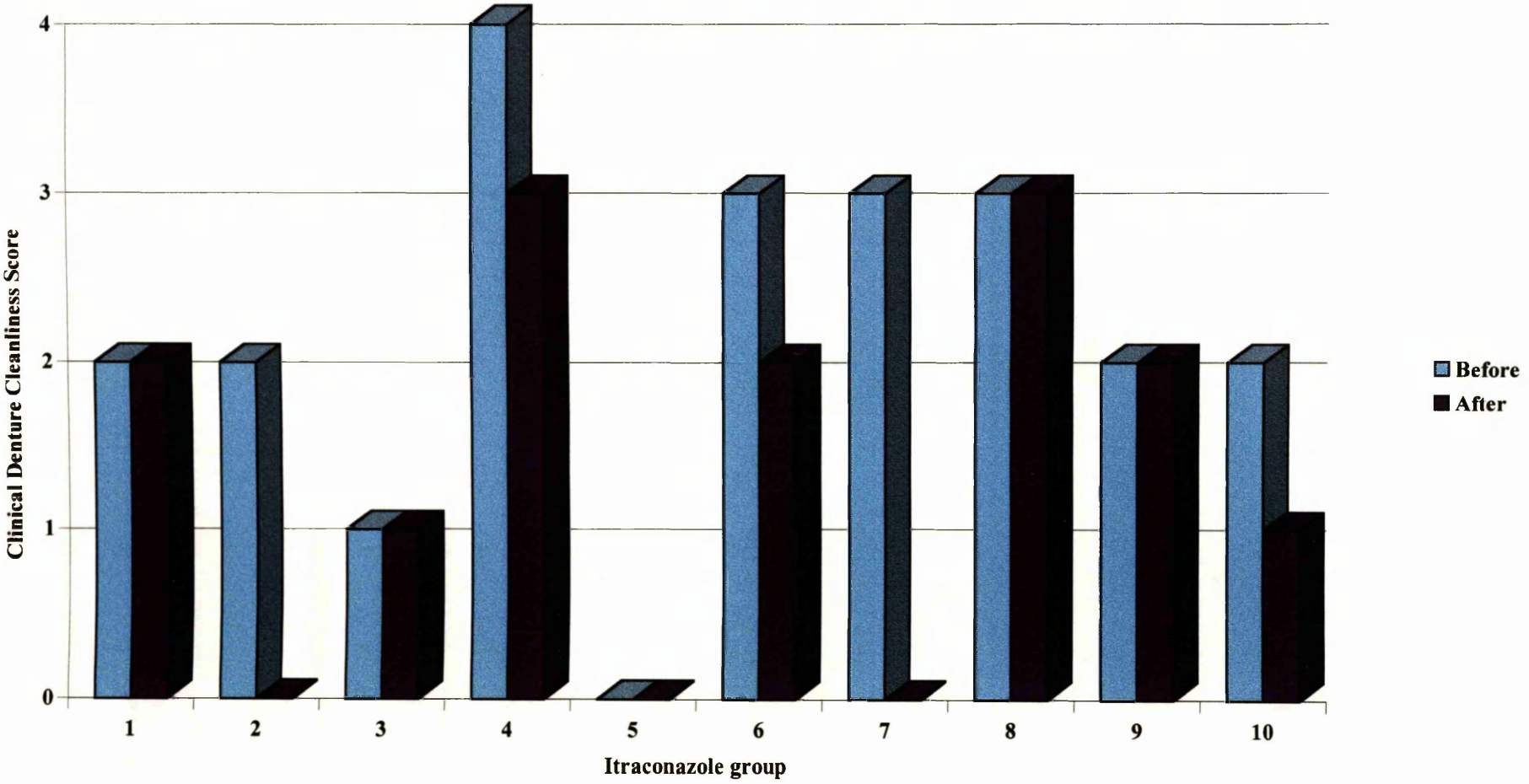


Figure 3.6. Clinical visual denture cleanliness scores for patients in the itraconazole group before and after treatment.



3.1.2.2. Microbiological Investigations.

A wide range of yeast species was isolated from the mouths of the denture stomatitis patients before treatment (Table 3.5). *C. albicans* was the most common isolate, present in samples from all 20 patients, with *C. glabrata* the second most commonly isolated species .

Table 3.5. **The species of yeast isolated from patients with denture stomatitis before treatment.**

Study group	***Species isolated***					
	C. albicans	*C. glabrata*	*C. tropicalis*	*C. parapsilosis*	*Sacch. cerevisiae*	*C. guillermondii*
Fluconazole (n = 10)	10	5	1	1	2	0
Itraconazole (n = 10)	10	1	1	0	1	1
Total	20	6	2	1	3	1

Following antifungal therapy, patients were classified as having responded to treatment if they were mycologically cured or if there was a reduction in yeast count (Table 3.6). A total mycological cure was achieved in five of the 20 patients, one in the fluconazole group and four in the itraconazole group. Eight of the 10 patients in the fluconazole group had reduced yeast counts by the second visit. A reduction in yeast count also occurred in three of the 10 patients treated with itraconazole. However, one patient in the fluconazole group and three in the itraconazole group had increased yeast counts after treatment. *C. albicans* was eradicated completely in four patients after treatment with fluconazole and in six

patients after treatment with itraconazole. When *C. glabrata* was one of the original yeast species it persisted, in all cases, after antifungal therapy.

Table 3.6. **The mycological response to treatment in each patient group.**

	Fluconazole (n = 10)	**Itraconazole (n = 10)**
Mycological cure	1	4
Reduced yeast count	8	3
Increased yeast count	1	3

Coliforms were isolated from seven patients, four in the fluconazole group and three in the itraconazole group. *S. aureus* was isolated from one patient and Group B ß haemolytic streptococci from another in the itraconazole group. No further investigations were carried out on these organisms.

The objective microbiological 'denture cleanliness scores' measured before and after the two week course of antifungal medication are illustrated in Figs. 3.7 and 3.8. These data indicate that denture cleanliness did not improve for the majority of the participants.

Figure 3.7. The objective microbiological denture cleanliness scores (log_{10} of total anaerobic count) for patients in the fluconazole group before and after treatment.

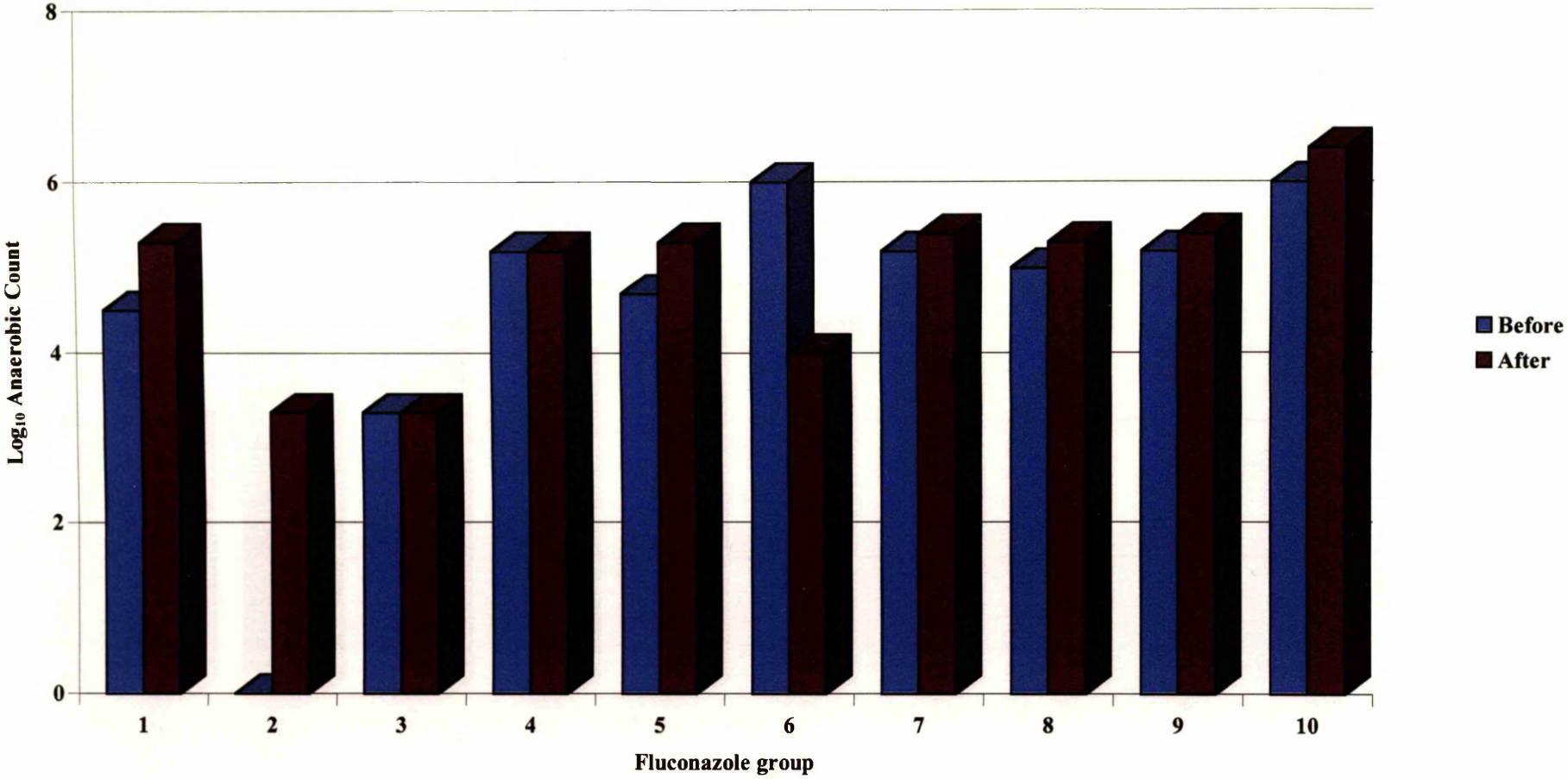
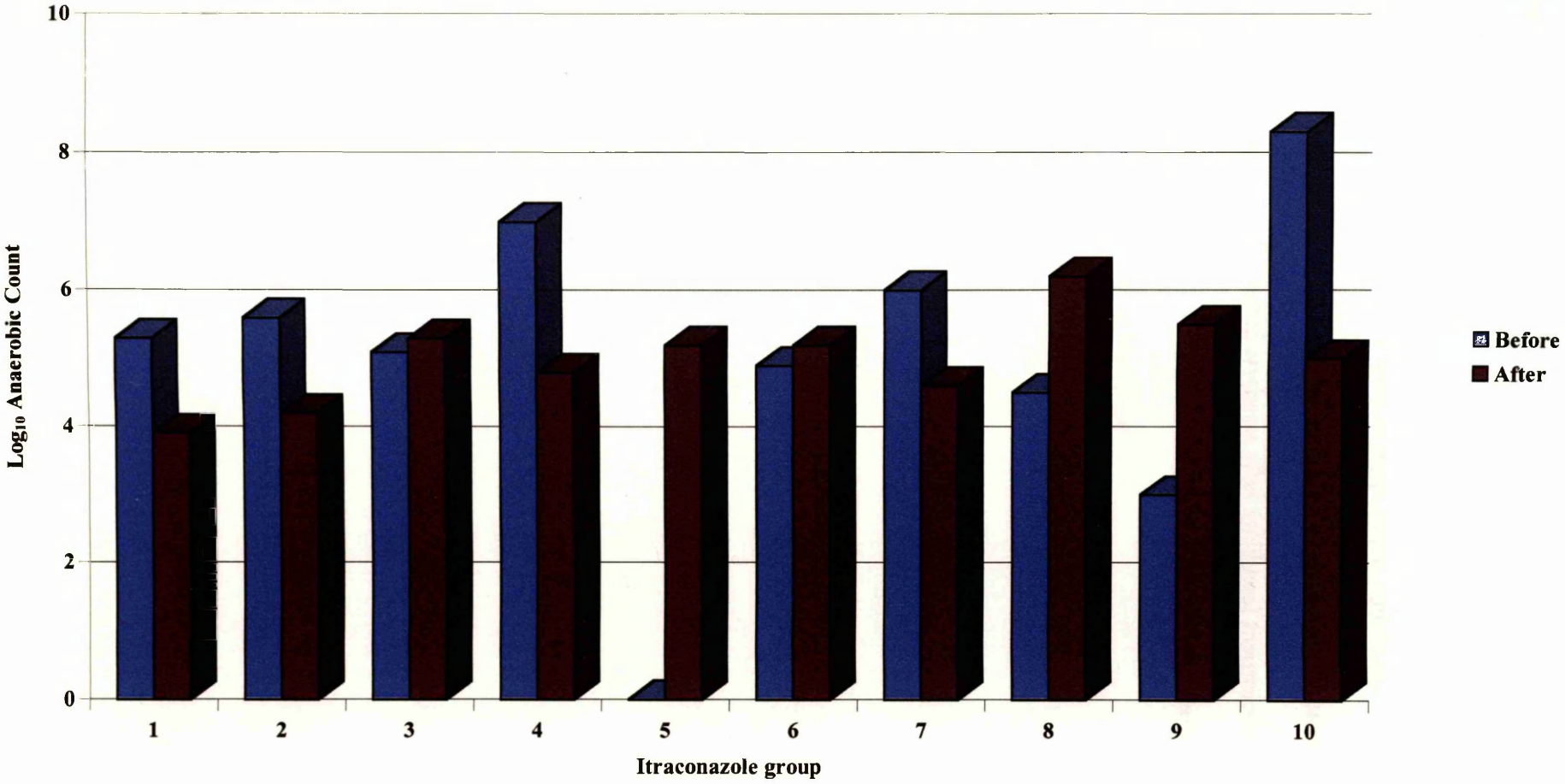


Figure 3.8. The objective microbiological denture cleanliness scores (log_{10} of total anaerobic count) for patients in the itraconazole group before and after treatment.



3.1.2.3. Haematological Investigations.

Six patients, three patients in each treatment group, were iron-deficient (serum ferritin <25ng/ml) at baseline and did not receive replacement iron therapy until after their antifungal treatment was completed. Despite this underlying haematinic deficiency, all six demonstrated reduced erythema after antifungal treatment and although none of these patients were mycologically cured, four had reduced yeast counts and had therefore responded to treatment.

3.2. RESULTS OF A CLINICAL TRIAL COMPARING THE EFFICACY OF CYCLODEXTRIN SOLUTION OF ITRACONAZOLE AND ITRACONAZOLE CAPSULES IN THE MANAGEMENT OF *CANDIDA*-ASSOCIATED DENTURE STOMATITIS.

3.2.1. Clinical Investigations.

The mean age of the patients' dentures was 9.9 years (range 0.2-27 years) in the cyclodextrin group and 10.5 years (range 0.3-30 years) in the capsule group. Sixty-two per cent of maxillary dentures in this study had retentive bases but 52% were unstable laterally or antero-posteriorly. Twenty-two per cent had under-extended bases and 12% had worn occlusal surfaces. The most common form of denture stomatitis seen was Newton's Type II, which affected seventeen patients in both the cyclodextrin and capsule groups respectively. Six patients in total had Newton's Type III denture stomatitis, three in each group. Type I denture stomatitis was not found in this study population. The majority of patients, 32 of 40 enrolled in the clinical trial, had attended the Dental Hospital for the provision of new complete dentures and were unaware of their palatal inflammation prior to the screening appointment. A further five of the 40 patients enrolled at baseline complained of oral dryness and three complained of a sore, burning tongue.

Two patients in the cyclodextrin group and two in the capsule group failed to complete the course of itraconazole. These patients were excluded from all efficacy analyses. Another two patients in the capsule group each missed one appointment during the trial - one at four weeks and the other at six months after

treatment commenced. However, results obtained from these patients were included in the efficacy analyses.

The mean reduction in *Erythema meter* indices recorded at each visit following antifungal therapy are given in Table 3.7. Using Repeated Measures Analysis of Variance on the reductions in mean *Erythema Meter* index from baseline, a significant reduction in erythema was found ($p = 0.002$) but there was no significant ($p = 0.31$) treatment effect with respect to the visit at which the mean *Erythema Meter* index was recorded. The cyclodextrin solution of itraconazole resulted in a constant additional reduction in palatal erythema over itraconazole capsules of an average of 10.1 units (95% confidence interval of 3.6, 16.4).

Table 3.7. **Mean reductions in *Erythema meter* indices following antifungal therapy.**

	Mean Reduction in Erythema Index (range)		
Visit	*2*	*3*	*4*
Cyclodextrin group	43.9 (6-93)	54.7 (26-100)	54.6 (-4-136)
	S.D. 24.6	*S.D. 22.6*	*S.D. 38.9*
	n = 18	n = 18	n = 18
Capsules group	36.6 (20-74)	36.1 (-30-77)	50.6 (-11-91)
	S.D. 16.8	*S.D. 27.3*	*S.D. 26.5*
	n = 18	n = 17	n = 17

The mean *Erythema meter* indices for each patient, at each visit, have been plotted for those in the cyclodextrin and capsule groups (Figs. 3.9 and 3.10).

Figure 3.9. Mean *Erythema Meter* Indices (EMI) across visits for patients in the cyclodextrin itraconazole group.

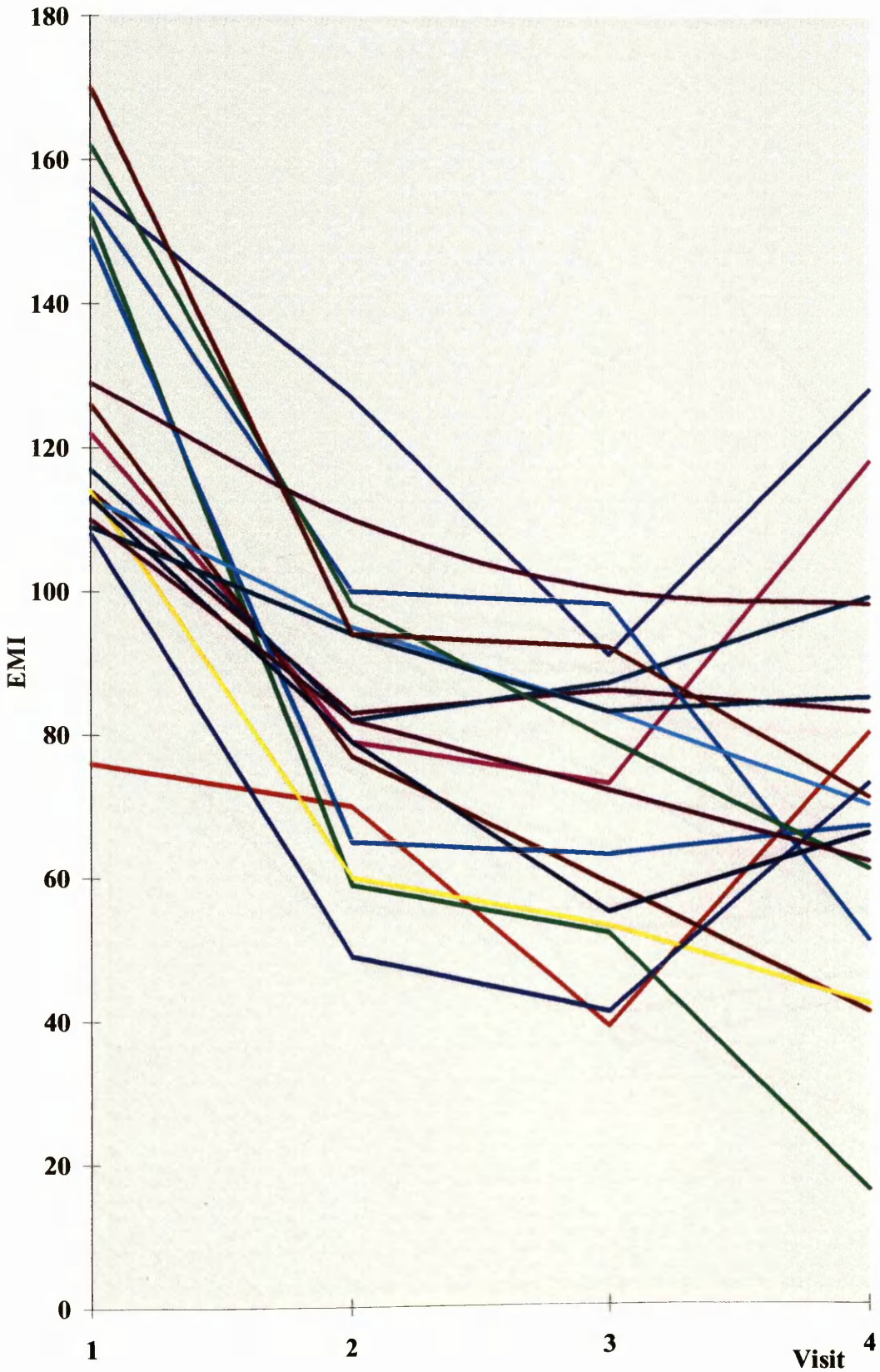
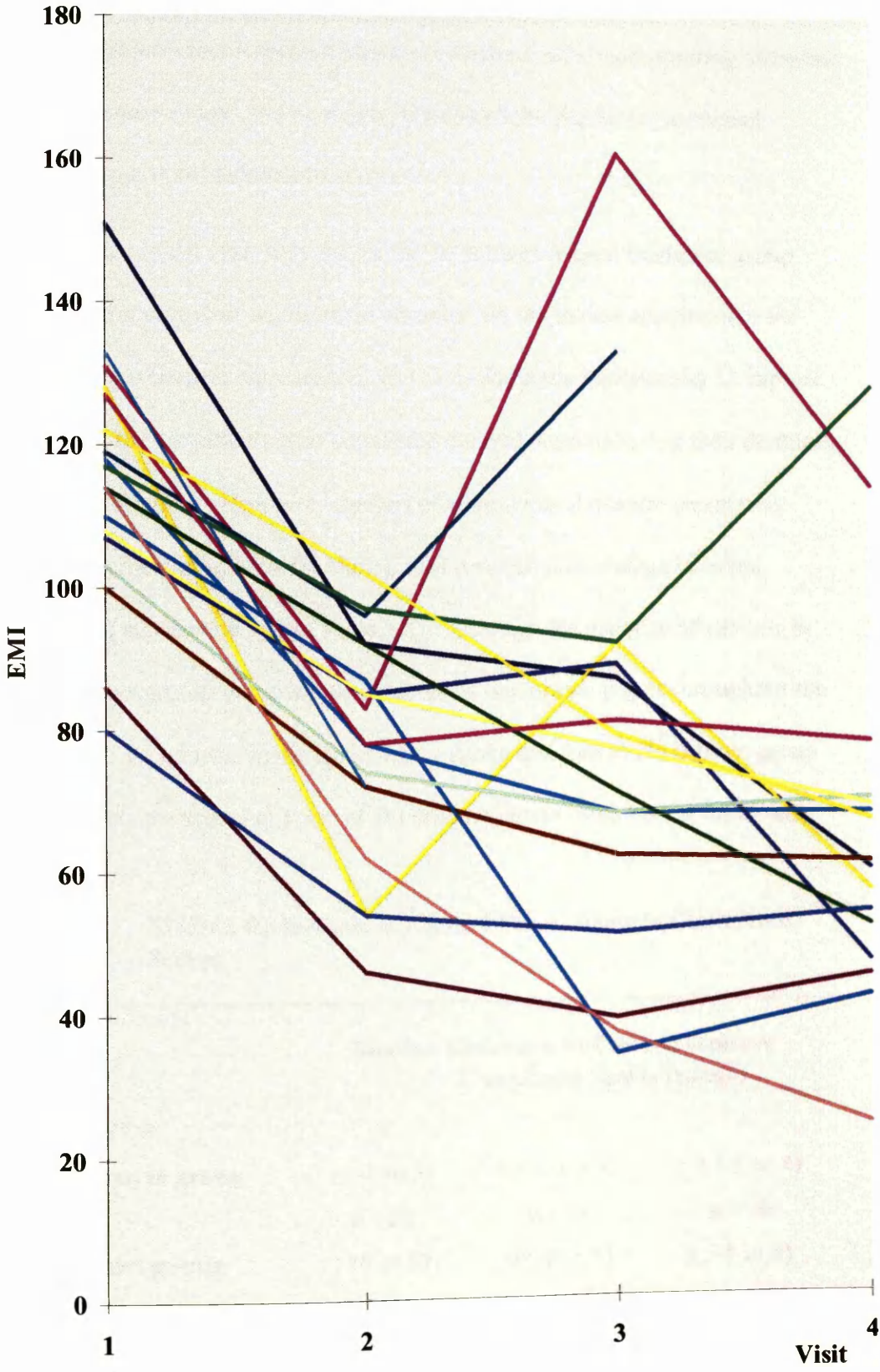


Figure 3.10. Mean *Erythema Meter* Indices (EMI) across visits for patients in the itraconazole capsule group.



Patients in the cyclodextrin group had a higher mean level of erythema at baseline than those in the capsule group. Consequently, when calculations were adjusted for the higher pre-treatment *Erythema meter* indices of the cyclodextrin group, the appropriate Repeated Measures Analysis, now incorporating 'Baseline *Erythema Meter* Index' as a covariate, eliminated the significant treatment difference ($p = 0.38$) referred to above.

At the initial visit, only five of the 20 patients in each treatment group admitted removing their dentures for sleeping. By the review appointment six months after treatment commenced, 25 (13 cyclodextrin patients and 12 capsule patients) of the 36 patients who completed the trial were removing their dentures at night for sleeping. However, analysis of clinical visual denture cleanliness scores recorded throughout the clinical trial revealed little change between baseline and subsequent visits (Table 3.8). Although the majority of patients in each treatment group improved their scores by one or two points throughout the clinical trial, six patients in the cyclodextrin group and four in the capsule group had higher plaque scores at some of the post-treatment visits than at enrolment.

Table 3.8. **Median Reductions in Clinical Visual Denture Cleanliness Scores.**

	Median Reduction in Clinical Denture Cleanliness Scores (range)		
Visit	*2*	*3*	*4*
Cyclodextrin group	1 (-1 to 3)	1.5 (-2 to 4)	1.5 (-2 to 4)
	n =18	n =18	n =18
Capsules group	1 (0 to 3)	0 (-2 to 3)	2 (-1 to 4)
	n =18	n = 17	n = 17

Six patients in the cyclodextrin group and nine in the capsule group received new dentures whilst enrolled in the clinical trial. These dentures were provided between four weeks and six months after treatment commenced. Table 3.9 summarises the mycological response of trial participants, six months after treatment commenced, in relation to whether they received new dentures or not whilst enrolled in the study. From this table it can be seen that there is some evidence of a higher rate of mycological cure in those patients who did receive new dentures, in contrast to those who continued to wear their existing dentures.

Table 3.9 **The mycological response six months after treatment commenced of patients in each treatment group in relation to the provision of new complete dentures.**

		New dentures	No new dentures	p-value
Cyclodextrin group	**mycologically cured**	3	1	
	not mycologically cured	3	11	**0.131**
Capsule group	**mycologically cured**	6	1	
	not mycologically cured	3	7	**0.137**

When the mycological results for both treatment groups were pooled, a *Chi*-squared test of these data revealed a significantly higher chance of a patient in this study remaining mycologically cured six months after treatment commenced if new dentures were provided following antifungal therapy ($p = 0.020$) (Table 3.10).

Table 3.10. **A comparison of the mycological cure rates of patients who received new dentures and those who did not, irrespective of treatment group, using a *Chi*-squared test (p = 0.020).**

	New dentures	No New dentures	Total
Mycologically cured	9	2	11
Not mycologically cured	6	18	24
Total	15	20	35

3.2.2. Microbiological Investigations.

A wide range of yeast species was isolated from the mouths of the denture stomatitis patients before treatment (Table 3.11). *C. albicans* was the most common isolate, present in samples from all 40 patients. *C. glabrata* was the second most commonly isolated species. It was found in 12 patients at baseline and persisted, post-treatment, in all but two of these cases - one from each treatment group.

Table 3.11. **The species of yeast isolated from patients with denture stomatitis before treatment.**

Study group	***Species isolated***						
	C. albicans	*C. glabrata*	*C. tropicalis*	*C. krusei*	*C. parapsilosis*	*Sacch. cerevisiae*	*C. guillermondii*
Cyclodextrin (n=18)	20	5	1	0	0	0	0
Capsules (n=18)	20	7	0	1	1	3	1
Total	40	12	1	1	1	3	1

Following antifungal therapy, patients were classified as having responded to treatment if they were mycologically cured or if there was a reduction in yeast count (Table 3.12). A total mycological cure was achieved in 17 of the 36 patients on completion of treatment (Visit 2). Four weeks after treatment commenced, of the 17 mycologically cured patients, 13 (eight in the cyclodextrin group and five in the capsule group) remained yeast free and seven of these patients (three cyclodextrin and four capsule) were still mycologically cured six months after treatment commenced. Reduced yeast counts were found, on completion of treatment, in seven patients in the cyclodextrin group and 11 in the capsule group. Four weeks after treatment commenced, four cyclodextrin patients and eight capsule patients had yeast counts lower than they had been at baseline and the same was found in eight cyclodextrin patients and seven capsule patients six months after treatment started. No patients had an increased yeast count on completion of the two week course of antifungal therapy, suggesting good patient compliance with both preparations of itraconazole. However, some patients, in each of the treatment groups, had higher yeast counts by four weeks and six months after treatment started than had been found prior to treatment (Table 3.12).

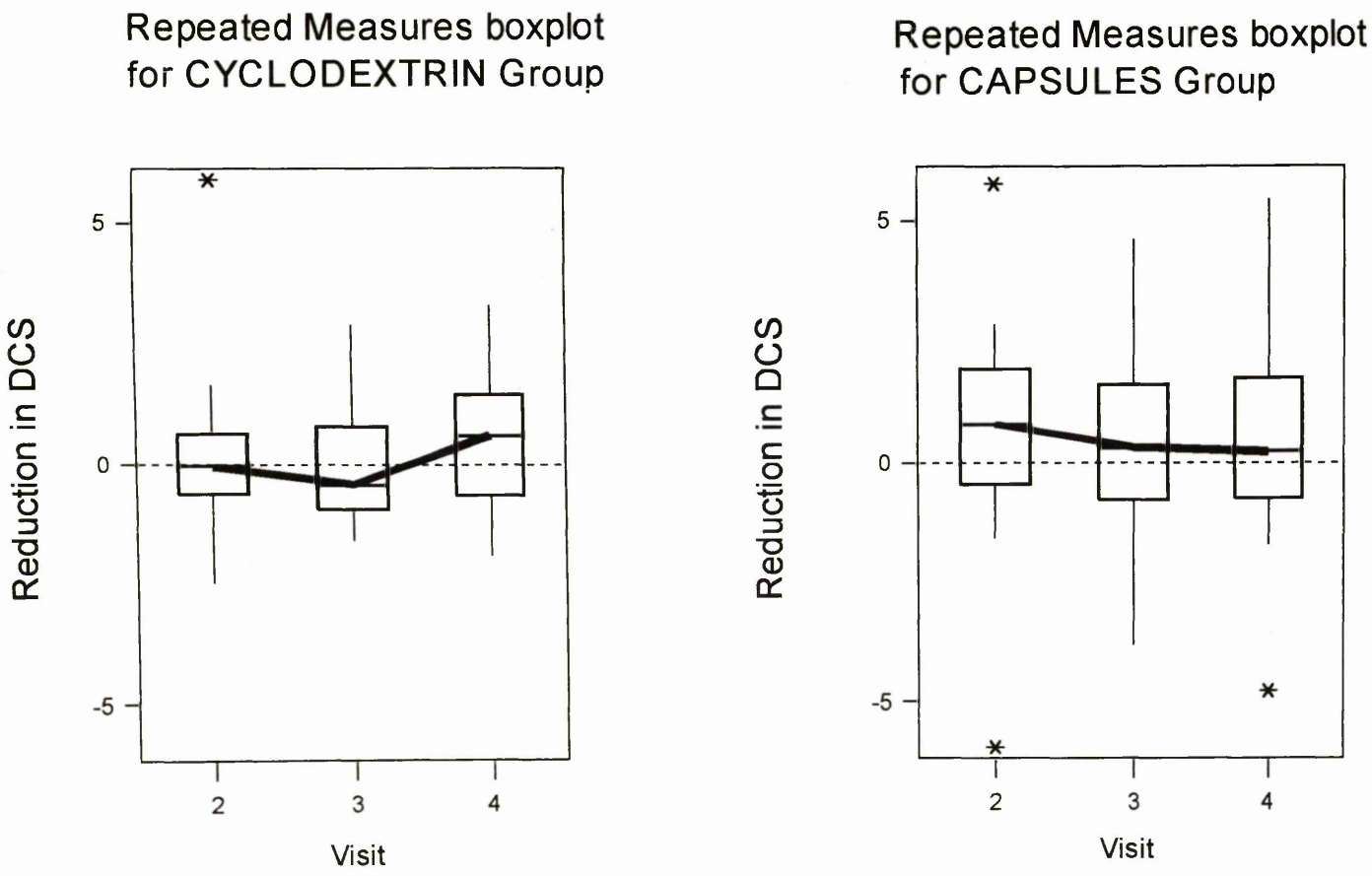
Table 3.12. **The mycological response to treatment in each patient group.**

	Cyclodextrin group			Capsule group		
Visit	*2* n = 18	*3* n = 18	*4* n = 18	*2* n = 18	*3* n = 17	*4* n = 17
Mycological cure	10	11	4	7	6	8
Reduced yeast count	7	4	8	11	8	7
Increased yeast count	0	3	6	0	3	2
No change	1	0	0	0	0	0

Coliforms were isolated from 16 of 36 patients who completed the clinical trial - seven in the cyclodextrin group and nine in the capsule group. *S. aureus* was isolated from only two patients throughout the trial. Following identification of these organisms, the number of colonies in each sample was noted but no further microbiological investigations were carried out. No ß haemolytic streptococci were isolated from this patient population.

Repeated Measures Analyses of the median reductions in objective microbiological 'denture cleanliness scores' measured from baseline for each treatment group are illustrated in figure 3.11. These data indicate that denture cleanliness in both treatment groups improved very slightly but significantly ($p = 0.01$), by an average value of 0.3 (95% confidence interval of 0.1, 0.5).

Figure 3.11. Box plots of reductions in microbiological denture cleanliness scores (ie. DCS = $\log_{10}$ of total anaerobic count) across visits.



3.2.3. Haematological Investigations.

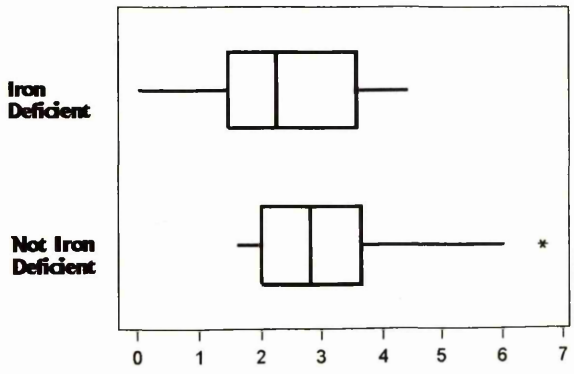
Eight patients, three in the cyclodextrin group and five in the capsule group, were iron-deficient (serum ferritin <25ng/ml) at baseline. All received appropriate investigation and replacement iron therapy and only one patient remained iron-deficient six months after treatment commenced. Despite this underlying haematinic deficiency at baseline, all eight demonstrated reduced erythema after antifungal treatment and three (two patients on cyclodextrin solution of itraconazole and one on itraconazole capsules) were mycologically cured on completion of treatment.

Figure 3.12 is a box-plot diagram comparing the total yeast concentration at baseline of patients with iron deficiency and those with normal serum ferritin levels, irrespective of treatment group. From this diagram it can be seen that patients with iron deficiency at baseline had, overall, a slightly lower yeast concentration than those with normal blood results. However, a two sample t-test (Table 3.13) was used to compare the mean total yeast concentrations of the two groups at baseline. This analysis revealed that although patients with iron deficiency did have slightly lower yeast concentrations at baseline than those with normal serum ferritin levels, this proved not to be statistically significant (p=0.19).

Table 3.13. **A two sample t-test to compare the differences in total baseline yeast concentration in those patients with iron deficiency and those with normal serum ferritin (p = 0.19 n.s.).**

	n	MeanYeast Concentration	S.D.	SE Mean
Normal serum ferritin	28	3.13	1.31	0.25
Iron deficient	8	2.36	1.40	0.49

Figure 3.12. A comparison of the total yeast concentration found in denture stomatitis patients with iron deficiency at baseline and those with normal blood results.



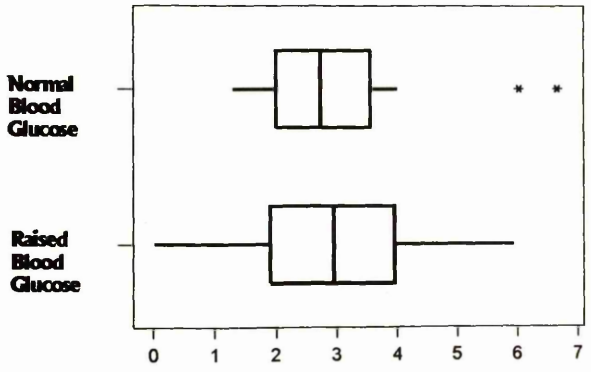
Twelve patients, four in the cyclodextrin group and eight in the capsule group, had high blood sugar levels (random blood glucose level > 6.0mmol/L) at enrolment. One of these patients failed to complete the course of itraconazole and was excluded from the trial. Despite corrective measures, six of the remaining eleven patients still had raised blood sugar levels six months after treatment commenced. However, all of the 11 patients had reduced erythema indices following antifungal therapy and six were mycologically cured on completion of treatment. Furthermore, two of these patients remained mycologically cured for the duration of the clinical trial.

Figure 3.13 is a box-plot diagram comparing the total yeast concentration at baseline of patients with raised blood glucose levels and those with normal blood glucose levels. From this diagram it can be seen that there was little difference in the total baseline yeast concentrations between the two groups. Consequently a two sample t-test found no significant difference between the baseline yeast concentrations of the two groups ($p = 0.99$ n.s.) (Table 3.14).

Table 3.14. **A two sample t-test to compare the differences in total baseline yeast concentration in those patients with raised blood glucose and those with normal blood glucose levels ($p = 0.99$ n.s.).**

	n	Mean Yeast Concentration	S.D.	SE Mean
Normal blood glucose	11	2.95	1.59	0.48
Raised blood glucose	25	2.96	1.26	0.25

Figure 3.13. **A comparison of the total yeast concentration found in denture stomatitis patients with raised blood glucose levels at baseline and those with normal blood glucose levels.**



There were no adverse changes in haematologic indices, liver function tests or urea and electrolyte measurements throughout the trial. C-reactive protein measurements were found to be normal (<10mg/L) at baseline and on completion of treatment in 21 of 36 trial participants. Nine patients had raised C-reactive protein levels at baseline but normal levels on completion of treatment. Of the remaining six patients, all had persistently higher than normal values of C-reactive protein before and after antifungal therapy.

3.2.4. Questionnaire.

Questionnaires from 18 patients in each treatment group who had completed the course of itraconazole were assessed. Only two patients in the cyclodextrin group and one in the capsule group found the time at which they had to take their medication inconvenient. With the exception of three patients in the cyclodextrin group and one in the capsule group, the study participants found their particular formulation of itraconazole pleasant to take. However, twelve patients in the cyclodextrin group complained of gastro-intestinal side-effects such as diarrhoea and stomach cramps. None of the patients who had been taking itraconazole capsules reported any side-effects. Lastly, 15 of the cyclodextrin patients and nine of the capsule patients said that their mouths "felt better" following the course of antimycotic treatment.

3.2.5. Itraconazole Levels.

Table 3.15 shows the results for serum and saliva levels measured for patients in each of the two study groups on completion of the 15 day course of treatment. The median serum itraconazole level in the cyclodextrin group was 0.74mg/L (interquartile range 0.46 to 1.18) compared to 0.61mg/L (interquartile range 0.37 to 0.93) in the capsule group. Using a two sample t-test, no significant difference was found in the serum itraconazole concentrations between the two formulations ($p = 0.39$). However, when considering the saliva itraconazole concentrations, the high incidence of zero values in the capsule group is very obvious (Table 3.15).

Table 3.15. **The serum and saliva itraconazole levels for each patient in each treatment group.**

Cyclodextrin solution (n = 18)		**Capsules (n = 18)**	
Serum(mg/l)	**Saliva(mg/l)**	**Serum(mg/l)**	**Saliva(mg/l)**
1.08	0.16	0.37	0.00
0.75	0.00	0.83	0.00
0.00	0.00	1.42	0.00
1.20	0.00	0.87	0.00
0.56	0.68	0.45	0.00
1.03	0.48	1.23	0.00
0.34	0.00	0.31	0.00
1.03	1.50	0.93	0.00
0.68	0.17	0.71	0.00
1.26	0.07	0.21	0.00
1.17	0.69	0.91	0.00
0.28	1.04	1.24	0.00
0.00	0.21	0.50	0.00
0.68	0.00	0.51	1.68
1.50	0.05	0.39	7.40
0.73	0.06	0.34	0.00
1.48	0.53	0.39	0.00
0.50	0.02	1.09	0.02
Median 0.74 Interquartile range 0.46, 1.18	**Median 0.12 Interquartile range 0.00, 0.53,**	**Median 0.61 Interquartile range 0.37, 0.93**	**Median 0.00 Interquartile range 0.00 0.00,**

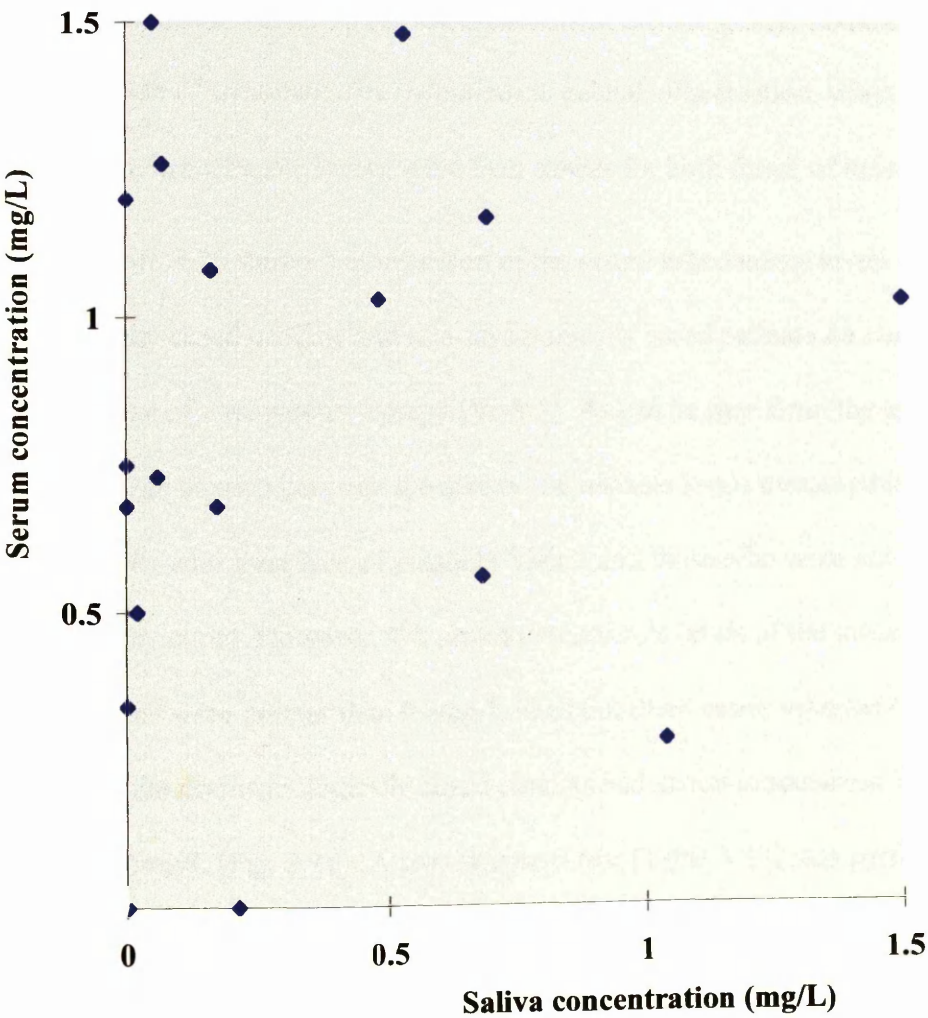
A confirmation of the difference in saliva concentration achieved in each of the groups was obtained by comparing the frequency of zero and non-zero values on each treatment using a *Chi*-squared test (Table 3.16). This provided a p-value of less than 0.001, representing a clear and significant difference in the proportion of patients in the cyclodextrin group with detectable itraconazole levels in their saliva on completion of treatment, compared to those in the itraconazole capsule group.

Table 3.16. **A comparison of the frequency of zero and non-zero saliva concentrations of itraconazole in each treatment group using a *Chi*-squared test ($p < 0.001$).**

	zero	**non-zero**
Cyclodextrin (n=18)	5	13
Capsules (n=18)	15	3

The serum and saliva itraconazole levels for the cyclodextrin group only were then analysed in order to test whether there was any correlation between these two concentrations for this preparation of itraconazole. Figure 3.14 shows little evidence of any relationship ($p = 0.704$) between the serum and saliva levels of those patients in the cyclodextrin group.

Figure 3.14. The relationship of saliva and serum itraconazole concentrations for patients in the cyclodextrin group



The relationship between the serum itraconazole levels and the mean reductions in *Erythema meter* index for patients in both treatment groups on completion of treatment (Visit 2) is shown in figure 3.15. This plot shows a significant ($p = 0.033$), moderately positive relationship (95% confidence interval of 0.03, 0.61) between the serum itraconazole concentrations and the mean reductions in *Erythema meter* index recorded at visit 2 for patients in both of the treatment groups. The sample correlation coefficients were 0.370 for the cyclodextrin group and 0.302 for the itraconazole capsule group, revealing that, on completion of treatment, the reductions in palatal inflammation, when related to the serum itraconazole levels, were very similar for both forms of itraconazole.

Figure 3.16 shows a comparison of the serum itraconazole levels of mycologically cured patients and non-mycologically cured patients on completion of the course of antimycotic therapy (Visit 2). As can be seen from the graph, there is a large overlap between the serum itraconazole levels measured for both those patients who were free of yeasts at Visit 2 and those who were not mycologically cured. However, the serum itraconazole levels of the mycologically cured patients were greater than 0.6mg/L in all but three cases, whereas the majority of the non-mycologically cured patients had serum itraconazole levels of less than 0.5mg/L (Fig. 3.16). A two sample t- test (Table 3.17) was performed to assess the significance of these higher drug levels. As a result it was found that the slightly higher serum itraconazole levels seen in the patients who were mycologically cured at Visit 2 were not significantly different, on average, from those not cured at Visit 2 ($p = 0.28$ n.s.).

Figure 3.15. The relationship between the serum itraconazole levels and the mean reductions in *Erythema meter* indices (EMI) for patients in both treatment groups at Visit 2. Diamond symbol: cyclodextrin group; square symbol: capsule group.

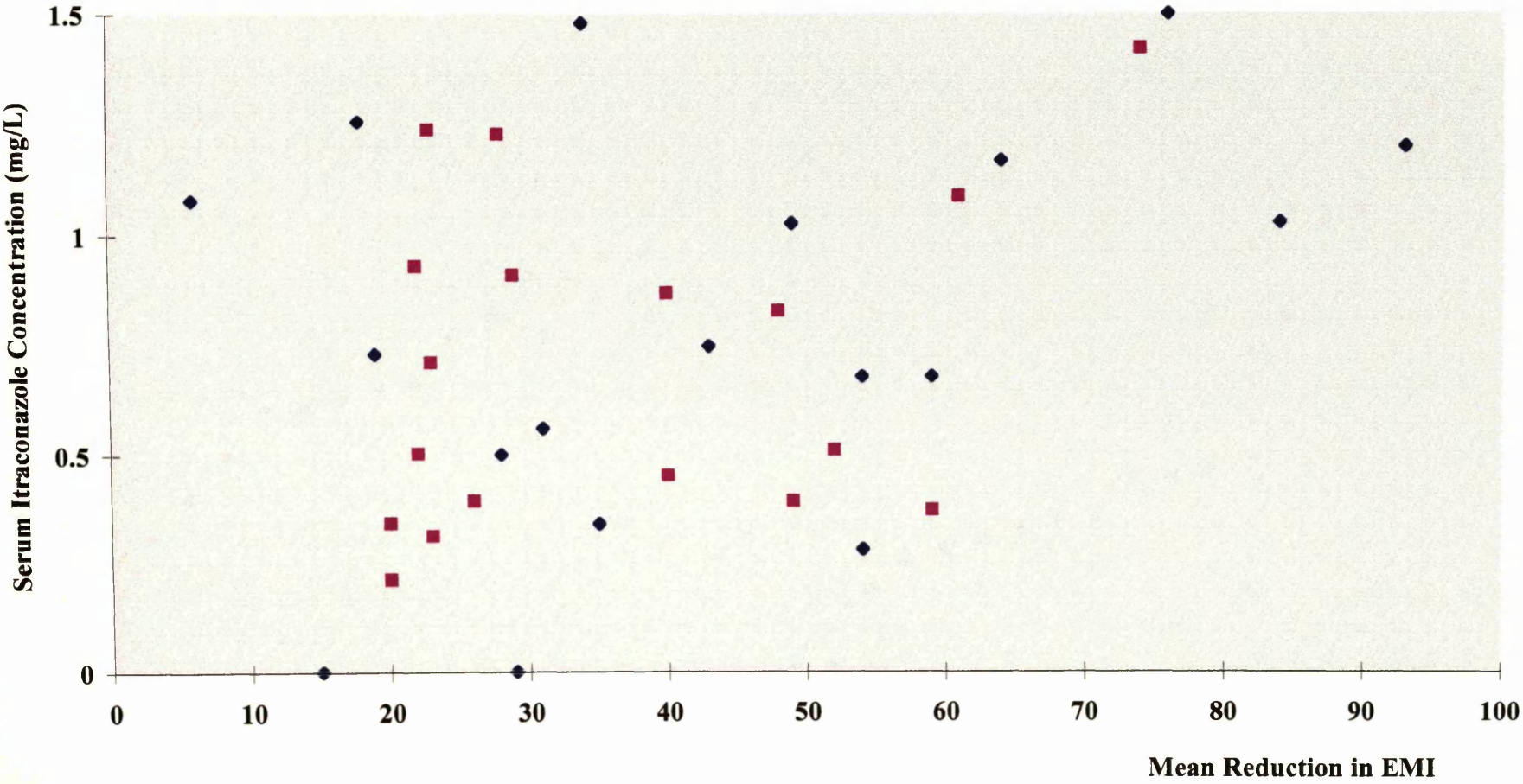


Figure 3.16. A comparison of the serum itraconazole levels of mycologically cured patients (diamond symbol) and non-mycologically cured patients (square symbol) on completion of treatment (Visit 2) and irrespective of treatment group.

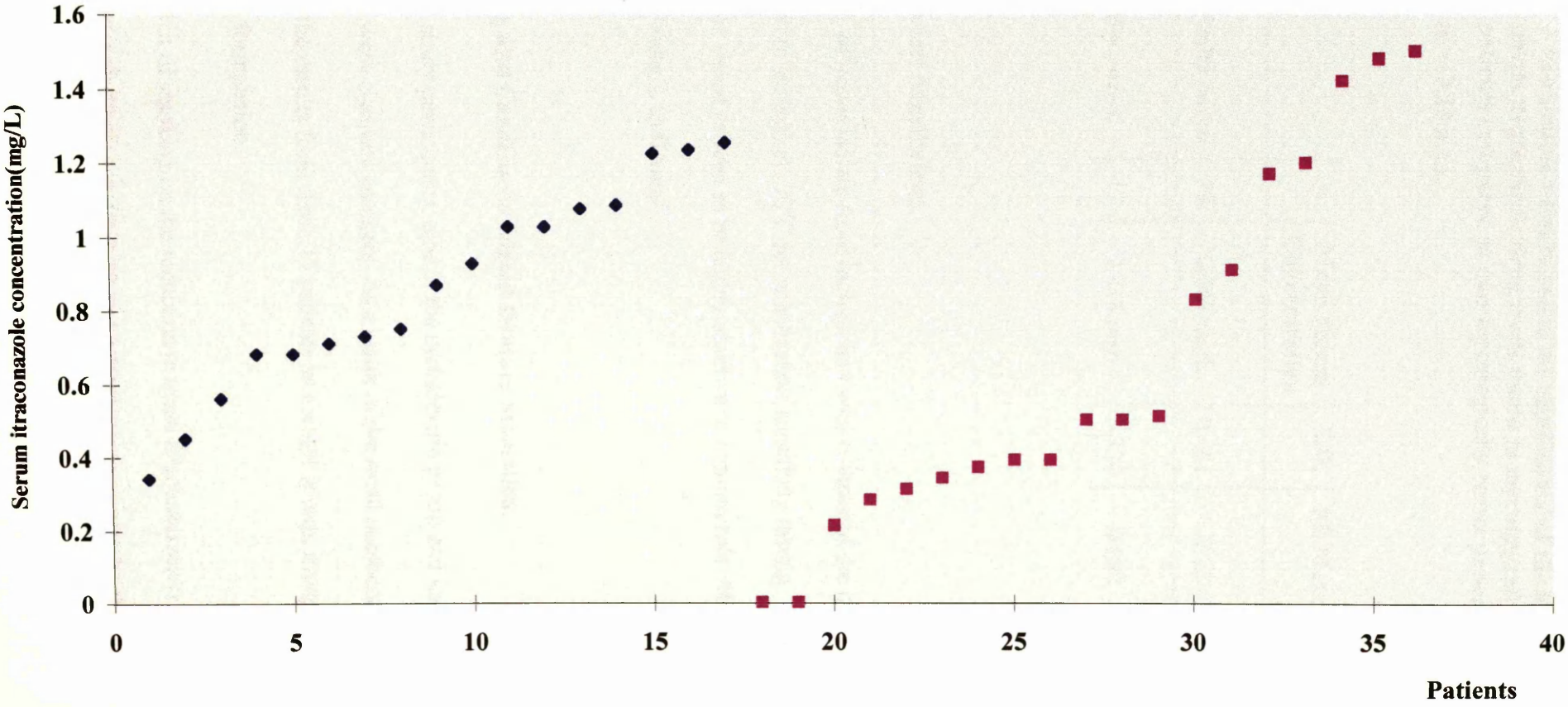


Table 3.17. **A two sample t-test to assess the significance of the higher serum itraconazole drug levels found in mycologically cured patients compared to non-mycologically cured patients (p = 0.28 n.s.).**

	n	Mean Serum Concentration	S.D.	SE Mean
Non-mycologically cured	19	0.663 mg/L	0.461	0.12
Mycologically cured	17	0.818 mg/L	0.370	0.083

3.2.6. Itraconazole Sensitivities.

One baseline *C. albicans* isolate from each patient who completed the clinical trial was removed from storage at -70°C for itraconazole sensitivity testing. All *C. albicans* isolates tested proved to be highly sensitive to itraconazole. MIC values ranged from 0.06mg/L to 0.5mg/L.

3.2.7. Smoking and *Candida*-associated Denture Stomatitis.

Thirteen of the study participants, nine in the cyclodextrin group and four in the capsule group, were cigarette smokers. As a result of the small numbers, analyses were made on the results from these 13 patients as a single group, irrespective of their treatment formulation.

The effect of smoking on the reduction in mean *Erythema meter* index was considered first of all. Figures 3.17, 3.18 and 3.19 show, respectively, the range of

Figure 3.17. **A box-plot comparing the baseline mean *Erythema Meter* Indices (EMI) of smokers versus non-smokers, irrespective of treatment group.**

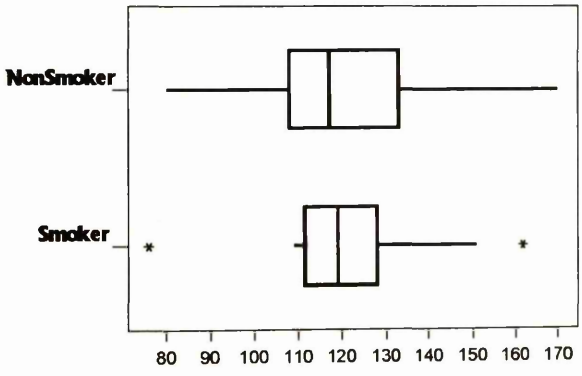


Figure 3.18. **A box-plot comparing the reduction in mean *Erythema Meter* Indices (EMI) on completion of treatment (Visit 2) of smokers versus non-smokers, irrespective of treatment group.**

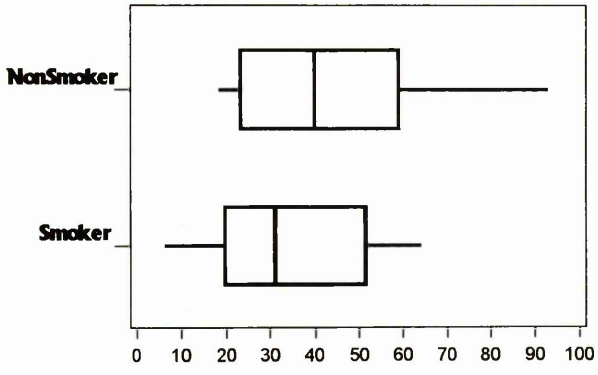
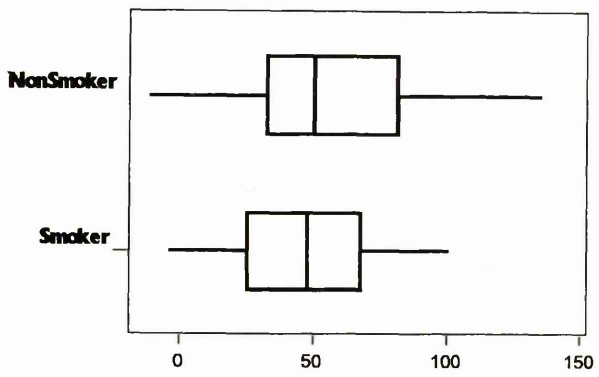


Figure 3.19. **A box-plot comparing the reduction in mean *Erythema Meter* Indices (EMI) six months after treatment commenced (Visit 4) of smokers versus non-smokers, irrespective of treatment group.**



mean erythema indices at baseline and reductions in mean erythema indices at two weeks (Visit 2) and six months (Visit 4) after treatment commenced. At baseline (Fig. 3.17), the non-smoking group had higher levels of erythema than the smokers. Following antifungal therapy at Visit 2 (Fig. 3.18), and also six months after treatment commenced (Fig. 3.19), the non-smokers had greater reductions in their mean erythema indices than the smokers. However, two-sample t-tests of the differences in erythema at baseline or the differences in mean reductions in erythema at subsequent visits of smokers against non-smokers did not prove significant (Table 3.18).

Table 3.18. **Two sample t-tests of the differences in erythema at baseline and the differences in mean reductions in erythema following antifungal therapy in smokers versus non-smokers.**

		n	Mean	S.D.	S.E. Mean	p-value
Baseline	**Smokers**	13	120.6	20.7	5.7	
	Non-smokers	23	121.5	22.7	4.7	**0.91**
Visit 2	**Smokers**	13	34.2	18.2	5.0	
	Non-smokers	23	43.7	22.2	4.6	**0.18**
Visit 4	**Smokers**	12	45.8	31.6	9.1	
	Non-smokers	23	56.3	33.9	7.1	**0.37**

Secondly, the effect of smoking on the chance of achieving a mycological cure following antifungal therapy was analysed. A Fisher Exact test was used because of the small numbers of trial patients who smoked. Although the p-value of the Fisher Exact test of 0.10 did not quite prove significant, it suggested,

although not conclusively, that smokers in this study had less chance of a long term (ie. 12 weeks after treatment commenced) mycological cure.

3.2.7. Molecular Typing of *C. albicans* Isolates.

3.2.7.1. DNA Extraction.

Eighteen of 36 patients who completed the clinical trial had *C. albicans* present at baseline and six months after treatment commenced. Genomic DNA was extracted from 36 stored *C. albicans* isolates in total, two isolates from each of the 18 patients, one which had been collected at baseline and another collected six months after treatment commenced. A one microlitre aliquot of each DNA sample was run on a 1% agarose gel to estimate the size of yeast DNA fragments extracted (Fig. 3.20). The majority of the DNA extracted was found to be in the region of 23.1 kilobases.

3.2.7.2. Restriction Enzyme Digestion.

Following overnight digestion with the enzyme EcoR1, a 30µl aliquot of each DNA sample was run out on a 0.8% agarose gel. Figure 3.21 shows a representative sample of the digested *C. albicans* DNA extracts. The first and last lanes in the gel contain the base pair marker λHind III-EcoR1 and the remaining lanes contain the DNA samples.

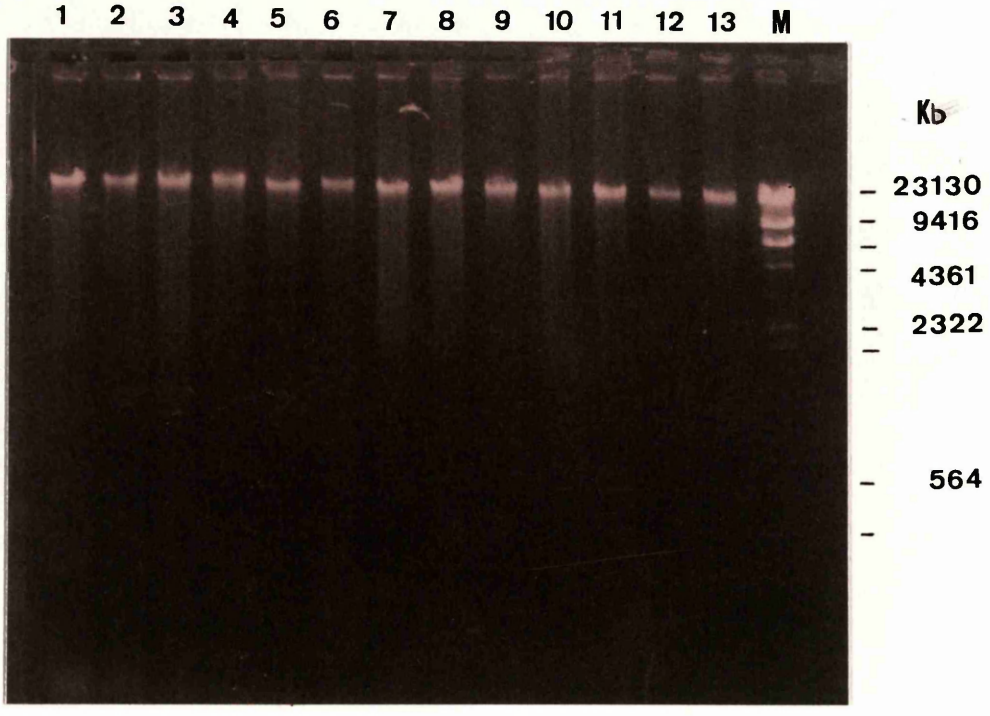


Figure 3.20. **A photograph of an ethidium bromide-stained 1% agarose gel. Lanes 1 to 13 each contain a one microlitre aliquot of extracted yeast DNA. Lane 14 contains six microlitres of the molecular base pair marker λHind III.**

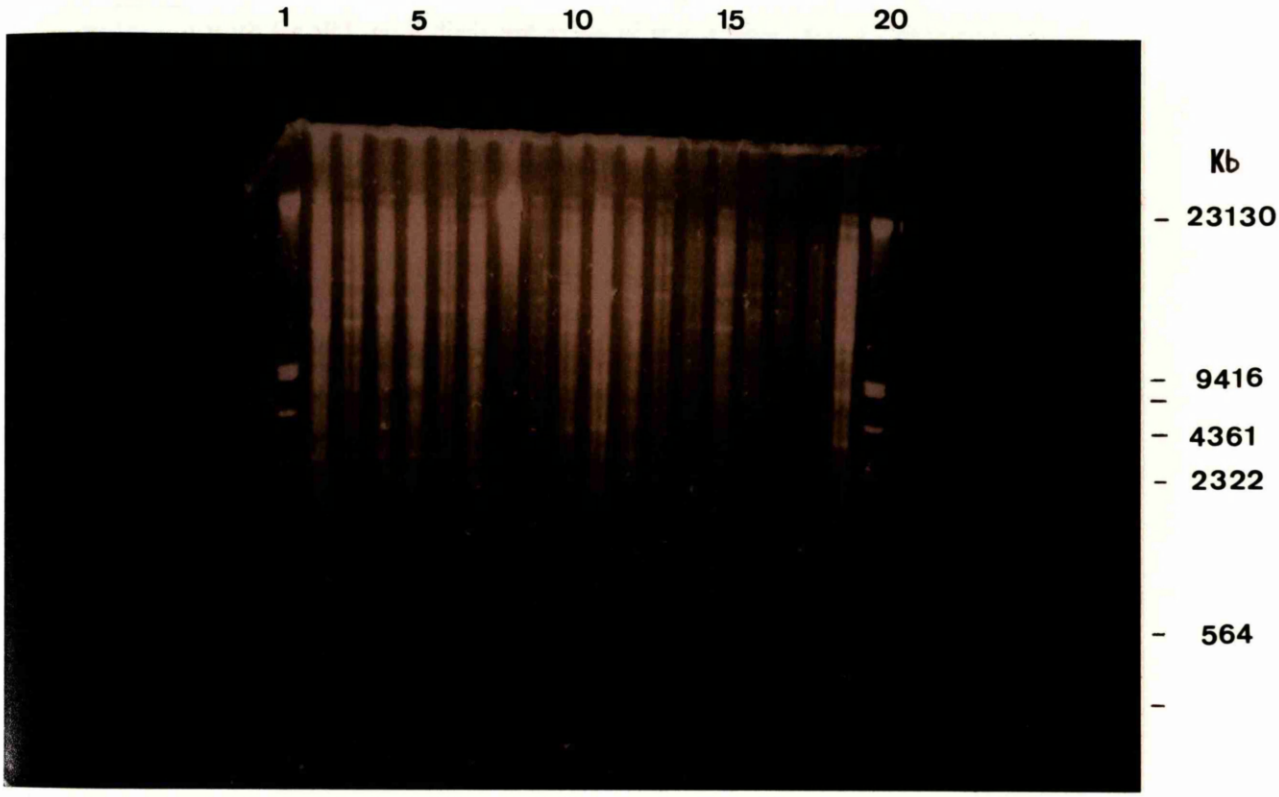


Figure 3.21. **A photograph of an ethidium bromide-stained 0.8% agarose gel. Lanes 1 and 20 contain the molecular base pair marker λHind III-EcoR1. Lanes 2 to 19 contain 30 microlitre aliquots of digested yeast DNA from a representative sample of trial patients.**

3.2.7.3. Southern Blotting and Hybridization.

DNA was transferred from a 0.8% agarose gel to a nylon membrane (Boehringer Mannheim) by Southern blotting. Hybridization of the 27A probe to the DNA samples cut with EcoRI gave differentiation of the strains of *C. albicans* (Figs. 3.22 and 3.23). The lanes labelled 'M' in figures 3.22 and 3.23 show the base pair marker λHind III-EcoR1. The sizes of the marker fragments are shown on the right. Lanes 1 to 12 in figure 3.22 and lanes 1 to 18 in figure 3.23 represent samples taken from six and nine patients respectively. Each pair of lanes represents the DNA of yeasts isolated from the same patient at baseline (first lane of any pair) and six months (second lane of any pair) after treatment commenced. Use of the *C. albicans* 27A repetitive element probe revealed only minor differences in band patterns between lane pairs indicating that, in any one patient, the *C. albicans* strains isolated prior to itraconazole therapy and six months after treatment commenced were the same strain. These differences in band pattern were subtle when compared to the major differences seen among strains from different patients. For example, in figure 3.22 lanes 9 and 10 are *C. albicans* isolates taken from the same patient at baseline (lane 9) and six months after treatment commenced (lane 10). The band patterns of these two isolates are almost identical and were considered to be the same strain. However, comparison of isolates taken from different patients, for example, lanes 5, 9 and 11 in figure 3.22 and lanes 1, 3 and 17 in figure 3.23, reveals a significantly different band pattern in each lane implying that different strains of *C. albicans* have been

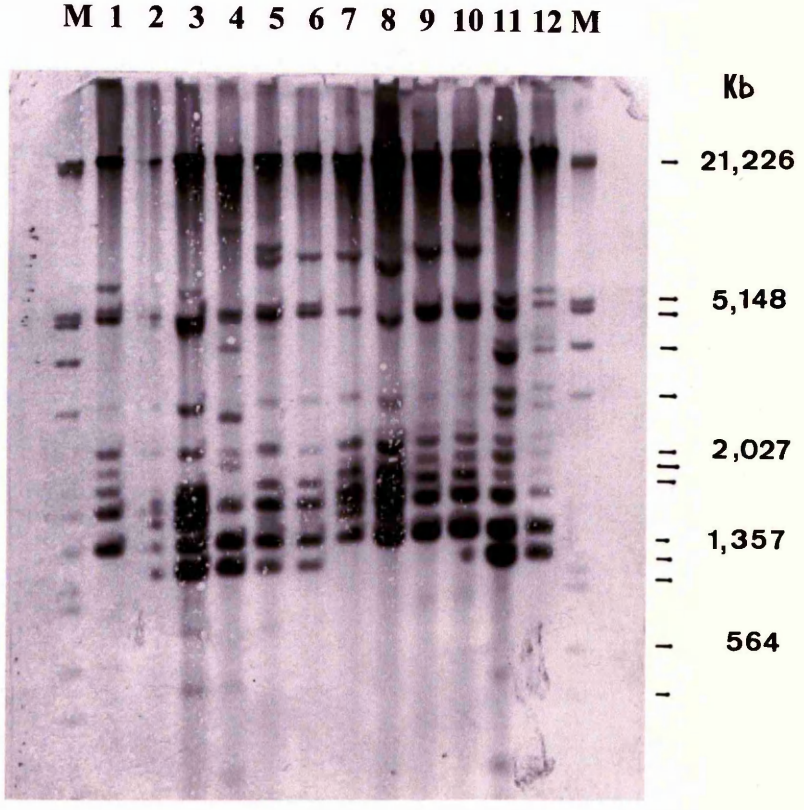


Figure 3.22. A scanned image of a Southern blot showing the *C. albicans* 27A repetitive element probe hybridized to EcoR1 digested *C. albicans* DNA.

The lanes 'M' at either side of the blot show the base pair marker λ Hind III / EcoR1. Lanes 1 to12 show a representative sample of digested DNA from *C. albicans* isolates taken from six of the study patients. Each pair of lanes represents *C. albicans* DNA extracted from isolates from the same patient. In any pair of lanes, the first lane represents digested DNA from yeasts isolated at baseline and the second lane represents DNA from yeasts isolated six months after treatment commenced. Lane 2 failed to hybridize completely.

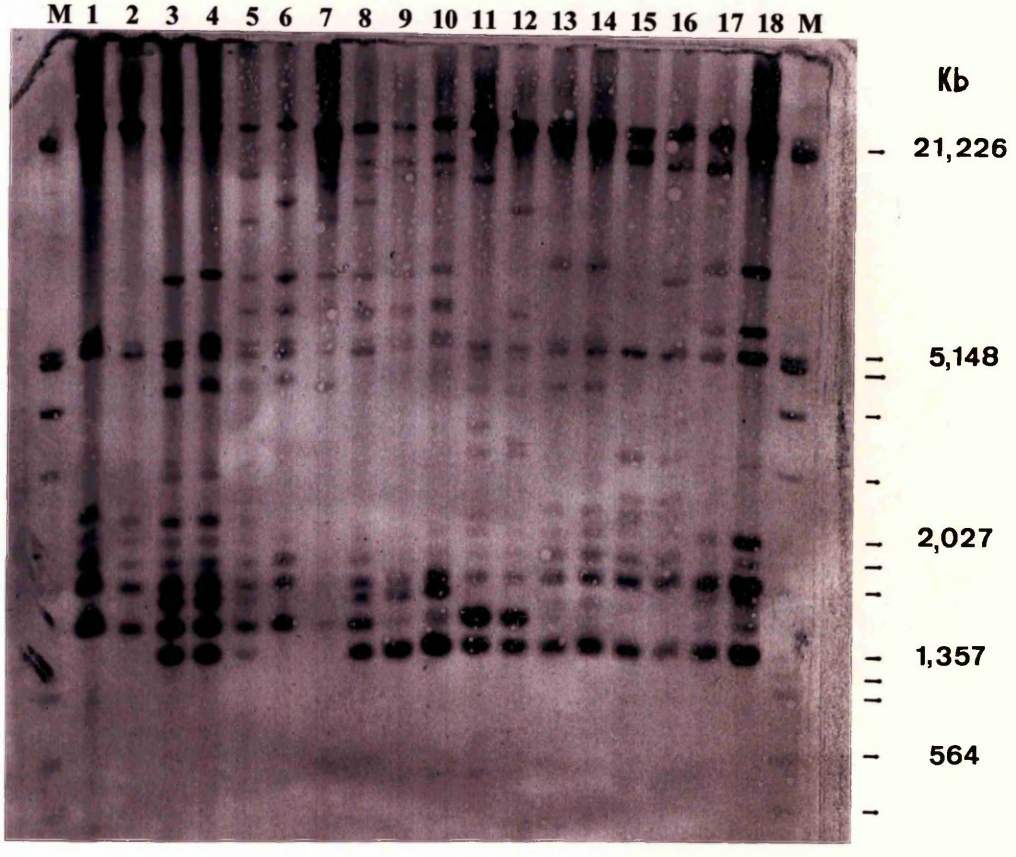


Figure 3.23. A scanned image of a Southern blot showing the *C. albicans* 27A repetitive element probe hybridized to EcoR1 digested *C. albicans* DNA.

The lanes 'M' at either side of the blot show the base pair marker λ Hind III / EcoR1. Lanes 1 to18 show a representative sample of digested DNA from *C. albicans* isolates taken from nine of the study patients. Each pair of lanes represents *C. albicans* DNA extracted from isolates from the same patient. In any pair of lanes, the first lane represents digested DNA from yeasts isolated at baseline and the second lane represents DNA from yeasts isolated six months after treatment commenced. Lane 7 failed to hybridize well.

isolated from each of these patients. Among this group of eighteen patients, thirteen completely different strains (arbitrarily labelled A to M) of *C. albicans* were found. Of these thirteen strains, four were found to be carried by more than one patient. The different genotypes of *C. albicans* isolated from eighteen study patients who had *C. albicans* present at baseline and six months after treatment commenced are illustrated in Table 3.19.

Table 3.19. **The range of *C. albicans* strains isolated from this study population.**

Strain	A	B	C	D	E	F	G	H	I	J	K	L	M
Patient	2 19 20	6	7	8	10	16	23	24 34	26 40	29	33 37	35	38

Lanes 2 (Fig. 3.22) and 7 (Fig. 3.23) failed to hybridize well.

CHAPTER FOUR

DISCUSSION

Denture stomatitis is recognised as a multifactorial disease (Budtz-Jörgensen, 1974). Whilst yeasts are known to play an important role in many cases, the value of antifungal medication in treatment is unclear. Walker *et al.* (1981) showed that mechanical cleansing of dentures was as effective as topical treatment with amphotericin B in treating *Candida*-associated denture stomatitis, and concluded that routine prescription of antimycotic drugs was unnecessary. However, although treatment should be directed primarily towards reducing levels of denture plaque (Arendorf and Walker, 1979), not all patients follow denture hygiene advice, as seen in this study. Overall, the results show a significant benefit of using antifungal medication as an adjunct to other measures. Antifungal treatment may also be indicated for patients who are at risk of disseminated infection (Warnock, 1992).

Of the systemic antifungals, itraconazole is the most recent addition to the orally-active triazole derivative agents. It has a very broad spectrum of activity (Odds, 1993a), but to date there have been no therapeutic trials of itraconazole in the treatment of denture stomatitis. Previous studies (Osterloh, 1992) of fluconazole treatment of *Candida*-associated denture stomatitis have shown a significant initial reduction in erythema and yeast cell concentration, although high rates of relapse have also been reported (Bissell *et al.*, 1993). Itraconazole is an effective agent for the treatment of both superficial and systemic mycoses (Smith *et al.*, 1991; Van t'Wout *et al.*, 1991; Odds, 1993a). The new cyclodextrin solution of itraconazole has been used successfully among a group of AIDS

patients with a history of failed treatment for oral candidosis using other azoles (Cartledge *et al.*, 1994).

Preliminary investigations were carried out prior to commencing the main clinical trial. These aimed to evaluate the sensitivity and reproducibility of an electro-optical instrument, the *Erythema meter*, as an objective means of assessing erythema of the palatal mucosa. Until this study, use of the *Erythema meter* had been mainly confined to the skin, where objective data were obtained following experimentally induced erythematous lesions (Farr & Diffey, 1984). The instrument had also been used to record intra-oral erythema in one patient with stomatitis as a result of intolerance to bovine products (Ferguson *et al.*, 1987).

Erythema meter readings taken from the check-standard used by graphic artists for colour reproduction permitted determination of the range of redness that the instrument could resolve. These non-clinical investigations showed the *Erythema meter* to be a very sensitive and reproducible means of differentiating varying shades of red, up to an erythema index reading of approximately 270. Above this level the instrument had difficulty in discriminating between stronger shades of red. However, as the clinical investigations have indicated, this does not pose a problem for measurement of mucosal erythema *in vivo*, since inflamed mucosa does not result in readings this high.

To determine the range of erythema indices that would be found intra-orally in both health and disease, a series of clinical experiments was performed, involving 40 patients with healthy palatal mucosa and 20 with denture stomatitis. The healthy denture wearers and denture stomatitis groups were well age-matched, although as would be expected, the non-denture wearing group had a

much lower mean age. Both the healthy denture wearing group and the non-denture wearers were well sex matched but there was a predominance of female patients in the denture stomatitis group. This will be discussed in relation to the pilot clinical trial of fluconazole versus itraconazole. Patients with healthy palatal mucosa gave a range of erythema indices of below 40, whereas the erythema indices for patients with denture stomatitis lay within a much higher range (46 to 117).

As a result of comparing the objective erythema indices with the more subjective visual scores, it was clear that the clinician could quite easily distinguish visually no inflammation, or even mild inflammation, from severe. However, there was some difficulty when discriminating mild inflammation from moderate, or moderate from severe. Such discrepancies pose a major problem in clinical trials, when an accurate end-point is essential. Use of the *Erythema meter* increases objectivity since degrees of inflammation can now be related to *Erythema meter* indices. For example, absence of inflammation of the palatal mucosa corresponds to an erythema index of less than 20, mild inflammation gives an index of 20 to 40, moderate 40 to 80 and severe inflammation corresponds to an index of greater than 80.

In addition to this, the study was also designed to assess the sensitivity and reproducibility of the instrument. The reproducibility coefficient of 88% is sufficiently exacting for clinical trial use. The *Erythema meter* proved to be quick and easy to use intra-orally. It was well tolerated by all patients. This highly sensitive and reproducible method of measuring oral mucosal erythema has many potential applications for both clinical research and diagnostic purposes. For

example, the instrument is currently being used in a study to assess the effect on the palatal mucosa of a new light-cured acrylic used in the base plates of upper removable orthodontic appliances. Adaptation of the *Erythema meter* to improve the portability of the instrument and reduce the size of the tip of the fibre optic cable, to facilitate easy use at any intra-oral site, could extend the use of the instrument in other dental specialities, such as Oral Medicine, Oral Surgery and Periodontology.

To test the usefulness of the *Erythema meter* in a clinical trial situation and to pilot the study design, a clinical trial was conducted, on an open basis, among 20 patients to examine the efficacy of itraconazole and fluconazole capsules in the treatment of denture stomatitis. All patients had long-standing denture stomatitis and were prescribed antifungals on the basis of both a clinical and mycological diagnosis. It should be noted that the fluconazole and itraconazole denture stomatitis groups, although well matched for age, were not well sex-matched. There was a predominance of females, as may be expected from the epidemiology of this condition (Budtz-Jörgensen, 1974). However, there was no difference between the sexes regarding compliance with the two antifungal agents or denture hygiene advice.

The tri-azoles are generally well tolerated antimycotic drugs (Osterloh, 1992; Bissell *et al.*, 1993; Odds, 1993a; Karyotakis and Anaissie, 1994). In the pilot study all patients completed their prescribed course of either fluconazole or itraconazole capsules and no adverse side-effects were reported.

The reduction in erythema after treatment with fluconazole was greater than the reduction seen in those patients treated with itraconazole, though it

should be noted that the erythema indices in the fluconazole group were higher to start with. The latter is a reflection of the small numbers in the pilot study. However, in both groups the median *Erythema meter* indices remained higher after antifungal treatment than the indices found in patients with clinically healthy palatal mucosa.

Patients who were mycologically cured or had a reduced yeast count at the end of their course of antifungal treatment were classed as having responded to treatment. Using these criteria, both itraconazole and fluconazole produced similar results, with nine patients in the fluconazole group and seven in the itraconazole group responding to their respective treatments (Table 3.6). Although the total mycological cure rates were low, these patients had improved clinically and it can be concluded that itraconazole and fluconazole do reduce inflammation in denture stomatitis.

Some workers have reported that although fluconazole has a relatively narrow spectrum of activity compared to previous azole derivatives (Odds, 1993a), itraconazole shows a high potency against all the commonly encountered pathogenic *Candida* species (Espinol-Ingroff *et al.*, 1984; Van Cutsem *et al.*, 1987; Lombardi *et al.*, 1989; Van Cutsem, 1989; Osterloh, 1992). Other studies, however, have reported that neither fluconazole nor itraconazole are active against *C. glabrata, C. krusei* and *Fusarium* species (Odds, 1993b; Karyotakis and Anaissie, 1994). This may explain why, in the pilot study, when *C. glabrata* was one of the yeasts isolated at baseline it persisted, in all cases, after antimycotic therapy.

One patient in the fluconazole group and three in the itraconazole group had increased yeast counts on completion of treatment (Table 3.6). As *Candida glabrata* was the species isolated from three out of four of these patients following treatment, the increased yeast count could be attributed to the reduced azole sensitivity of this species (Odds, 1993b; Karyotakis and Anaissie, 1994). However, the isolation of *C. albicans* in two of the patients with increased yeast counts is more difficult to explain. This may be the result of poor patient compliance either with the drug or, more likely, with denture hygiene procedures.

Of the five patients who were mycologically cured, three were colonised by *C. albicans* alone and the remaining two patients had a mixed growth of *C. albicans* and *C. guillermondii* in one case and *C. albicans*, *C. parapsilosis* and *Sacch. cerevisiae* in the other. *C. albicans* was present in samples from all 20 patients before treatment but was not always eradicated completely by antifungal medication, persisting in six patients on completion of a course of fluconazole and four patients after treatment with itraconazole.

The pilot study ended after a review appointment which coincided with the completion of the two week course of antifungal therapy and no data were obtained to allow assessment of the rate of re-establishment of oral yeasts in this study population, an aspect which received attention in the main clinical trial.

The relationship between denture cleanliness and inflammation in denture stomatitis is well established (Walker *et al.*, 1981). As part of the overall management of denture stomatitis in this pilot study, patients were advised to remove their dentures for sleeping and to immerse them overnight in an alkaline peroxide cleanser. Patients were also asked to clean their dentures regularly after

each meal with a soft brush and soap. Although every patient followed the advice to remove their dentures at night, analysis of the clinical denture cleanliness scores relating to the amount of plaque visible on the denture fitting surfaces before and after treatment indicated that most patients were not complying with the additional advice. Furthermore, the standard of denture cleanliness was quantified by a microbiological assay technique devised by Blair *et al.* (1995). This confirmed the lack of improvement in denture hygiene for many of the patients over the two week trial period. Interestingly, two of the three patients whose erythema indices showed little change from one visit to the next, in spite of treatment with antifungal medication, had higher microbiological denture cleanliness scores at the second visit. The persistence of significant amounts of plaque on the denture fitting surfaces in many patients at the second visit may help to account for the higher median erythema index recorded post-treatment in this group compared to the median erythema index that was previously determined for denture wearers with healthy palatal mucosa (Cross *et al.*, 1998).

Itraconazole and fluconazole produced similar results in the pilot study. Both drugs were found to reduce palatal inflammation in patients with denture stomatitis although they rarely resulted in a mycological cure. The *Erythema meter* proved to be a valuable means of objectively measuring changes in erythema of the palatal mucosa. The design of the main clinical trial was modified slightly as a result of the pilot study. For example, in the latter study *Erythema meter* readings were taken from two sites on either side of the palate in an attempt to obtain a level of redness for the whole of the palate. However, analysis of the preliminary results suggested that these repeated measurements offered no

advantage and so only one site (that which appeared most inflamed on visual examination) was recorded with the *Erythema meter* in the main clinical trial. Also as a result of the pilot study, itraconazole appeared to be of comparable efficacy to fluconazole in the treatment of denture stomatitis but as it had received relatively little attention in relation to treatment of oral candidosis in immunocompetent hosts, it was felt that this drug merited further study. In addition, the recent introduction of the new cyclodextrin solution of itraconazole meant that a clinical trial of these two forms of itraconazole in the management of denture stomatitis would be timely. In particular, it was postulated that the new cyclodextrin solution of itraconazole may provide a major therapeutic advantage over traditional treatment of oral candidosis as it combines the effect of topical application to the mucosa and systemic effects after absorption (Blatchford, 1990).

A randomised single blind clinical trial was, therefore, conducted among 40 patients to examine the efficacy of the cyclodextrin solution of itraconazole and itraconazole capsules in the treatment of denture stomatitis. All patients had long-standing denture stomatitis and were prescribed antifungals on the basis of both a clinical and mycological diagnosis. The two treatment groups, were well matched for age and sex, but as in the pilot study there was a predominance of females. However, as before, no difference was found between the sexes regarding compliance with the two antifungal agents or denture hygiene advice.

A questionnaire was used in the clinical trial to help assess patient compliance and the incidence of side-effects in each treatment group. Both forms of itraconazole were similar as regards ease of use and patient compliance. Only

four of the 40 patients enrolled in the study, two from each treatment group, failed to complete the two week course of itraconazole. The two patients in the cyclodextrin group who failed to complete the course found they could not tolerate the solution, the taste of which they both described as 'sickly'. The two patients excluded from the capsule group did not give any particular reason for failing to complete their course of medication. An important difference between the two forms of itraconazole was seen in the incidence of gastro-intestinal side-effects. Two-thirds of the patients using the cyclodextrin liquid preparation complained of diarrhoea, stomach cramps or nausea whilst taking the course of itraconazole compared to the capsule group who did not complain of any problems. The symptoms experienced by patients in the cyclodextrin group were transient and resolved on completion of the course of itraconazole. A study carried out for Janssen-Cilag (1996) has also reported an increased stool frequency, loose stools and abdominal cramps in nine of 12 volunteers taking 200mg cyclodextrin solution twice daily for fifteen days (ie. double the dosage prescribed in the denture stomatitis trial). These abnormalities were not observed with the capsule formulation of itraconazole. β-cyclodextrin is a chemically modified cylinder-shaped oligosaccharide starch derivative, which when combined with the otherwise insoluble itraconazole molecule increases the solubility of the latter in aqueous media. The gastro-intestinal side-effects noted in relation to the liquid preparation have been attributed to an osmotic effect of high levels of hydroxy-propyl-β-cyclodextrin in the gut following administration of the cyclodextrin solution of itraconazole (Vandewoude *et al.*, 1997). None of the denture stomatitis patients failed to complete their course of cyclodextrin solution

as a result of the side-effects. However, since the response to treatment was similar with both preparations, the itraconazole capsules would be preferred to the cyclodextrin solution of itraconazole for treatment of denture stomatitis.

Denture stomatitis is generally regarded as a symptomless condition (Arendorf and Walker, 1987) although some studies have recorded oral complaints in 28% to 70% of cases (Ritchie *et al.*, 1969; Budtz-Jörgensen and Bertram, 1970a; Olsen, 1975a; Dorey *et al.*, 1985). In this study only 22% of patients complained of oral symptoms at baseline. These patients complained either of oral dryness or a painful burning tongue. However, 64% of the patients who completed the clinical trial, 15 of 18 patients in the cyclodextrin group and eight of 18 patients in the capsule group, reported that their mouths felt better on completion of the course of antimycotic treatment. The higher level of subjective improvement reported by the cyclodextrin group is possibly related to the fact that this is a 'mouthwash' formulation which may be associated with general oral health and a feeling of oral cleanliness, in addition to the fact that the cyclodextrin solution of itraconazole has a topical as well as systemic effect.

The reduction in erythema after treatment with the cyclodextrin solution of itraconazole appeared greater than the reduction seen in those patients treated with itraconazole capsules. However, it should be noted that the erythema indices in the cyclodextrin group were higher to start with and when appropriate corrections were made to account for this, there was no statistically significant difference between the two preparations. This problem was encountered in the pilot study, where the itraconazole capsule group had greater levels of erythema at baseline than the fluconazole group. It seems difficult to avoid such loading in

studies of this design where patients are recruited from various clinics and randomly assigned to a particular treatment group. In both groups the median *Erythema meter* indices remained higher after antifungal treatment than the indices found in patients with clinically healthy palatal mucosa (Cross *et al.*, 1998), though with time they may reduce further. The presence of residual erythema regardless of the preparation of itraconazole used emphasises the multifactorial nature of denture stomatitis (Budtz-Jörgensen, 1974).

Patients who were mycologically cured or had a reduced yeast count at the end of their course of antifungal treatment were classed as having responded to treatment. Using these criteria, both forms of itraconazole produced similar results, with 17 patients in the cyclodextrin itraconazole group and 18 in the itraconazole capsule group responding at the end of each treatment course (Table 3.12). Although one patient in the cyclodextrin group had no change in yeast count on completion of the antifungal therapy, all patients in each treatment group had improved clinically by this stage in the trial and it can be concluded that both preparations of itraconazole reduced inflammation in denture stomatitis. This is an obvious benefit, as more accurate impressions may then be taken of the less inflamed tissues, enabling the provision of new dentures, an important step in the long-term management of denture stomatitis. In this study, patients who received new dentures during the trial had a significantly greater chance ($p = 0.020$) of remaining mycologically cured six months after treatment commenced than those patients who continued to wear their existing dentures following the antimycotic therapy.

As found in the pilot study, when *C. glabrata* was one of the yeasts isolated at baseline from either the cyclodextrin group or the itraconazole capsule group, it persisted, in all but two cases, after antimycotic therapy. *C. krusei*, although isolated from only one patient at baseline, was also present in that patient's samples at each post-treatment visit. These data reflect the relative resistance of these two species to itraconazole.

Eight patients in the cyclodextrin group and four in the capsule group had increased yeast counts at either four weeks, six months or at both four weeks and six months after treatment commenced. Six of these twelve patients were regular smokers which may have been a contributory factor (Arendorf and Walker, 1980; Cumming *et al.*, 1990) or it may be the result of poor patient compliance with denture hygiene procedures, since five of the twelve (four of whom were non-smokers) had poorer levels of denture cleanliness at four weeks or six months after treatment commenced.

Of the 17 patients who were mycologically cured on completion of treatment, 12 were colonised by *C.albicans* alone. Two of the remaining five patients had a mixed growth of *C. albicans* and *C. glabrata*, two had *C. albicans* and *Sacch. cerevisiae* and one had *C. albicans* and *C. guillermondii*. *C. albicans* was present in samples from all 40 patients before treatment but was not always eradicated completely by antifungal medication, persisting in five patients on completion of a course of cyclodextrin solution and seven patients after treatment with itraconazole capsules. However, although yeasts may not have been completely eradicated in some patients, on completion of therapy yeast counts

were reduced in all patients except one in the cyclodextrin group, whose yeast count remained unchanged between baseline and the end of treatment.

Progressive recolonisation of the mucosa and the denture fitting surface by yeasts and the consequent recurrence of denture stomatitis after completion of antifungal treatment has been reported (Bergendal *et al.*, 1979; Budtz-Jörgensen *et al.*, 1983a; Bissell *et al.*, 1993; Karyotakis and Anaissie, 1994). In the present study, seven patients remained mycologically cured after completing the course of antifungal therapy until the end of the trial. A further patient who was free of yeasts four weeks after treatment commenced remained so six months later. However, 50% of patients who had been mycologically cured at two or four weeks after treatment commenced were recolonised by yeasts at six months. Genetic typing of *C. albicans* strains isolated from these patients at baseline and six months after treatment has shown that, in the majority of cases, the same strain of *C. albicans* was present before treatment as at the end of the clinical trial. This will be discussed in more detail below.

Seventy per cent of patients followed the advice to remove their dentures at night. However, the clinical denture cleanliness scores showed that in many cases the level of denture hygiene did not improve, despite written and verbal instructions. The standard of denture cleanliness was quantified by an objective microbiological assay technique (Blair *et al.*, 1995). These data also showed only a small improvement, though overall it was significantly better than at baseline. These findings were similar to those seen in the pilot study. This suggests that treatment of denture stomatitis by educating patients to improve their denture hygiene without adjunctive antifungal therapy is unrealistic.

Baseline haematological investigations of the denture stomatitis patients revealed eight patients with iron deficiency and 12 patients with a raised blood sugar level. Although these haematinic abnormalities did not adversely affect the patients' response to antifungal therapy, the relatively high proportion of patients in this study population with some underlying haematological problem highlights the importance of the routine screening of patients with denture stomatitis for such problems. For example, two patients had elevated blood glucose levels at baseline of 14mmol/L and 16.5mmol/L respectively. As a direct result of their denture stomatitis and subsequent enrolment in this clinical trial, these patients were diagnosed with maturity onset diabetes mellitus. Consequently, corrective measures were started before other more serious signs of this condition became apparent.

In a study by Jennings and MacDonald (1990), the density of candidal colonisation in denture stomatitis patients with haematological deficiencies was found to be lower than that found in denture stomatitis patients with normal blood results. Mucosal atrophy is commonly associated with iron deficiency and so patients with such a deficiency may be expected to have clinically obvious palatal inflammation with lower yeast concentrations than those with normal blood results. In this study the only form of haematological deficiency discovered at baseline was iron deficiency, affecting eight patients. Although the number of patients with low serum ferritin levels (ie. <25ng/ml) was small, this group of trial paticipants was found to have slightly lower yeast concentrations at baseline than those patients with normal serum ferritin levels. However, the difference in yeast concentration between these two groups was found not to be statistically

significant ($p = 0.19$ n.s.), possibly due to the small number of patients involved. A similar analysis carried out on those patients with elevated blood glucose levels (ie. >6.0mmol/L) at baseline found this group of patients to have similar yeast concentrations to patients with normal blood sugar levels ($p = 0.99$ n.s.).

C-reactive protein measurement is a useful indicator of the presence of underlying inflammatory disease and the response to treatment. As a result, C-reactive protein levels would be expected to fall following the administration of a course of antifungal therapy. In this clinical trial, only nine of the 36 trial participants had such a response. However, 58% of the study patients had normal C-reactive protein levels both before and after antifungal therapy. This may suggest that the area of inflammation associated with denture stomatitis is too small to be reflected in the C-reactive protein measurement and that perhaps the patients who did have elevated C-reactive protein levels had some other underlying disease process in addition to their denture stomatitis. In fact, the medical histories of those patients with elevated C-reactive protein levels did record a range of inflammatory conditions including chronic bronchitis, ulcerative colitis and osteoarthrosis.

The solubility and subsequent absorption of itraconazole following oral administration of the capsule formulation is dependent on gastric acidity, the bioavailability of the itraconazole being reduced in the fasting state or under conditions of hypochlorhydia. One study involving healthy volunteers found the bioavailability of itraconazole to be significantly reduced when the gastric pH was increased by administration of ranitidine but also showed that this effect could be counteracted by the co-administration of an acidic cola beverage (Lange *et al.*,

1997). In contrast to the capsules, the cyclodextrin solution of itraconazole is more bioavailable when taken optimally with or before food (Janssen-Cilag, 1996). To ensure optimal bioavailability of each formulation of itraconazole in this clinical trial, patients were requested to take the cyclodextrin solution of itraconazole one hour before food and the itraconazole capsules with food. However, it is claimed that the cyclodextrin solution of itraconazole offers improved solubility over itraconazole capsules resulting in improved absorption and enhanced bioavailability (Janssen-Cilag, 1996). To investigate this claim, patients from each treatment group in this clinical trial provided serum and whole saliva samples on completion of the 15 day course of itraconazole. Serum and saliva itraconazole concentrations were then measured using reversed-phase high-performance liquid chromatography (HPLC). No significant difference was found in the serum itraconazole concentrations achieved in patients from the two treatment groups. The reduction in palatal inflammation resulting from the itraconazole therapy was found to be very similar for both formulations of the medication. The serum itraconazole levels for those patients who were mycologically cured on completion of treatment were then compared to the levels found in those patients from whom yeasts could still be isolated. These patients were grouped according to their mycological response, irrespective of treatment group, as it was now apparent that both formulations of itraconazole had similar efficacy in the treatment of denture stomatitis. Some overlap was found between the serum itraconazole levels of the mycologically cured and non-mycologically cured patients in this trial. However, patients who were free of yeasts on completion of treatment did have slightly higher levels of serum itraconazole,

although this proved not to be statistically significant ($p = 0.28$ n.s.). Nevertheless, it is interesting to note that the majority of mycologically cured patients in this clinical trial had serum itraconazole levels of greater than 0.6mg/L whereas most of the non-mycologically cured patients had serum itraconazole levels of 0.5mg/L and less. In contrast to this, Cartledge *et al.* (1997) found higher serum drug concentrations and better clinical response rates with the cyclodextrin solution of itraconazole than with itraconazole capsules in AIDS patients. The bioavailability of itraconazole capsules in immunocompromised patient groups is variable and known to be lower than in non-immunocompromised indivduals, largely resulting from low gastric acidity or chemotherapy-induced gastro-intestinal toxicity associated with patients in the former group (Janssen-Cilag, 1996). Cartledge *et al.* (1997) also suggested that a serum itraconazole concentration of less than 1.0mg/L on day seven was predictive of therapeutic failure. Therapeutic success was achieved with much lower serum itraconazole concentrations in the denture stomatitis patients. This may reflect the immuno-competent status of the patients enrolled in this study or the multifactorial aetiology of denture stomatitis.

C. albicans species isolated from trial participants at baseline all proved to be sensitive to itraconazole. The MIC values (range 0.06 - 0.5mg/L) related well to the serum itraconazole levels discussed above.

Patients in the cyclodextrin group had significantly higher ($p < 0.001$) levels of itraconazole in their saliva, compared to patients in the itraconazole capsule group, the majority of whom had no itraconazole detectable in their saliva at all. Only two patients in the capsule group had unusually high salivary

itraconazole levels. These results were so aberrant that they must be attributed to laboratory or sampling error. The detection of itraconazole in the saliva of patients treated with the cyclodextrin formulation suggests an additional topical effect of cyclodextrin which would provide an obvious advantage of this preparation in the treatment of oropharyngeal candidosis. However, in this study, despite the significantly higher salivary itraconazole concentrations achieved with the cyclodextrin solution of itraconazole, the advantage of a topical effect was not apparent as both formulations were equally effective in the management of denture stomatitis in terms of mycological response and reduction in erythema.

When the serum and saliva itraconazole levels of the group on the cyclodextrin preparation were considered, no relationship was found ($p = 0.704$). This implies that the presence of itraconazole in the saliva following administration of the cyclodextrin solution is due to persistence of the drug following direct contact with the buccal mucosa to which itraconazole adheres and is subsequently released (Janssen-Cilag, 1996), rather than due to systemic absorption followed by secretion of itraconazole into saliva. This is confirmed by the fact that no active metabolite has been found in saliva (Janssen-Cilag, 1996).

The association between tobacco smoking and an increased prevalence of denture stomatitis has been reported previously (Hand and Whitehill, 1986; Cumming *et al.*, 1990). In this study the proportion of trial participants who were cigarette smokers was quite small (13 out of 36 patients). However, analysis of the results from these patients, considered as a group irrespective of their treatment formulation, revealed some interesting facts. For example, at baseline, the smokers had lower levels of erythema than the non-smokers. This could be

due to the vasoconstrictive action of nicotine affecting the blood vessels of the palatal mucosa and resulting in lower levels of palatal erythema. However, perhaps because of the small numbers, these differences in erythema at baseline and the differences in mean reductions in erythema at subsequent visits of smokers versus non-smokers did not prove to be statistically significant.

Smoking is known to be a major risk factor for periodontal disease (Haber, 1994) and would appear to have an adverse effect on the outcome of periodontal treatment (Preber and Bergstrom, 1985; Preber and Bergstrom, 1990). Interestingly, the results from this clinical trial, although not fully conclusive, suggest that denture stomatitis patients in this study who were cigarette smokers had less chance of a long-term mycological cure than their non-smoking counterparts. Thus part of the overall management of *Candida*-associated denture stomatitis, as in periodontal disease, must include counselling for those patients who smoke in order to help them to stop.

One of the aims of this study was to determine, by molecular typing of *C. albicans* species isolated before and after treatment, whether there had been a recurrence of infection with the same strain, selection of particular strains or infection with new strains of *C. albicans*. In the past it was difficult to answer this type of question as strain comparisons were based on phenotypes rather than genotypes, with the assumption that the underlying genetic differences that produced the phenotypic changes were the same in different isolates (Scherer and Stevens, 1988).The advent of recombinant DNA technology has meant that genetic assessments of strain relatedness are now possible, overcoming the risk of grouping together genetically unrelated strains with similar phenotypes and

separating related strains with different phenotypes. One of the most effective of these techniques has been Southern blot hybridization with DNA probes containing moderately repetitive sequences (Hellstein *et al.*, 1993). The *C. albicans* middle repetitive 27A sequence was chosen as this has been shown to be a very sensitive method of analysing restriction digested DNA samples (Magee *et al.*, 1992). EcoR1 was chosen as the enzyme for the digestion of the DNA as this has been suggested as the enzyme of choice for the 27A probe (Magee *et al.*, 1992; Anthony *et al.*, 1995).

For every patient enrolled in the trial, at least five yeast isolates per visit had been stored at -70°C for further investigation. Although *C. albicans* was not the only species isolated from this study population it is considered to be the main pathogen in oral candidosis and, as corresponding species-specific repeat sequence probes have not been developed for other *Candida* species (Sullivan *et al.*, 1993), it was decided to concentrate only on *C. albicans* for the molecular investigations. In this study there were 18 patients from whom *C. albicans* was isolated six months after treatment commenced as well as at baseline. Two isolates from each patient, one collected at baseline and one six months after treatment commenced, were removed from storage. Although some studies have found that the majority of *C. albicans* isolates from different sites within the same oral cavity have been found to belong to the same genetic group (Mathaba *et al.*, 1995), other studies have reported that some patients may carry more than one strain of *C. albicans* (Anthony *et al.*, 1995). In this study, as far as possible, the isolate collected six months after treatment commenced was from the same intra-oral site as the baseline isolate with which it was to be compared. This was to

overcome the chance of 'sampling error' which may have given the impression that a different strain was present at the six month review than at baseline, when actually multiple strains may have been present at different intra-oral sites for much of the time.

The genetic typing of *C. albicans* isolates proved to be a very time-consuming procedure. From the extraction of DNA to the end stage of detection of the 27A probe at least seven days would be required to complete ten samples. If a sufficient yield of DNA were not obtained in the first instance, this would lead to poor digestion and incomplete or uninterpretable hybridizations. The labelled 27A probe was stored at -20°C and could be re-used confidently up to six times, after which the signals became very faint and difficult to interpret. Even when optimal conditions had been achieved and the method followed to the letter, there was no guarantee that every sample would hybridize to the same degree. Examples of poor hybridizations can be seen in lane two of figure 3.22 and lane seven of figure 3.23.

In this study, 13 different strains of *C. albicans* were isolated from the 18 patients. This genetic diversity is similar to that found in other studies of *C. albicans* strains isolated from the oral cavities of non-immunocompromised individuals (Hellstein *et al.*, 1993; Mathaba *et al.*, 1995). For each volunteer with a mycological relapse, the strain of *C. albicans* isolated six months after itraconazole treatment commenced was considered to be genetically the same as the original strain isolated at baseline. In the study by Mathaba *et al.* (1995) the *C. albicans* strains isolated from the dentures during the primary infection were the same as those isolated during the secondary infection, following a course of

antifungal therapy, in nine out of 11 patients. This implies that the presence of *C. albicans* in these patients is typically due to a recolonisation of the original infecting strain rather than re-infection with a different strain. The primary reservoir of *Candida* species in healthy individuals is generally considered to be the gastro-intestinal tract (MacFarlane, 1990). Consequently, the patient's denture must be considered a potential source of re-infection, a view shared by other authors (Budtz-Jörgensen and Bertram, 1970b; Nairn, 1975; Bissell *et al.*, 1993). This further supports the conclusion that the provision of new dentures is an important factor in the long- term resolution of denture stomatitis.

The variety of genotypes identified in this study, as in the study by Mathaba *et al.* (1995), suggests that no one particular virulent strain of *C. albicans* is associated with denture stomatitis. However, it would be interesting to compare the genotypes of *C. albicans* isolates from denture wearers with healthy palatal mucosa and those from denture wearers with denture stomatitis, in an attempt to determine whether commensal strains of *C. albicans* are capable of causing an infection or whether, in the transition from a healthy to an infected mouth, there is strain substitution.

Itraconazole is clearly a very effective drug for use in the treatment of denture stomatitis. Both the new cyclodextrin solution of itraconazole and itraconazole capsules produced similar reductions in palatal erythema and yeast colonisation in this clinical study. However, the high incidence of gastro-intestinal complications with the cyclodextrin solution of itraconazole and its significantly higher cost compared to the capsule formulation, suggest that the latter is more appropriate for management of denture stomatitis.

Conclusions.

The following conclusions have been drawn from the results of this study:

1. The *Erythema meter* is a useful instrument for measuring oral mucosal erythema in a clinical trial situation. It is quick and easy to use and is well tolerated by patients.

2. Fluconazole and itraconazole capsules have similar efficacy in the treatment of denture stomatitis. Both antifungal agents reduced palatal erythema but rarely resulted in a mycological cure. Neither drug is associated with any serious side-effect.

3. Itraconazole capsules and the cyclodextrin solution of itraconazole have similar efficacy in the treatment of denture stomatitis. However, the high incidence of gastro-intestinal side-effects associated with the cyclodextrin formulation of itraconazole contra-indicates its use in the management of out-patient oral candidosis. The high cost of the cyclodextrin formulation, at twice the cost of itraconazole capsules, further limits its use in the management of this condition.

4. The establishment and maintenance of good denture hygiene habits, including the removal of dentures for sleeping, are important in the long-term resolution of denture stomatitis. However, despite careful instruction, not all patients achieve

acceptable levels of denture hygiene and so denture hygiene advice alone is not an acceptable means of managing denture stomatitis.

5. Cigarette smoking may reduce the chance of patients with denture stomatitis achieving a long-term mycological cure following itraconazole therapy.

6. Denture stomatitis may be an early indication of some underlying haematinic deficiency or elevated blood glucose level. All patients presenting with denture stomatitis should be routinely screened haematologically and biochemically.

7. The patient's denture must be considered a potential source of re-colonisation. As a result, the provision of new complete dentures soon after completion of a course of antifungal therapy greatly improves the chance of a long-term mycological cure and resolution of the condition.

8. The strain of *C. albicans* isolated from denture stomatitis patients six months after antifungal therapy commenced is likely to be the same as the original strain isolated at baseline. This implies that a recurrence of denture stomatitis is due to a re-colonisation by the original infecting strain, rather than re-infection with a different strain.

9. To obtain long-term clinical success in the treatment of *Candida*-associated denture stomatitis the multifactorial aetiology of denture stomatitis must be borne in mind. As a result, the management of *Candida*-associated denture stomatitis should include:

i) Routine haematological and biochemical screening.

ii) Itraconazole capsules - 100mg twice daily for 15 days, taken with food.

iii) Comprehensive denture hygiene instructions, provided in a written form. The importance of following this advice in relation to treatment success should be stressed.

iv) Denture stomatitis patients who smoke should be strongly advised to give up the habit. The reduced chances of successful treatment for those who continue to smoke should be emphasised.

v) Once the health of the palatal mucosa has been restored, impressions should be taken for the provision of new complete dentures.

vi) Patients should be reviewed regularly to screen for any evidence of relapse and to encourage the maintenance of good denture hygiene habits.

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APPENDICES

APPENDIX I

Patient Consent Form

GLASGOW DENTAL HOSPITAL AND SCHOOL
PATIENT INFORMATION SHEET

A COMPARATIVE TRIAL OF ITRACONAZOLE TABLETS (SPORANOX) WITH A MOUTHWASH FORM OF ITRACONAZOLE (CYCLODEXTRIN) FOR TREATMENT OF DENTURE STOMATITIS.

I ..... of
.....
freely and voluntarily agree to participate in a clinical research study entitled "A comparative trial of itraconazole tablets (Sporanox) with a mouthwash form of itraconazole (cyclodextrin) for treatment of denture stomatitis".

I have read the accompanying information sheet. The nature and purpose of the study have been explained to me by ..... I have had the opportunity to ask questions and I understand fully what is proposed.

I recognise that I may receive no benefit from the study. I accept that there may be other risks associated with the procedure which are not directly attributed to negligence on the part of those undertaking the procedure.

I understand that I am free to withdraw my consent at any time without prejudice to me or my dental care.

I have been assured that any information obtained from me will not be disclosed without my permission to any other party in any manner which will reveal my identity.

Signature .....

Date .....

I confirm that I, ..., have explained the nature and purpose of the clinical research study and the procedure in respect of which consent has been given by the above named.

Signature .....

Date .....

APPENDIX II

Patient Details Forms

CYCLODEXTRIN TRIAL.
Screening Visit.

PATIENT DETAILS

Name _____ Hospital Number _____

D.O.B. _____

Medical History relevant / not relevant
(*see medical history sheet*)

Smoker / Non-smoker

CLINICAL EXAMINATION.

Dentures Age _____

Fit _____

_____

Removed for sleeping Y / N

Oral Mucosa Denture Stomatitis Localised ☐

Diffuse ☐

Hyperplastic granular ☐

Microbiology Swab a) palate ☐

b)tongue ☐

Signature _____ Date _____

Enrol Y / N

CYCLODEXTRIN TRIAL.

Visit 1 - Enrolment.

PATIENT DETAILS

Name _____ Hospital Number _____

D.O.B. _____ Study Number _____

CLINICAL EXAMINATION.

Denture Cleanliness Score (clinical) _____

Palatal Mucosa i) Visual Examination (circle)

0	No inflammation	(pink and healthy)
1	Slight inflammation	(pinkish-red)
2	Moderate inflammation	(red)
3	Severe inflammation	(florid redness)



ii) Erythema Meter Reading

1. _____ 2. _____ 3. _____

HAEMATOLOGY & BLOOD CHEMISTRY

FBC	Serum Fe	Serum Folate	Vit B_{12}	Random glc	Us&Es	LFTs	CRP
☐	☐	☐	☐	☐	☐	☐	☐

IMMUNOLOGY

10mls Whole Saliva ☐

10mls Venous Blood ☐

MICROBIOLOGY

i) Imprint Culture a) Palate b) Tongue c) Denture

ii) Oral Rinse ☐ iii) Denture Disc ☐

Signature _____ Date _____

CYCLODEXTRIN TRIAL.

Visit 2 - Completion of Course of Antifungals .

PATIENT DETAILS

Name _____ Hospital Number _____

D.O.B. _____ Study Number _____

Date and Time of Last Dose of Antifungals _____ _____

CLINICAL EXAMINATION.

Denture Cleanliness Score (clinical) _____

Palatal Mucosa i) Visual Examination (circle)

0	No inflammation	(pink and healthy)
1	Slight inflammation	(pinkish-red)
2	Moderate inflammation	(red)
3	Severe inflammation	(florid redness)



ii) Erythema Meter Reading

1. _____ 2. _____ 3. _____

HAEMATOLOGY & BLOOD CHEMISTRY

FBC ☐ Us&Es ☐ LFTs ☐ C-reactive protein ☐

IMMUNOLOGY

10mls Whole Saliva ☐

10mls Venous Blood ☐

DRUG LEVELS

Saliva ☐

Serum ☐

Time _____

MICROBIOLOGY

i) Imprint Culture a) Palate b) Tongue c) Denture

ii) Oral Rinse ☐

iii) Denture Disc ☐

Signature _____ Date _____

CYCLODEXTRIN TRIAL.

Visit 3 - Four Weeks After Antifungal Treatment Commenced .

PATIENT DETAILS

Name _____ Hospital Number _____

D.O.B. _____ Study Number _____

CLINICAL EXAMINATION.

Denture Cleanliness Score (clinical) _____

Palatal Mucosa i) Visual Examination (circle)

0	No inflammation	(pink and healthy)
1	Slight inflammation	(pinkish-red)
2	Moderate inflammation	(red)
3	Severe inflammation	(florid redness)



ii) Erythema Meter Reading

1. _____ 2. _____ 3. _____

IMMUNOLOGY

10mls Whole Saliva ☐

10mls Venous Blood ☐

MICROBIOLOGY

i) Imprint Culture a) Palate b) Tongue c) Denture

ii) Oral Rinse ☐ iii) Denture Disc ☐

Signature _____ Date _____

CYCLODEXTRIN TRIAL.

Visit 4 - Six Months After Antifungal Treatment Commenced .

PATIENT DETAILS

Name _____ Hospital Number _____

D.O.B. _____ Study Number _____

CLINICAL EXAMINATION.

Denture Cleanliness Score (clinical) _____

Palatal Mucosa i) Visual Examination (circle)

0 No inflammation (pink and healthy)

1 Slight inflammation (pinkish-red)

2 Moderate inflammation (red)

3 Severe inflammation (florid redness)



ii) Erythema Meter Reading

1. _____ 2. _____ 3. _____

HAEMATOLOGY & BLOOD CHEMISTRY

FBC Serum Fe Serum Folate Vit B_{12} Random glc

☐ ☐ ☐ ☐ ☐

IMMUNOLOG Y

10mls Whole ☐ Saliva

10mls Venous ☐ Blood

MICROBIOLOGY

i) Imprint Culture a) Palate b) Tongue c) Denture

ii) Oral Rinse ☐

iii) Denture Disc ☐

Signature _____ Date _____

APPENDIX III

Patient Information Sheets

A COMPARATIVE TRIAL OF ITRACONAZOLE TABLETS (SPORANOX) WITH A MOUTHWASH FORM OF ITRACONAZOLE (CYCLODEXTRIN) FOR TREATMENT OF DENTURE STOMATITIS.

You have been given two boxes of the Itraconazole tablets (Sporanox). This course will last two weeks.

Please take two tablets daily - the first should be taken just after breakfast and the second just after your evening meal.

It is important to complete the course.

If you require further advice please contact Dr. J. Bagg on (0141) 211 9741 (daytime) or (0141) 357 5802 (evenings).

Thank you for your help in this study.

A COMPARATIVE TRIAL OF ITRACONAZOLE TABLETS (SPORANOX) WITH A MOUTHWASH FORM OF ITRACONAZOLE (CYCLODEXTRIN) FOR TREATMENT OF DENTURE STOMATITIS.

You have been given two bottles of the mouthwash form of itraconazole (Cyclodextrin). This course will last two weeks.

Please take 10mls of this liquid twice daily on an empty stomach - take the first dose approximately 1 hour before breakfast and the second approximately 1 hour before your evening meal.

Please remove your dentures before taking the medicine and swish it around your mouth before swallowing it.

It is important to complete the course.

If you require further advice please contact Dr. J. Bagg on (0141) 211 9741 (daytime) or (0141) 357 5802 (evening).

Thank you for your help in this study.

GLASGOW DENTAL HOSPITAL AND SCHOOL
PATIENT INFORMATION SHEET

A COMPARATIVE TRIAL OF ITRACONAZOLE TABLETS (SPORANOX) WITH A MOUTHWASH FORM OF ITRACONAZOLE (CYCLODEXTRIN) FOR TREATMENT OF DENTURE STOMATITIS.

CARE OF YOUR DENTURES.

Your dentures may accumulate food debris, stain and tartar so keeping them clean is important.

You should clean them regularly after each meal using a **SOFT BRUSH** and **SOAP**.

Your dentures should be **IMMERSED** overnight in **LUKE-WARM WATER** containing a tablet (or powder) of an alkaline peroxide denture cleanser such as *Milton* or *Steradent*.

DO NOT WEAR YOUR DENTURES OVERNIGHT.

APPENDIX IV

Questionnaire

GLASGOW DENTAL HOSPITAL AND SCHOOL
QUESTIONNAIRE

A COMPARATIVE TRIAL OF ITRACONAZOLE TABLETS (SPORANOX) WITH A MOUTHWASH FORM OF ITRACONAZOLE (CYCLODEXTRIN) FOR TREATMENT OF DENTURE STOMATITIS.

NAME_____ STUDY NUMBER _____

1. Which form of itraconazole did you take? tablets / mouthwash
(please circle)
2. Did you complete the course? Yes / No
(please circle)
3. Did you find the time of day you were asked to take the medicine convenient?

Yes / No
(please circle)

If inconvenient, please state why.

_____

_____

4. Was your medicine pleasant to take? Not at all / Quite / Very
(please circle)

If unpleasant, please state why.

_____

_____

5. Did you suffer any of the following side-effects whilst taking the medicine?

stomach cramps	Yes / No
diarrhoea	Yes / No
sickness	Yes / No

other problems _____

_____

6. Did your mouth feel better after taking the medicine? No / Same / Yes
(please circle)

Thank you very much for your help in this study.

APPENDIX V

Buffers, Solutions and Media

Buffers, Solutions and Media

Denaturation Solution 1000ml

5M NaOH	100ml
5M NaCl	300ml
Autoclaved Distilled Water	600ml

Neutralisation Solution 1000ml

1M Tris HCl	500ml
Na Cl (solid)	175.4g
Autoclaved Distilled Water	to make up to 1000ml

Buffer 1

NaCl	150mmol/l
Maleic acid	100mmol/l, pH 7.5

Adjusted with solid or concentrated NaOH
Autoclaved

Blocking Reagent

Blocking Reagent is dissolved in buffer 1 to a final concentration of 10% (w/v) with shaking and heating in a microwave oven. The blocking Reagent must be heated while it dissolves in the maleic acid buffer (buffer 1). Boiling will cause the reagent to coagulate, so care should be taken to avoid boiling.
Autoclave.
Store at room temperature, +4°C or - 20 °C.

Buffer 2

Blocking Reagent stock solution diluted 1:10 in buffer 1
(final concentration = 1% blocking reagent)

Buffer 3

Tris-HCl	100mmol/l
NaCl	100mmol/l
$MgCl_2$	50mmol/l

TE Buffer

Tris HCl	10mmol/l
EDTA	1mmol/l

10 x TBE Stock Solution

Tris Base	108g
Boric Acid	55g
EDTA	7.46g

Made up to 1 litre with autoclaved distilled water

20 x SSC

3M NaCl	350.4g
300mM Sodium citrate	176.5g
Autoclaved Distilled Water	to make up to 2000ml

Standard Hybridization Buffer

5 x SSC
0.1% sodium-lauroylsarcosine
0.02% SDS
1% Blocking Reagent from 1% blocking reagent stock solution

Stringency Washes

I 2 x SSC
0.1% SDS

II 0.1 x SSC
0.1% SDS

Colour Substrate Solution

45µl NBT solution and 35µl X-Phosphate in 10ml Buffer 3.
Use directly.

1% Agarose Gel

Agarose	2g
1 x TBE	200ml
Ethidium Bromide	35µl

CHROMagar® *Candida*

Peptone	10.0g/l
Special chromogenic mix	22.0g/l
Chloramphenicol	0.5g/l
Agar	15.0g/l

MOPS Buffer

MOPS	34.53g
Glucose	20.00g

Dissolved in 1 litre distilled water
pH adjusted to 7.0

RPMI

RPMI powder	1 bottle
MOPS buffer	1 litre

Itraconazole Stock Solution

Itraconazole powder	10mg
DMSO	1ml
RPMI	9ml

Heat to 60°C to dissolve

GLASGOW UNIVERSITY LIBRARY