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# Immune Response and Candidal Colonisation in Denture Associated Stomatitis

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## Abstract

Denture stomatitis (DS) is an inflammatory oral condition affecting most frequently the palatal mucosa covered by the fitting surface of a denture. The aim of this study was to determine the local cytokine profile generated in DS, as well as the level and identity of *Candida* involved. Palatal inflammation was determined by clinical indices in 93 upper denture-wearing subjects (42 with DS). The level of selected cytokines in palatal fluid was measured by cytometric bead array. Grouping of the cytokines associated with Th1, Th2, Th17 and regulatory T cell (Treg) responses, showed significant increases particularly for Th1 and Th17 responses. Using imprint culture, *Candida* was isolated from 48 patients (29 with DS). DS patients had significantly higher numbers of *Candida* isolated from the fitting surface of the denture (P=0.0113). However, there was significantly (P=0.03) higher numbers of *Candida* on the palate of non-DS patients. In summary, it would appear that an appropriate immune response is elicited in DS patients and whilst this appears to reduce *Candida* levels on inflamed mucosa, it is probable that *Candida* on the fitting surface of the denture remain protected from local immune responses which results in a continued reservoir of infection.

**Keywords:** Immune response; Candida; Denture stomatitis

## Introduction

Denture stomatitis (DS), also known as denture-related stomatitis, denture induced stomatitis or chronic erythematous candidosis, is an oral condition that presents clinically as erythematous and inflamed areas of mucosa exclusively limited to those sites covered by a denture. It has been reported that the majority (67%) of denture wearers suffer from some degree of DS [1]. DS is usually asymptomatic, although some patients may complain of itching, burning, discomfort and occasional bleeding. In its mildest form, DS may arise purely from a poor fit of the denture. However, the aetiology of DS is likely to be complex in more clinically severe cases. In addition to physical irritation [2], potential precipitating factors include, poor denture hygiene [3], continuous wearing of dentures [4], poor denture quality, undiagnosed or poorly controlled diabetes mellitus [5], reduced salivary gland function [6], tobacco use [7], hypersensitivity reactions to denture materials, immune-suppression or immune-compromised states [8], and *Candida* infection [9]. With regards to the latter, DS is considered to be the most prevalent form of oral candidosis [1,10].

Importantly, it has been shown that the visible mucosal inflammatory changes associated with DS actually reduce following treatment with either topical or systemic antifungal therapy [11]. This response provides strong evidence for the role of *Candida* in the aetiology of DS. However, inflammation usually rapidly recurs following cessation of such antifungal treatment, an observation that, in part, probably reflects the failure to eradicate the presence of *Candida*, on the denture surface. Therefore, to fully control DS, it is likely to be necessary to simultaneously reduce mucosal inflammation and the microbial colonisation of the denture. As a consequence, dentures should be removed during sleep, since this will reduce any physical irritation and permit access of saliva to the mucosa. It is also important to keep the dentures in a clean storage container so that the denture is not re-colonised. In addition, adequate denture hygiene should be regularly maintained, such as through the regular soaking and brushing of dentures using a denture cleanser in an attempt to eliminate the *Candida* biofilm.

An appropriate host immune response is considered essential in the control of *Candida* infection at oral mucosal surfaces. The key factors involved in the protection of mucosal infection by *Candida* are those associated with cell-mediated immunity. Host cells first recognise *Candida* by detection of pathogen-associated molecular patterns (PAMPs) on the surface of *Candida* cells and this is mediated by pattern recognition receptors (PRRs), such as Dectin-1, TLR2 and TLR4 on the host cells including those of the oral epithelium [12-14]. Following this interaction, phagocytic clearing of *C. albicans* by polymorphonuclear cells (PMNs) and macrophages occurs.

Host immune cells in the mucosa, particularly those involved in innate immunity, such as dendritic cells, macrophages and neutrophils recognise *Candida* PAMPs and produce cytokines to drive adaptive immune responses involving T cells and B cells in candidosis [15]. All these cells are able to produce cytokines and generate a tissue environment that can either promote or suppress the immune mechanisms involved in controlling *Candida*. $CD4^+$ T cells are the most influential host immune cells in this respect, and dictate immunity against *Candida* by secreting cytokines, such as those associated with Th1 (IFNγ and IL-2), Th17 (IL-17a and IL-22) and Treg cells (IL-10 and TGF-β1) [16]. It is now generally accepted, that Th1/Th17-type responses are protective against candidosis [17,18], whilst a Th2 response is non-protective [19]. Interestingly, Treg cells are positively associated with elevated Th17 cells in the skin [16]. Tregs can secrete

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TGFβ1 which is a critical cytokine for Th17 development and can also suppress Th1 cells and dendritic cell function, resulting in immune suppression [20,21]. Therefore, inflammatory and anti-inflammatory cytokine production may fluctuate, resulting in a delicate balance of host immunity and pathogen growth. Importantly, excessive Th1 and Th17 responses could also contribute to inflammation and tissue damage.

The role of T helper cells in response to fungal infection has been widely investigated using animal models [22,23]. As mentioned previously, Th1 and Th17 responses have been suggested to play a protective role during *Candida* infection, whilst Treg and Th2 responses are associated with a *Candida*-related pathology. Consequently, in acute *Candida* infection of immunocompetent individuals, as might be expected to occur in the early stages of DS, it would be hypothesised that elevated Th1 and/or Th17 responses would occur, compared with Th2 responses. As such, an increased production of IFNγ, IL-2 and TNFα, together with a simultaneously low level of IL-4, IL-5 and IL-10 cytokines (associated with Th2 responses) might be anticipated. Conversely, for subjects who have DS with chronic inflammation induced by *Candida*, a Th2 response might be anticipated (indicating an inability to raise a protective immune response) with an appropriate cytokine profile reflecting this. However, despite the existence of a broad knowledge base concerning host immune responses during *Candida* infection, limited studies have been undertaken concerning the local inflammatory response associated with mucosal infection by *Candida* in humans.

The aim of the present study was to investigate the local cytokine response elicited by the host, in addition to the extent of *Candida* colonisation in denture wearing patients, with and without DS. It was envisaged that the results would provide information on the role of host immune status in combating DS and potentially highlight diagnostic markers that could indicate DS prognosis.

## Materials and Methods

### Patients groups studied

A total of 93 patients from Cardiff and Vale NHS Trust were recruited through an existing subject database, clinician referrals and the local media. The study had previously received ethical approval main REC reference number 09/H0206/32 and all subjects provided written informed consent. The study population comprised of 55 males (mean age 69.4 years) and 38 females (mean age 69.1 years). All patients wore a complete upper maxillary denture and the majority (n=91) were Caucasian.

### Classification of DS

Following removal of the patient's upper denture, the appearance of the palatal mucosa was scored according to the Newton classification scale [24]. This was 0=none, 1=pin-point hyperaemic lesions (localized erythema), 2=diffuse erythema (generalized simple inflammation), and 3=hyperplastic granular surface (inflammatory papillary hyperplasia). For standardisation, all examiners had previously been calibrated to score the extent of erythema.

### Isolation and identification of *Candida*

The presence of *Candida* on the fitting surface of the denture, the palate and the tongue was determined using an imprint culture technique that also permitted quantification of *Candida* at the test site [25]. Briefly, sterile foam squares (2 $cm^2$) were initially moistened in sterile water and placed on the target site for 30 s. The foam squares were then transferred to CHROMagar® *Candida* agar plates for 30 min and then removed. The agar plates were then incubated at 37°C for 48 h. The number of *Candida* colony forming units (CFUs) was recorded and expressed per $cm^2$. Representative colony types were selected for definitive identification based on standard morphological testing (germ-tube test), biochemical profiling using the Auxocolour 2 system (Biorad), and sequence analysis of PCR amplified ribosomal DNA, as previously described (ITS1 and ITS4; [26]).

### Cytokine collection and analysis

Fluid from the palatal mucosa was collected by absorption on to filter paper strips. A piece of filter paper (2 × 3 cm, Whatman Chromatography grade No. 3 mm) was placed on each side of the fitting surface of the upper denture, which was then placed in the mouth for 5 min. The denture was then removed and filter paper placed in a microcentrifuge tube containing 400 µl of phosphate buffered saline, 5% FBS, 0.05% sodium azide, protease inhibitor and Tween (0.002%). The vial was then agitated gently for 2 h at 4°C and after this period, contaminating particles were removed by centrifugation and the solution stored at -70°C until analysis.

To determine the efficiency of cytokine recovery from the filter paper, positive controls of buffer, which had been artificially contaminated with a known quantity of specific cytokines was analyzed. The BD™ Cytometric Bead Array (CBA; BD) Human Th1/Th2/Th17 Cytokine Kit was used to quantify cytokines (IFNγ, IL-2, TNFα, IL-4, IL-6, IL-10, IL-17a) in single tissue fluid samples (minimum sensitivity estimated at 2 pg/ml for the cytokines), as described in the manufacturer's recommended protocol. Single plex flex kits (CBA; Becton) were used to quantify TGF-β1 and IL-5. An Enzyme-Linked Immunosorbent Assay (ELISA, ebioscience) was used to quantify IL-22 in the palatal fluid samples. The CBA and single plex kit samples were analysed on a FACSCalibur machine (BD Biosciences) and the ELISA results were measured on a fluostar optima machine.

### Statistical methods

Summary statistics (means, standard deviation (SDs), medians, maxima, minima) were obtained for the study variables. P-values for differences in patients with DS versus patients without DS in logarithmic *Candida* counts as well as cytokine levels were calculated using the non-parametric Wilcoxon Rank Sum test. All tests were performed at a 5% significance level. Correlation values between the different cytokines measured were also calculated.

## Results

Of the recruited patients, 30 males and 21 females had no clinical evidence of DS (Newton score 0), whilst 25 males (mean age 59.5 years) and 17 females (mean age 40.5) had varying degrees of erythematous changes in the palate consistent with DS (Newton score 1, 2 and 3). Baseline demographics of the patients studied also revealed that of the 93 patients, 22 were smokers (10/42 with DS and 12/51 without DS).

### Isolation and identification of *Candida*

Table 1 summarises the extent of *Candida* colonisation at specific sites related to Newton's Classification of DS. *Candida* was isolated from 48 patients (29/42 with DS, and 19/51 without DS). The mean *Candida* count for patients without DS (Newton's Classification=0) was 9.97 cfu/$cm^2$ on the palate, 12.8 cfu/$cm^2$ on the tongue, and 9.93 cfu/$cm^2$ on the denture. In comparison, for patients categorised with DS (Newton's class=1, 2 and 3) the mean palatal count was 8.46 cfu/$cm^2$, tongue count was 16.64 cfu/$cm^2$ and fitting surface of denture count

| | | Newton's classification | | | |
|---|---|---|---|---|---|
| **Sample site** | **Statistical measure** | **0** | **1, 2 and 3** | **1** | **2 and 3** |
| Denture | N | 51 | 42 | 27 | 15 |
| | mean | 9.93 | 24.23 (P=0.01) | 19.44 | 32.85 |
| | SD | 22.74 | 31.67 | 30.67 | 32.65 |
| | CV (%) | 229.1 | 130.7 | 157.8 | 99.4 |
| | Median | 0.00 | 5.00 | 0.50 | 24.50 |
| | Minimum | 0.00 | 0.00 | 0.00 | 0.0 |
| | Maximum | 75.0 | 75.0 | 75.0 | 75.0 |
| Palate | | | | | |
| | Mean | 9.97 | 8.46 (P=0.03) | 5.32 | 14.12 |
| | SD | 24.26 | 19.84 | 15.44 | 25.63 |
| | CV (%) | 243.3 | 234.4 | 290.0 | 181.5 |
| | Median | 0.00 | 1.00 | 0.25 | 1.50 |
| | Minimum | 0.00 | 0.00 | 0.0 | 0.0 |
| | Maximum | 75.0 | 75.0 | 75.0 | 75.0 |
| Tongue | | | | | |
| | Mean | 12.80 | 16.64 (P=0.004) | 13.35 | 22.55 |
| | SD | 27.27 | 26.11 | 24.747 | 28.31 |
| | CV (%) | 213.1 | 157.0 | 185.3 | 125.5 |
| | Median | 0.00 | 4.38 | 3.00 | 10.50 |
| | Minimum | 0.00 | 0.00 | 0.0 | 0.0 |
| | Maximum | 75.0 | 75.0 | 75.0 | 75.0 |

CV(%): Coefficient of variation.
P-value is based on the Wilcoxon Rank Sum test.

**Table 1:** *Candida* growth (cfu/cm$^2$) obtained from three sites and Newton's classification of denture stomatitis in 93 patients.

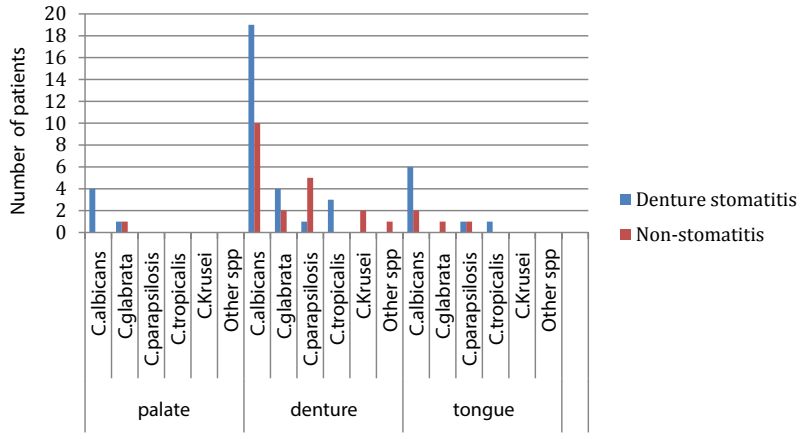


**Figure 1:** The frequency of *Candida* species from the three different oral sites in two patient groups. *C. albicans* was the most prevalent on the denture out of the three sites in denture stomatitis patients (19/42; 45%), followed by *Candida glabrata* (4/42; 10%) and *C. tropicalis* (3/42; 7%). In the non denture stomatitis group, *C. albicans* was the most prevalent again at the denture site, found in 10/51 (20%) followed by *C. parapsilosis* (5/51; 10%) *C. glabrata* (2/51; 4%), and *C. krusei* (2/51; 4%). When the results for the three sites were combined, *C. albicans* was again the most prevalent species in the denture stomatitis group (24/42; 57%) and in the non denture stomatitis group (11/51%; 22%).

was 24.23 cfu/cm$^2$. A statistically higher *Candida* count was evident on the tongue (P=0.043) and fitting surface of the denture (P=0.0113) for patients with DS compared to those without DS. Interestingly, the palatal *Candida* count of DS patients was significantly lower (P=0.0301) compared with those without DS.

Figure 1 illustrates the frequency of *Candida* species isolated from different oral sites in patients with and without DS. It was evident that *C. albicans* was the most prevalent species in DS patients (24/42; 57%), with *Candida glabrata* (5/42; 12%), *C. tropicalis* (4/42; 10%) and *C. parapsilosis* (2/42; 5%) also encountered. Mixed *Candida* species were recovered from 7 (17%) of the 42 DS patients. In patients without DS, the recovered *Candida* species were *C. albicans* (11/51; 22%), *C. glabrata* (4/51; 8%), *C. parapsilosis* (5/51; 10%), and *C. krusei* (2/51; 4%). Four patients (8%) without DS were colonized by mixed *Candida* species. In a number of patients, different *Candida* species were found at multiple sites.

## Cytokine profiling at the mucosal surface of DS and non DS patients

A summary of the level of detected cytokines is presented in Table 2 and Figure 2. Statistical analysis showed that the following cytokines were significantly elevated in patients with DS (Newton's class=1, 2

| | | Newton's Classification | | | |
|---|---|---|---|---|---|
| **Cytokine** | **Statistic** | **0 (n=47)** | **1, 2 and 3 (n=42)** | **1 (n=27)** | **2, 3 (n=15)** |
| IFN-γ | Mean | 0.24 | 3.83 (P=0.0004) | 0.47 | 9.88 |
| | SD | 1.39 | 11.9 | 1.39 | 18.71 |
| | CV (%) | 590.9 | 310.8 | 298.9 | 189.4 |
| | Median | 0.00 | 0.07 | 0.00 | 0.74 |
| | Minimum | 0.0 | 0.0 | 0.0 | 0.0 |
| | Maximum | 9.6 | 64.4 | 6.8 | 64.4 |
| IL-2 | Mean | 0.22 | 0.42 (P=0.058) | 0.36 | 0.55 |
| | SD | 0.40 | 0.54 | 0.49 | 0.63 |
| | CV (%) | 180.5 | 127.9 | 136.5 | 115.5 |
| | Median | 0.00 | 0.00 | 0.00 | 0.46 |
| | Minimum | 0.0 | 0.0 | 0.0 | 0.0 |
| | Maximum | 1.2 | 2.2 | 1.7 | 2.2 |
| IL-4 | mean | 0.33 | 0.46 (P=0.139) | 0.52 | 0.34 |
| | SD | 0.56 | 0.566 | 0.622 | 0.444 |
| | CV (%) | 168.3 | 124.1 | 119.9 | 129.5 |
| | Median | 0.00 | 0.16 | 0.00 | 0.32 |
| | Minimum | 0.0 | 0.0 | 0.0 | 0.0 |
| | Maximum | 2.0 | 1.6 | 1.6 | 1.4 |
| IL-5 | Mean | 0.56 | 0.80 (P=0.0744) | 0.68 | 1.01 |
| | SD | 0.76 | 0.74 | 0.77 | 0.640 |
| | CV (%) | 136.3 | 92.4 | 113.6 | 63.3 |
| | Median | 0.00 | 0.16 | 0.63 | 1.09 |
| | Minimum | 0.0 | 0.0 | 0.0 | 0.0 |
| | Maximum | 2.6 | 1.6 | 2.6 | 2.4 |
| IL-6 | Mean | 3.48 | 28.32 (P<0.0001) | 9.16 | 62.82 |
| | SD | 5.21 | 40.06 | 10.64 | 63.40 |
| | CV (%) | 149.8 | 162.6 | 116.2 | 100.9 |
| | Median | 2.08 | 8.63 | 5.49 | 37.67 |
| | Minimum | 0.0 | 0.0 | 0.0 | 3.7 |
| | Maximum | 30.5 | 215.7 | 41.9 | 215.7 |
| IL-10 | Mean | 0.08 | 1.06 (P<0.0001) | 0.47 | 2.13 |
| | SD | 0.16 | 2.14 | 0.92 | 3.15 |
| | CV (%) | 204.9 | 200.8 | 195.7 | 147.4 |
| | Median | 0.00 | 0.17 | 0.00 | 1.09 |
| | Minimum | 0.0 | 0.0 | 0.0 | 0.0 |
| | Maximum | 0.6 | 10.2 | 3.9 | 10.2 |
| IL-17a | Mean | 1.02 | 8.35 (P=0.026) | 2.78 | 18.39 |
| | SD | 2.37 | 18.45 | 4.67 | 28.07 |
| | CV (%) | 233.1 | 220.9 | 168.3 | 152.7 |
| | Median | 0.00 | 0.00 | 0.00 | 6.89 |
| | Minimum | 0.0 | 0.0 | 0.0 | 0.0 |
| | Maximum | 9.0 | 89.2 | 13.7 | 89.2 |
| IL-22 | Mean | 9.90 | 76.70 (P=0.0004) | 33.39 | 154.65 |
| | SD | 13.66 | 124.13 | 74.50 | 157.14 |
| | CV (%) | 138.0 | 161.8 | 223.1 | 101.6 |
| | Median | 0.00 | 24.72 | 7.81 | 91.11 |
| | Minimum | 0.0 | 0.0 | 0.0 | 0.0 |
| | Maximum | 39.3 | 411.4 | 321.8 | 411.4 |
| TGF-β1 | Mean | 258.67 | 360.39(P<0.0001) (P<0.0001) | 321.37 | 430.64 |
| | SD | 495.52 | 308.77 | 297.32 | 326.81 |
| | CV (%) | 191.6 | 85.7 | 92.5 | 75.9 |
| | Median | 186.89 | 280.11 | 273.48 | 343.90 |
| | Minimum | 10.1 | 93.4 | 93.4 | 167.1 |
| | Maximum | 3537.0 | 1705.5 | 1705.5 | 1335.7 |
| TNF-α | Mean | 0.02 | 0.69 (P=0.0009) | 0.61 | 0.84 |
| | SD | 0.048 | 2.079 | 2.26 | 1.765 |
| | CV (%) | 301.3 | 300.0 | 371.6 | 209.1 |
| | Median | 0.00 | 0.00 | 0.00 | 0.15 |
| | Minimum | 0.0 | 0.0 | 0.0 | 0.0 |
| | Maximum | 0.2 | 11.6 | 11.6 | 6.1 |

CV (%): Coefficient of Variation.
P-value is based on the Wilcoxon Rank Sum test.

**Table 2:** Level of 10 different cytokines and Newton's classification of denture stomatitis in 93 patients.

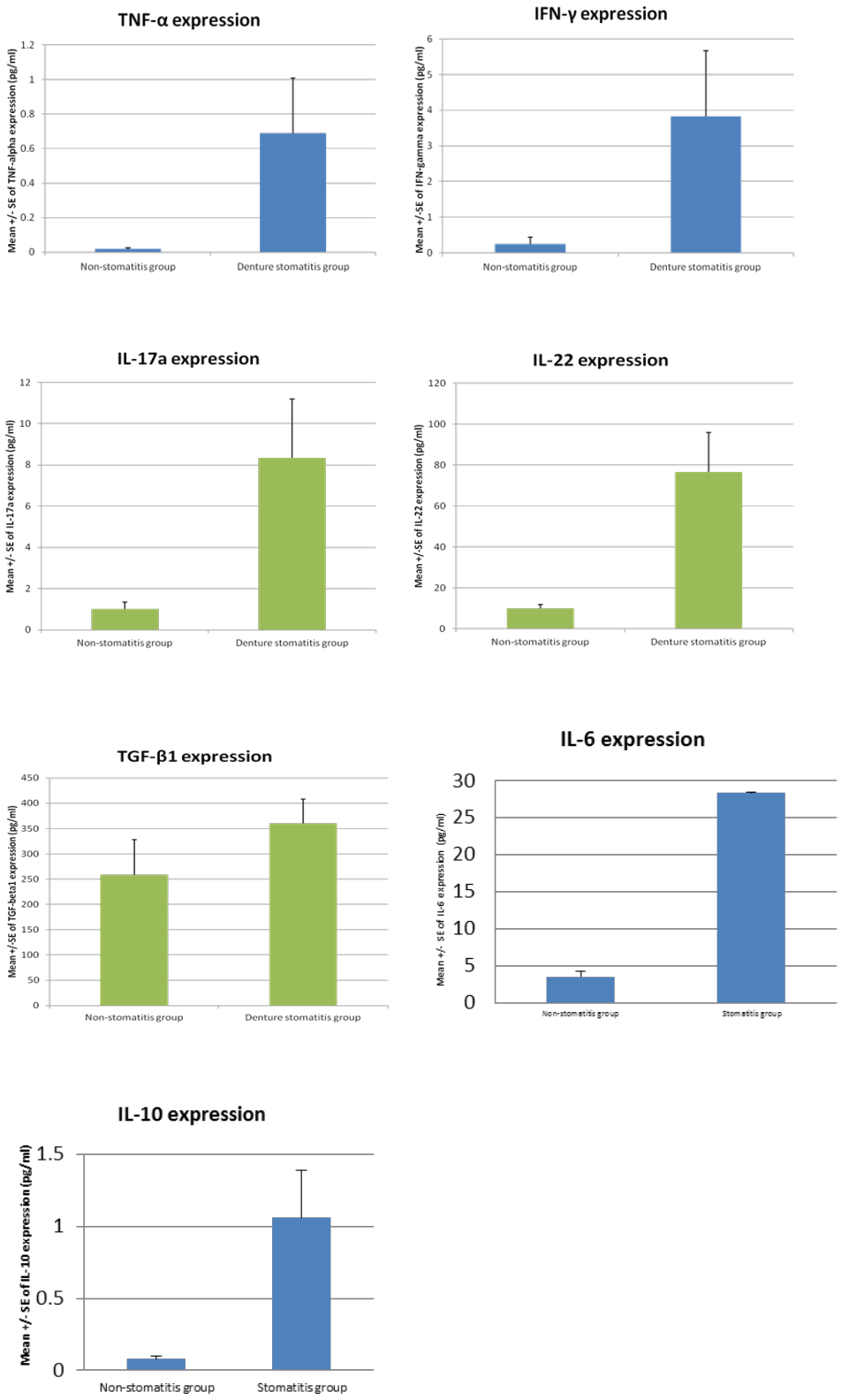


**Figure 2:** Levels of Th1 cytokines (IFNγ, P=0.0004, TNFα, P=0.0009) measured from the palatal secretions were significantly higher in the denture stomatitis group compared to the non-stomatitis group. IL-2 did not show a significant increase (data not shown). There was no significant increase in the Th2 cytokine levels (IL-4 and IL-5, data not shown). The levels of Th-17 cytokines (IL-17a and IL-22) were significantly increased in the denture stomatitis group compared to the non-stomatitis group (IL-17a, P=0.0026 and IL-22, P=0.0004). A significant (P=0.0001) increase in TGF-β1 was also evident and this is a critical cytokine for Th17 cell differentiation. IL-6 (P<0.0001) and IL-10 (P<0.0001) are also significantly increased in denture stomatitis patients.

and 3) compared with those with no DS (Newton's class=0): IFN-γ (P=0.0004), IL-6 (P<0.0001), IL-10 (P<0.0001), IL-17a (P=0.026), IL-22 (P=0.0004), TGF-β1 (P<0.0001) and TNF-α (P=0.0009). Grouping of the cytokines associated with Th1 (IFN-γ, TNF-α, and IL-2), Th2 (IL-4, IL-5 and IL-10) and Th17 (IL-17a, IL-22 and TGF-β1) responses, showed significant increases in Th1 (P=0.0008), Th2 (P=0.0028) and Th17 (P<0.0001) responses in DS patients. Of the cytokines in the Th2 group, only IL-10 was significantly increased in stomatitis, IL-4 and IL-5 were not. The Treg cytokines IL-10 and TGFβ-1 were also significantly elevated in the stomatitis group. The most significant changes were however, with Th1 and Th17 responses.

### Correlation between the different cytokine levels in DS patients

In the DS group, it was evident that the levels of IL-17a strongly correlated with those of IL-22 ($R^2$=0.556). Since both of these cytokines are produced by Th-17 cells this finding was anticipated. Associations were also seen between IFNγ and IL-17 ($R^2$=0.590), and IFNγ and IL-22 ($R^2$=0.541). As IFNγ production is strongly associated with Th-1 cells, this would further suggest that Th-1 and Th-17 responses against *Candida* co-exist in DS patients. There was no correlation between TNFα/IFNγ ($R^2$=0.101), nor between TNFα/IL-10 ($R^2$=0.247). Although IL-10 and TGFβ-1 may both originate from Treg cells, in DS patients, IL-10 levels were not associated with TGFβ-1 ($R^2$=0.001). This may indicate that TGFβ-1 and IL-10 in DS patient samples had been generated by other cells in the mucosa, such as fibroblast cells, keratinocytes and macrophages.

## Discussion

DS is considered the most prevalent form of oral candidosis, reportedly occurring in 65% of all denture wearers, often without clinical symptoms [1]. There are several contributory aetiological factors along with *Candida* colonisation that are associated with DS and these include: reduced salivary flow and function [6], denture trauma through a poorly fitting denture [2], and an impaired or senescent immune response [27].

A number of previous investigations have examined the microbiology of DS, particularly with respect to the *Candida* species colonisation of the oral cavity [28-30]. In such studies, *C. albicans* is reported as being the most prevalent *Candida* species on the dentures of stomatitis patients, with an incidence of 54-75%, and a lower incidence evident on the dentures on non DS patients (39%) [28-30]. In the present study, similar findings were reported, with a 57% incidence of oral colonisation by *C. albicans* in DS patients and a 22% incidence in non-DS patients. Of additional interest was the apparent absence of *Candida* in the oral cavity of 13 (31%) DS patients. This finding supports the view that other microorganisms apart from *Candida* could contribute to DS and indeed previous studies have implicated a range of Gram-negative [31] and Gram-positive bacteria [32] with the condition.

Innate immune responses are integral components in host defense against mucosal infection by *Candida*. The oral epithelium is the first line of host protection against *Candida* and as mentioned earlier the 'passive barrier function' of epithelium protection can be impaired by trauma from a poor fitting denture. However, the cells within the oral epithelium can detect the presence of an invading *Candida* through the possession of PRRs (*e.g.* Dectin 1 and TLRs) which interact with PAMPs on the surface of the fungi [33,34]. Following this recognition, host cells can release a range of cytokines/chemokines and immune effector molecules to initiate the adaptive immunity, such as the T cell response. T cells will migrate into sites of infection to produce cytokines to drive the innate immune cells, such as macrophages and neutrophils towards candidal phagocytosis. The cytokines produced by $CD4^+$ T helper cells largely determine the local inflammatory environment to control infection and promote phagocytosis. Th1 cells produce mainly IFNγ, and Th17 produce IL-17 and IL-22 in response to *Candida* leading to a protective effect. Th2 produce IL-4 and IL-5, and Treg cell responses generate TGFβ1, IL-10 and IL-35 which have an immunosuppressive response. In terms of cytokine production in DS, very few studies have been undertaken. This is important as determining whether or not an appropriate immune response is being generated in this condition provides key information in understanding the pathology of DS.

It has previously been found that elevation of IL-4 in the serum of elderly DS patients is coupled with reduced levels of IL-12 in saliva. In younger DS patients, an increase in salivary IL-10 levels has also been reported, together with higher levels of IFN-γ in both the saliva and the serum [35]. In our present study the levels of a number of cytokines including IFN-γ, IL-10, IL-17a, IL-22, TGF-β1 and TNF-α were all found to be locally increased. When grouped according to the type of T-helper cell response, perhaps unsurprisingly, compared with patients without inflammatory responses, Th1 (IFN-γ an IL-2), and Th17 (IL-17a and IL-22) responses were significantly elevated. The levels of anti-inflammatory cytokines, (IL-10 and TGFβ1), were seen to have increased in palatal mucosa. These cytokine may be associated with increased numbers of Treg cells during the infection. Treg cell ($CD4^+CD25^+Foxp3^+$ T cell) responses are normally considered to suppress Th1 and Th17 responses. A recent study has, however, showed that Treg cells can promote Th17 for IL-17 and IL-22 production in mouse oral *Candida* infection [18]. This present study is the first to suggest this same phenomenon also happens during *Candida* mucosal infection in humans. These results imply that patients with DS generate a local T-helper cell response, particular for Th1, Th17 and Treg responses that would be considered protective against infection by *Candida*. Indeed it was interesting to note that significantly higher numbers of *Candida* occurred on the dentures of DS patients, although on the palate itself, lower counts were reported compared with non-DS patients. This finding would support the view that in the DS patients, whilst an effective and appropriate local inflammatory response was indeed present to combat palatal colonisation by *Candida*, this was not able to lower candidal levels on the denture. Importantly however, such a response might effectively block *Candida* penetration into the mucosa and limit systemic infection. The likely situation of poor denture hygiene would contribute to the high *Candida* colonisation of the denture in DS patients, which could then provide a continuous reservoir of infecting *Candida* or their virulence factors to the palatal mucosa. Since by definition, the non DS patients had no palatal inflammation, a protective immune response was not being raised, potentially allowing higher palatal *Candida* colonization in these patients. These findings would be supported and validated by further larger studies.

Based on these findings, the importance of reducing the number of *Candida* on the denture is clearly evident. The results of this study suggested that whilst Th1/Th17/Treg response may be of benefit in controlling *Candida* levels at mucosal sites in DS, such responses alone do not facilitate resolution of the infection.

## Funding

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## Conflict of Interests

Potential conflict of interests: HR, MAOL, XQW, DWW, JSR and RVW have all received research funding from GlaxoSmith Kline as part of a larger clinical trial. BM and AG are both employed by GlaxoSmithKline. VP no conflict of interest.

## Meetings and Presentations

Part of this work has been presented as a poster at the PER in Helsinki, Finland, September 2012 and will be presented as a poster at the BSODR in Bath, UK, September 2013.

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