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ORIGINAL ARTICLE

# The association of *Candida* and antifungal therapy with pro-inflammatory cytokines in oral leukoplakia

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## Abstract

**Objectives** To study the association of *Candida* and antifungal therapy with pro-inflammatory cytokines (PIC) in oral leukoplakia (OL).

**Materials and methods** A prospective observational study where immunocompetent adult subjects with OL (30 homogenous (HL), 30 non-homogenous (NHL)) and 30 age and sex-matched healthy controls (C) with no predisposing factors for oral *Candida* infection were recruited. Sterile cotton swabs and ophthalmic sponges were used to sample the lesion surface in OL and buccal mucosa in C, for direct microscopy and culture for *Candida* and to determine levels of PIC (IL-6, IL-8. IL-17, TNF-α) by ELISA, respectively. Sampling for PIC was repeated at same sites in OL, 2 weeks after antifungal therapy.

**Results** *Candida* was associated with 55.3% of NHL, 23.3% of HL and 13.3% of C. The oral secretary levels of PIC were raised in NHL as compared to HL and C. The levels of IL-6, IL-8, TNF-α ($p<0.001$) and IL-17 ($p<0.01$) were significantly raised in *Candida* positive NHL while IL-6 ($p<0.05$) and TNF-α ($p<0.01$) were significantly raised in *Candida* positive HL before antifungal treatment. After antifungal treatment, there was significant reduction in PIC in *Candida* positive NHL and HL.

**Conclusions** *Candida* infection contributes to the inflammatory milieu in *Candida* associated OL which increases the risk of carcinogenesis. Antifungal therapy reduces the PIC in *Candida* associated OL.

**Clinical relevance** Identification and elimination of predisposing factors for *Candida* infection, like cessation of harmful habits, maintenance of oral/denture hygiene, surveillance for *Candida* and antifungal therapy at intervals, are recommended in OL.

**Clinical trial registration** ClinicalTrials.gov Identifier: NCT04712929

**Keywords** *Candida* · Oral leukoplakia · IL-6 · IL-8 · IL-17 · TNF alpha · Antifungal agents · Pro-inflammatory cytokines

## Introduction

The role of persistent inflammation induced by infection and chemical irritants is important in carcinogenesis as it contributes up to 25% of all cancer cases [1]. The link between chronic inflammation and oral cancer has regained renewed importance after various epidemiological studies have revealed the prominent role it may play in its development [2]. The hypothesis that was first proposed by Virchow in 1863 has been strengthened by molecular connections between

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inflammation and oral cancer that have been discovered since [3]. The various inflammatory mediators that are implicated are nuclear factor kappa B (NF-kB), VEGF, Inflammatory cytokines, Prostaglandins, p53, nitric oxide (NO), reactive oxygen species (ROS) and nitrogen species and specific microRNAs. The pro-inflammatory cytokines (PIC) produced in a dysregulated manner, play a role in cell growth, invasion, interruption of tumour suppression, immune status and survival by providing a permissive environment for the growth of cancer. PIC generated by local inflammatory reaction may cause DNA damage and cell proliferation, thereby predisposing potentially malignant lesions to neoplasia. They are also implicated in inducing further growth, angiogenesis, invasion, and metastasis in malignant tumours [4].

Previous in vitro and in vivo studies with samples from tissue, serum and saliva have shown that NF-kB dependent (IL-1 alpha, IL-6, IL-8, TNF-alpha, GM-CSF) and IL-17 inflammatory cytokines are increased in OSCC [5–10]. The same repertoires of cytokines are also raised in tissue, serum and saliva of patients with oral potentially malignant disorders (OPMDs) like oral leukoplakia (OL) [11–17]. These cytokines can therefore act as relevant diagnostic and prognostic indicators of carcinogenesis in OL [5, 11].

OL is a common potentially malignant lesion of the oral cavity usually associated with tobacco and alcohol use and with a high malignant transformation rate (0.3–34%) into oral squamous cell carcinoma (OSCC) [18]. The different types of OL are Homogenous leukoplakia (HL) with a uniform, flat thin, smooth/wrinkled/corrugated surface throughout the lesion whereas non-homogenous leukoplakia (NHL) has a mixture of red and white lesions with an irregularly speckled/nodular/verrucous surface [19].

The etiological role of *Candida* in OL has been a matter of debate over the years but candidal invasion has been associated with significant risk factor for oral cancer and malignant transformation in OL [20, 21]. The association has not been proven to be causal but believed to be incidental due to favourable environmental factors and surface provided for adherence of *Candida*. The ability of *Candida* to colonise, penetrate and invade the tissues depends on its virulence factors and host immune responses. The carcinogenic potential of *Candida* in OL has been found to be due to the nitrosation potential of *Candida* which has been found to be higher from organisms isolated from NHL than HL. The ability of *Candida* to produce acetaldehyde from precursors in oral cavity and the upregulation of inflammatory and proliferative markers in epithelium are others contributing factors [21–23]. *Candida* infection has been found in 6.6–100% of OL and is higher in NHL (87.5%). OL with *Candida* is also associated with epithelial atypia (14.3–56.3%), dysplasias (55.9%) and malignant transformation (2.5–28.7%) [21].

In vitro studies have shown that in oral epithelial cells, the innate immune response to *Candida* (a common commensal of oral cavity) is by producing the same PIC (IL-6, IL-8, IL-17, TNF-α) which are seen in OSCC and OL [24, 25]. Oral *Candida* therefore, could be contributing to the inflammatory milieu in OL and increasing the risk for carcinogenesis. Antifungal therapy has been recommended in OL which results in clinical improvement and reduction in oral *Candida* counts [26–29]. The correlation between *Candida* in OL with the levels of PIC and the change in levels of these cytokines after antifungal therapy has not been explored before. This study aims to study the association of *Candida* with antifungal therapy and PIC in OL.

## Materials and methods

A prospective case-control pilot study was conducted to study the association of *Candida* with antifungal therapy and PIC in OL. Ethical clearance from Institute Ethics committee (Reference Number: IEC-492/07.09.2018, RP-21/2018) and written informed consent was obtained before recruitment of subjects. The study subjects were recruited from the Out-Patient Department (OPD) of Oral Medicine and Radiology CDER at All India Institute of Medical Sciences New Delhi. The study included 60 adult immunocompetent subjects with clinical diagnosis of OL (30 homogenous leukoplakia (HL), 30 non-homogenous leukoplakia (NHL) (speckled/verrucous/nodular)) who had not been under treatment for the same or any antifungal therapy for past 6 months. The control group (C) included 30 approximately age and sex matched healthy subjects reporting to the OPD for other routine dental problems, who did not have any history of tobacco, areca nut or alcohol habits and did not have any OPMD. Only subjects with simplified oral hygiene index score (OHI) 0–3, periodontal screening and recording (PSR) code 0–2 were included in the study. Subjects with any history of significant and serious uncontrolled systemic disease, chronic inflammatory diseases, allergy to fluconazole, malignancy, antifungal therapy and pregnant women were excluded. Subjects with history of recent major/minor surgery and predisposing factors for oral candidiasis like diabetes/endocrine disorders, xerostomia, poor oral hygiene, removable prosthesis, prolonged corticosteroid/antibiotic/immunosuppressant/antibacterial mouthwash therapy, radiation/chemotherapy, auto immune disorders, primary/secondary immune deficiencies, known genetic conditions with susceptibility to *Candida* infections, nutritional deficiencies and hospitalised debilitated patients were also excluded. All subjects with current tobacco, areca nut or alcohol habits were counselled for habit cessation. The clinical characteristics and staging/grading for OL was done as per OLEP system (van der Waal 2000). Routine blood investigations were done to rule out undiagnosed systemic condition. On the day of sampling, firstly sterile swabs were used to collect samples for *Candida* detection from the surface of

the lesions. After 30 minutes, sampling with Merocel ophthalmic sponges was done from the surface of the lesions for PIC estimation. This was followed by incisional punch biopsy under local anaesthesia.

## Sample collection from oral lesions

The subjects were asked to abstain from eating, drinking and rinsing the mouth at least 2 hours prior to sampling. Two sterile swabs for each study subject (pre-wet with sterile normal saline) were taken from the oral lesion in OL, buccal mucosa from the control subjects and coded before further processing for Microbiology. The collected swabs were examined by direct microscopy with 10% KOH, cultured on Sabouraud dextrose agar (SDA) plates at 37°C followed by further speciation as per standard mycological procedures. Four Sterile Polyvinyl Alcohol (PVA) ophthalmic sponges (Merocel) were used to collect samples of oral secretions for four pro-inflammatory cytokine study from the oral lesion (other than biopsy site) by method described before and coded before further processing [30]. This is a non-invasive method and makes it site-specific which is an advantage over other serum and saliva estimations. In addition, assessment of local cytokine levels via longitudinal sampling in patients undergoing antifungal therapy in OL may allow for an understanding of the temporal nature of cytokine elevation and clinical response to antifungals.

The sponges were pre-wet with sterile normal saline, kept in contact with the lesion without rubbing or movement for 1 minute and then immediately stored in sterile containers at −80 °C until further analysis. A photographic and clinical record of the sample sites was kept as reference for future sampling of lesion in OL. This procedure was repeated at the same sites (avoiding $1cm^2$ area around biopsy site) after 14 days of antifungal therapy in OL study group.

## Incisional biopsy for histopathological examination:

The site for incisional biopsy was determined by clinical examination, inspection under white light and palpation. Lesions that were visibly ulcerated and indurated on palpation were considered highly suspicious of malignancy and excluded from the study. The area of the lesion with the worst clinical appearance (speckled/verrucous/nodular) in the highest risk site (floor of mouth/ventral/lateral surface of tongue/soft palate/maxillary retromolar areas) in non-homogenous oral leukoplakia, and areas from high-risk sites and/or the most symptomatic area when present in homogenous leukoplakia was selected as the site for biopsy. 4mm punch biopsy with adequate depth into connective tissue was done under local anaesthesia and haemostasis achieved using pressure pack and local measures. Post-operative clinical photograph of lesion with biopsy site was kept as record for future reference. Routine post-operative instructions were given and Tab Paracetamol 500mg (maximum daily dose of 2gm) was advised as a rescue medicine for symptomatic pain relief. The H & E histopathological features with PAS staining were recorded and grading of dysplasia was done as per standard WHO criteria.

## Antifungal therapy

The study group was treated with antifungal therapy (Tab Fluconazole 100 mg as a mouthwash (tablet dissolved in 10 ml of drinking water and used as a mouth rinse for 2 minute and swallowed) once a day for 14 days). Dispersible form of Fluconazole was selected as it provided a standard single dose per day regimen as against other topical antifungals which have limited time of contact with lesion and require multiple doses affecting patient compliance. The procedure was demonstrated to each study subject before starting the treatment. The subjects were advised to start Fluconazole treatment 24 hours after biopsy.

## Estimation of IL-6, IL-8, IL-17 and TNF-α levels by ELISA

The ophthalmic sponges (Merocel) were thawed at room temperature for 10 min. Sponges were then inserted into a microcentrifuge tube containing a 0.2 μm filter (SpinX centrifuge tube), equilibrated by adding 300 μl of extraction buffer and incubated for 30 min at 4 °C, followed by centrifugation at 4 °C for 30 min at 14,000 rpm. After centrifugation, the resultant supernatant was collected for the estimation of IL-6, IL-8, IL-17 and TNF-α levels by ELISA method with commercially available kits. The supernatant was stored at −80 °C until use.

A monoclonal antibody against the antigens (IL-6, IL-8, IL-17 and TNF-α) had been pre-coated onto the wells of the microtiter strips provided. Antigens present in the sample or standard were incubated with the plates to allow binding of antigens to the antibody. This was followed by the addition of a primary monoclonal anti-IL-6, IL-8, IL-17 and TNF-α antibody respectively conjugated to biotin in respective microtiter plates. An avidin-HRP conjugated antibody specific for primary antibody wad then added to the wells. After incubation and following a wash, to remove any unbound antibody enzyme reagent, a TMB one-step substrate reagent reactive with HRP was added to the wells. The color development was terminated by adding acid and absorbance was measured at 450 nm. A reference curve was obtained by plotting the different concentrations of standard samples versus absorbance and levels of the antigens in samples tested were calculated by the standard plot.

## Statistical analysis

A comparison of the levels of PIC (IL-6, IL-8, IL-17 and TNF-α) between the study (HL, NHL) and control groups before antifungal therapy and in HL and NHL after antifungal therapy was done. The comparison of the cytokines in *Candida* positive and *Candida* negative HL and NHL before and after antifungal therapy was also done. The chi-square and the Mann-Whitney *U* tests were used to estimate the statistical significance of difference observed between the groups.

## Results

The ages of the subjects in the study group (HL and NHL) ranged from 25 to 69 years (Mean 45.85years) and were mostly males (91.6%). Smoking tobacco was the most common habit (81.6%) while usage of smokeless tobacco (38.3%) and areca nut containing products (55%) was also frequently seen. There was no difference in the frequency and duration of the habits between HL and NHL. The most common site was the buccal mucosa (92%) and commissures (63%) in this study. Nearly 97% of the lesions were size $L_2$ and $L_3$ as per OLEP classification (mean size: 4.6 cm). Most of the lesions were bilateral (83%) and unilateral lesions were mostly non-homogeneous type (Size: $L_3$) on the tongue. Thirty percent of NHL showed mild/severe dysplasia and only one showed presence of *Candida* hyphae on PAS staining. There was significant differences in the *Candida* positivity between the three groups ($p<0.01$) with 55.3% of NHL, 23.3% of HL and 13.3% of Controls showing presence of *Candida*. *Candida albicans* was the most common phenotype in all the three groups sensitive to Fluconazole. No adverse effects to Fluconazole were reported by any of the study subjects.

The oral secretary levels of IL-6, IL-8, IL-17 and TNF-α in HL, NHL and the control group before and after Fluconazole treatment are shown in Table 1. The levels of IL-6 and IL-8 were observed to be significantly high ($p<0.05$) in NHL as compared to HL and controls before Fluconazole treatment. IL-17 levels were also found to be significantly higher in NHL as compared to controls ($p<0.05$) before Fluconazole treatment. The levels of IL-6, IL-8, IL-17 and TNF-α reduced after Fluconazole treatment but results were significant for TNF-α ($p<0.05$) in NHL.

The oral secretary levels of pro-inflammatory cytokines (IL-6, IL-8, IL-17 and TNF-α) in *Candida* positive and *Candida* negative NHL and HL before and after antifungal treatment are shown in Table 2. Comparison between levels of IL-6, IL-8, IL-17 and TNF-α in *Candida* positive and *Candida* negative NHL before and after antifungal treatment is shown in Fig. 1. The levels of IL-6, IL-8, TNF-α ($p<0.001$) and IL-17 ($p<0.01$) were significantly raised in *Candida* positive NHL before Fluconazole treatment. After Fluconazole treatment the levels of all the cytokines in *Candida* positive NHL reduced and there was no statistical difference in the levels of the cytokines between *Candida* positive and *Candida* negative NHL.

Comparison between levels of IL-6, IL-8, IL-17 and TNF-α in *Candida* positive and *Candida* negative HL before and after antifungal treatment is shown in Fig. 2. The levels of IL-6 ($p<0.05$) and TNF-α ($p<0.01$) were significantly raised in *Candida* positive HL before Fluconazole treatment. After Fluconazole treatment, the levels of IL-6 and TNF-α in *Candida* positive HL reduced and there was no difference between the levels of the cytokines between *Candida* positive and *Candida* negative HL.

All the cytokines were raised in *Candida* positive NHL before Fluconazole treatment. The levels of IL-6, IL-8, TNF-α ($p<0.001$) and IL-17 ($p<0.01$) were significantly reduced after Fluconazole treatment in *Candida* positive NHL.

All the cytokines were raised in *Candida* positive HL before Fluconazole treatment. There was reduction in the levels of all cytokines after Fluconazole treatment in *Candida* positive HL but it was statistically significant for IL-6 ($p<0.05$) and TNF-α ($p<0.01$).

**Table 1** The oral secretary levels of IL-6, IL-8, IL-17 and TNFα in NHL[a], HL[b] and Control group before and after antifungal treatment

| Study subjects (*n*) | IL-6 (pg/ml) Median (range) | IL-8 (pg/ml) Median (range) | IL-17 (pg/ml) Median (range) | TNF-α (pg/ml) Median (range) |
|---|---|---|---|---|
| NHL[a] Pre[c] (30) | 7.1 (4.6–17.8) | 167.0 (18.4–12205.0) | 58.38 (33.75–202.0) | 172.8 (20.5–730.0) |
| NHL[a] Post[d] (30) | 5.9 (3.6–8.7) | 83.9 (21.25–1424.0) | 46.75 (32.75–117.0) | 105.5 (22.5–213.0) |
| HL[b] Pre[c] (30) | 5.2 (3.7–8.4) | 45.75 (16.75–274.5) | 52.63 (26.75–162.0) | 112.5 (23.5–2776.0) |
| HL[b] Post[d] (30) | 4.3 (3.3–7.0) | 38.50 (15.75–355.0) | 40.25 (27.0–119.0) | 92.5 (20.5–173.0) |
| Controls (30) | 4.1 (3.1–7.9) | 36.50 (18.5–124.0) | 41.0 (30.0–80.0) | 94.5 (24.5–188.0) |

[a] Non-homogenous leukoplakia

[b] Homogenous leukoplakia

[c] Before antifungal treatment

[d] After antifungal treatment

**Table 2** The oral secretary levels of pro-inflammatory cytokines (IL-6, IL-8, IL-17 and TNFα) in Candida positive and Candida negative NHL[a] and HL[b] before and after antifungal treatment

| Study subjects (*n*) | | IL-6 (pg/ml) Median (range) | IL-8 (pg/ml) Median (range) | IL-17 (pg/ml) Median (range) | TNF-α (pg/ml) Median (range) |
|---|---|---|---|---|---|
| NHL[a] Pre[c] | *Candida* positive (*n*=16) | 9.2 (5.7–17.8) | 713.0 (22.8–12205.0) | 82.0 (48.8–202.0) | 220.0 (143.0–730.0) |
| | *Candida* negative (*n*=14) | 6.1 (4.6–10.3) | 68.5 (18.4–195.0) | 49.8 (33.8–73.0) | 89.0 (20.5–260.0) |
| HL[b] Pre[c] | *Candida* positive (*n*=7) | 6.4 (5.2–8.4) | 66.1 (26.5–274.5) | 64.1 (48.5–162.0) | 178.5 (129.5–2776.0) |
| | *Candida* negative (*n*=23) | 4.8 (3.7–7.3) | 41.4 (16.8–147.0) | 47.2 (26.8–84.0) | 92.0 (23.5–284.0) |
| NHL[a] Post[d] | *Candida* positive (*n*=16) | 6.0 (4.0–8.0) | 193.0 (26.5–1424.0) | 55.0 (34.5–88.0) | 134.0 (22.5–197.0) |
| | *Candida* negative (*n*=14) | 5.9 (3.6–8.7) | 67.0 (21.2–318.0) | 38.5 (32.8–117.0) | 104.0 (23.0–213.0) |
| HL[b] Post[d] | *Candida* positive (*n*=7) | 4.3 (4.2–7.0) | 32.6 (16.5–143.0) | 33.4 (28.5–77.0) | 109.5 (46.0–173.0) |
| | *Candida* negative (*n*=23) | 4.2 (3.3–7.0) | 40.0 (15.8–355.0) | 48.0 (27.0–119.0) | 74.0 (20.5–154.0) |

[a] Non-homogenous leukoplakia

[b] Homogenous leukoplakia

[c] Before antifungal treatment

[d] After antifungal treatment

The difference in levels of IL-6, IL-8, IL-17 and TNF-α in *Candida* negative NHL and HL after Fluconazole treatment was not statistically significant.

## Discussion

Tobacco (smoking/smokeless), areca nut containing products and alcohol are the most important risk factors for OSCC and OPMDs like OL. They expose the oral mucosa to both chronic physical and chemical irritation due to the carcinogens/alkaloids in them leading to formation of ROS which activate the Nf-kB dependent inflammatory cytokines (IL-6, IL-8, TNF-α) [4]. These cytokines have also been found to be related to poor periodontal health which is common in subjects with these habits and dysplasia in OL [14]. They contribute to the local oral environment for a pro-oncogenic inflammatory response through changes in cell proliferation, cell death, cellular senescence, DNA mutation, DNA methylation and angiogenesis. They lead to tumour growth, which further stimulates the inflammatory response in a cyclic progression leading to increased tumour vessel density and poorer outcomes in OSCC [4, 11, 14].

Neutrophils are the major component of the immune response to any neoplastic process and secrete IL-17A which further stimulate the secretion of IL-1β, IL-6 and TNF-α by macrophages and angiogenic factors like VEGF by neoplastic cells [8]. IL-17 has also been found to be upregulated in OSCC and OPMDs like Oral Lichen Planus (OLP) [8, 16]. The levels of the inflammatory cytokines are upregulated and found to correlate with the progression and grading of tumours in OSCC and to clinical severity of the lesions in OPMD like Oral Lichen Planus [5, 16]. The raised oral secretary levels of these cytokines may therefore lead to malignant transformation in OPMDs like OL [4, 11].

The oral epithelial cells being in constant contact with *Candida albicans* constitute the first line of defence, recognising the fungus in the commensal or pathogenic stage leading to either passive or active immune response. Colonisation and commensal growth on mucosal surface depends on adhesion between the fungus cell wall and epithelial cells in the yeast form which further stimulates the germ tube/hypha formation. The hypha formation attachment and induced endocytosis is followed by invasion causing epithelial penetration and tissue damage in the form of necrosis and apoptosis. The secretion of cytotoxic peptide Candidalysin by *Candida albicans* causes host cell damage and induction of PIC by epithelial and endothelial cells. Induction of innate immune response to *Candida albicans* by oral epithelial cells occurs by NF-kB and a bi phasic MAPK signalling response resulting in the production of PIC like IL-1alpha/beta, IL-6, G-CSF, TNF-α, RANTES, IL-8 and CCL20. The initial response is by NF-kB and a transient MAPK response which is independent of morphology, is activated by both yeast and hyphal forms. A second stronger danger response caused by activation of the three MAPK pathways is associated with increased burden of the hyphal form of the fungus. The PIC produced by oral epithelial cells result in the recruitment, differentiation and activation of immune cells like neutrophils, macrophages, dendritic cells, and T cells. The processed fungal antigen when presented to T cells in the local lymph node induces the Th1/2/17 and T regulatory (Tregs). Th17 cells secrete IL-17A, IL-17F and IL-22 and play an important role in antifungal immunity [31, 32]. The expressions of these cytokines are also related to the virulence factors of *Candida* [33]. The same repertoires of pro-inflammatory cytokines are found to be raised in OSCC and OL as well.

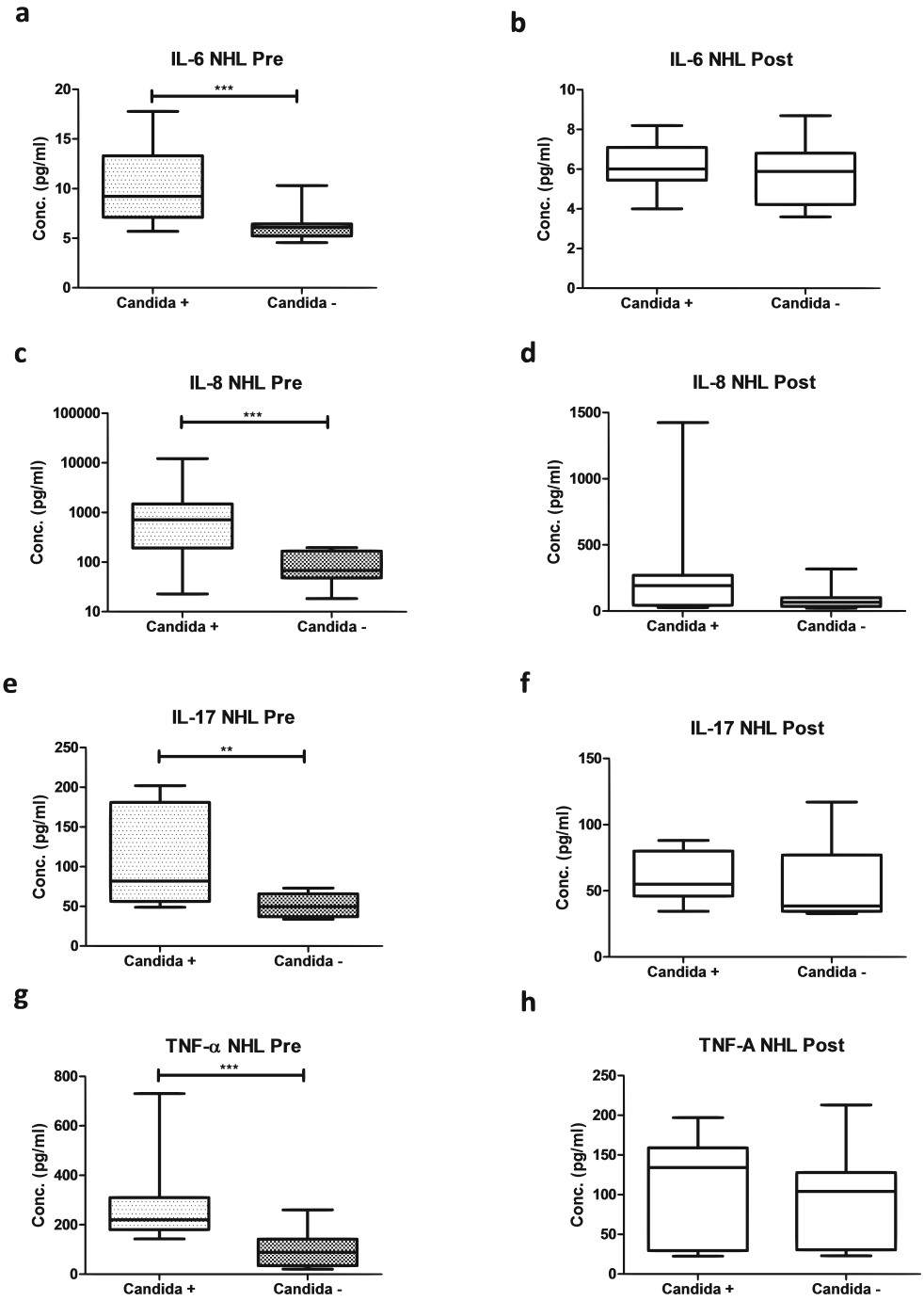


**Fig. 1** Comparison between levels of IL-6 (**a**, **b**), IL-8 (**c**, **d**), IL-17 (**e**, **f**) and TNF-α (**g**, **h**) in *Candida* positive and *Candida* negative non-homogenous leukoplakia before and after antifungal treatment (NHL, non-homogenous leukoplakia; Pre, before antifungal treatment; Post, after antifungal treatment; $^{**}p<0.01$; $^{***}p<0.001$)

Kaur et al. have reported higher levels of IL-6, IL-8, and TNF-α in OL which relates to the severity of epithelial dysplasia but they did not differentiate between NHL and HL [11]. We found the oral secretary levels of IL-6, IL-8, IL-17 and TNF-α to be highest in NHL followed by HL as compared to controls but no significant relation with dysplasia. The levels were related to the clinical severity of the lesions as they were raised in high-risk NHL as compared to low-risk HL which is similar to findings in erosive and non-erosive OLP [16]. Brailo et al. found increased levels of IL-6 and TNF-alpha in OL but they found no correlation with size, high/low-risk sites or smoking habits. They had also excluded patients with periodontitis which is an important confounder for salivary inflammatory cytokines [12]. Sharma et al. found IL-6 levels to be significantly higher in OL with periodontitis when compared to subjects with periodontitis and controls and found tobacco habits to be an independent risk factor [14]. Due to the oral health profile of our patients we could not exclude OL patients with poor periodontal health. However we only included subjects with simplified oral hygiene index

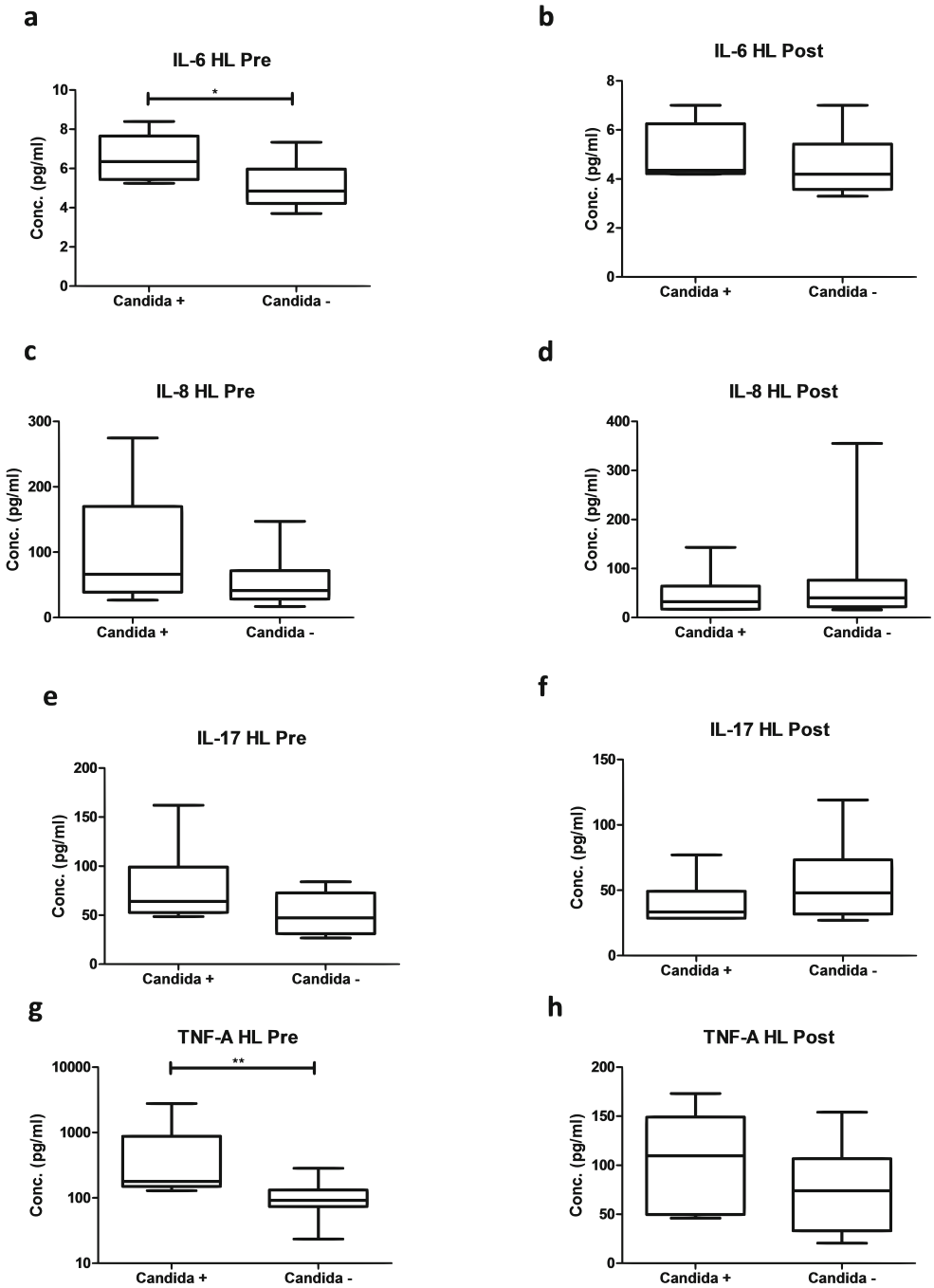


**Fig. 2** Comparison between levels of IL-6 (**a**, **b**), IL-8 (**c**, **d**), IL-17 (**e**, **f**) and TNF-α (**g**, **h**) in *Candida* positive and *Candida* negative homogenous leukoplakia before and after antifungal treatment (HL, homogenous leukoplakia; Pre, before antifungal treatment; Post, after antifungal treatment; *$p<0.05$; **$p<0.01$)

score (OHI) 0–3, periodontal screening and recording (PSR) code 0–2 in the study. We also found IL-6 and TNF-alpha to be increased in OL but majority of the lesions in this study were large in size (> 2cm) and associated with smoking habit and most were present on the relatively low-risk areas like buccal mucosa and commissures. Buccal mucosa is however one of the common sites for OSCC in Indian patients. Most of the studies did not differentiate between NHL and HL while estimating PIC in OL [11, 12, 14, 17]. None of the studies have estimated PIC in *Candida* associated NHL and HL. The effect of PIC after antifungal treatment in NHL and HL or *Candida* associated NHL and HL have also not been reported.

The raised levels were also related to the association of *Candida* as *Candida* associated NHL and HL showed higher levels of PIC indicating that they are at even higher risk for malignant transformation than *Candida* negative NHL and HL. The upregulation of these PIC in *Candida* associated OL give credence to the in vitro observations of induction of these cytokines by *Candida* in oral mucosal epithelial cells. This study also shows the reduction of these PIC after antifungal therapy in *Candida* associated NHL and HL.

Topical antifungals like nystatin and imidazoles and systemic antifungals like Fluconazole in immunocompetent patients have shown clinical improvement in OL and have been recommended to prevent malignant transformation [26–28]. There are recommendations about the need to treat OL with antifungal therapy for 2–4 weeks providing an interval to observe the possible regression or disappearance of the white lesion in OL after elimination of possible causative factors including smoking habits before taking a biopsy [27]. Candidal leukoplakia/hyperplastic candidiasis also shares the same predisposing factors like tobacco smoking as OL and hence it is possible that many diffuse lesions of OL may have co-existing Candidal leukoplakia which regress after antifungal therapy [34]. The oral lesions that persist after antifungal therapy are the true OL [27]. Complete elimination of *Candida* hyphae in cytosmear and reduction of salivary *Candida* colony counts after antifungal therapy in both HL and NHL have been reported before [29]. Fluconazole 100–200mg as a once daily dose for 11–14 days has been recommended in immunocompetent patients with oral candidiasis [35]. We therefore followed a uniform regimen of antifungal therapy (2 weeks) in both HL and NHL to observe for possible regression of lesions and rule out hyperplastic candidiasis. We also saw significant clinical improvement and down-staging of high-risk NHL into HL after Fluconazole treatment, but none of the lesion resolved completely. Fluconazole is a broad-spectrum triazole that is commonly used in oral candidiasis with antifungal activity against most of the species of *Candida* with excellent bioavailability, low toxicity, affordability and rare adverse effects. It also offers the convenience of single daily dose regimen in a dispersible form that improves patient acceptability and compliance as seen in this study. The significant reduction of PIC in OL after Fluconazole treatment shows its efficacy in reducing *Candida* infection in OL in this study. The duration of this beneficial and desirable effect in *Candida* associated OL after antifungal treatment was not investigated in this study. Re-colonisation of OL surface with *Candida* and elevation of PIC is possible after completion of antifungal treatment. Identification and elimination of predisposing factors for *Candida* infection in OL is required. Preventive measures like emphasis on complete cessation of tobacco/areca nut/alcohol habits, maintenance of proper oral/denture hygiene, surveillance for *Candida* infection with antifungal sensitivity and antifungal therapy at intervals can be employed. The PIC can be used as diagnostic and prognostic indicators in the long-term surveillance of *Candida* associated OL. Since this is a preliminary investigation with a 2 week follow-up, further prospective large-scale double-blinded randomised clinical trials in different populations with other common antifungal agents should be carried out to corroborate the results. Long-term follow-up studies to determine the beneficial effect of antifungal intervention in reducing malignant transformation rate in *Candida* associated OL are also required.

## Conclusions

The PIC (IL-6, IL-8, IL-17, TNF-α) are raised in *Candida* associated OL and can contribute to the inflammatory milieu in OL, thus increasing the risk for carcinogenesis. Surveillance for *Candida* infection and elimination/reduction of predisposing risk factors is recommended in OL. Management of OL should aim at maintenance of oral hygiene, homeostasis and salivation, regular oral prophylaxis, maintenance of dental, periodontal and systemic health, reinforcement of tobacco/areca nut/alcohol habit cessation through counselling and regular follow-up. Antifungal therapy can reduce the super-infection and oral secretary PIC in *Candida* associated OL and can be used as a therapeutic and prophylactic adjunct in the long-term management of *Candida* associated OL.

**Abbreviations** OL, Oral leukoplakia; OPMD, Oral potentially malignant disorder; OSCC, Oral squamous cell carcinoma; PIC, Pro-inflammatory cytokines; HL, Homogenous oral leukoplakia; NHL, Non-homogenous oral leukoplakia; C, Controls; OHI, Oral hygiene index; PSR, Periodontal screening and recording; OLEP, Classification and staging system for oral leukoplakia; IL-6, Interleukin-6; IL-8, Interleukin-8; IL-17, Interleukin-17; TNF-α, Tumour necrosis factor-alpha

**Author contribution** Dr. SR Gupta: (1) the conception and design of the study, acquisition of data, analysis and interpretation of data; (2) funding acquisition; investigation, methodology; project administration; resources; supervision; validation; (3) drafting the article, revising it critically for important intellectual content; (4) final approval of the version to be submitted.

Dr. Nidhi Gupta: (1) the conception and design of the study, acquisition of data, analysis and interpretation of data; (2) revising article critically for important intellectual content; (3) final approval of the version to be submitted.

Prof. Alpana Sharma: (1) the conception and design of the study, acquisition of data, analysis and interpretation of data; (2) revising article critically for important intellectual content; (3) final approval of the version to be submitted.

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Dr. Kalaivani Mani: (1) analysis and interpretation of data; (2) revising article critically for important intellectual content; (3) final approval of the version to be submitted.

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**Data availability** The datasets generated during and/or analysed during the current study are available from the corresponding author on request.

## Declarations

STROBE guidelines were adhered to in the reporting of this study.

**Ethics approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by Institute |Ethics Committee All India Institute of Medical Sciences New Delhi Ref No. IEC-492/07.09.2018, RP-21/2018. The methodology for this study was peer reviewed and approved by the Research Section of All India Institute of Medical Sciences New Delhi.

**Informed consent** Informed consent was obtained from all individual participants included in the study. No patient identifying data or photographs have been included.

**Conflict of interest** All authors, Dr. Shalini R Gupta, Dr. Nidhi Gupta, Prof. Alpana Sharma, Prof. Immaculata Xess, Dr. Gagandeep Singh and Dr. Kalaivani Mani, declare that they have no conflict of interests.

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