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Title: Immunological response and clinical profile in patients with recurrent dermatophytosis

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Abstract

Background: An alarming increase in the number of patients with chronic and recurrent dermatophytosis has invoked the need to study the immunological parameters of the host.

Objectives: To evaluate delayed type of hypersensitivity (DTH) response and immediate hypersensitivity (IH) response by flow cytometry evaluation of immune cells from peripheral blood and intradermal trichophytin skin test in patients with recurrent dermatophytosis.

Methods: A hundred patients with recurrent dermatophytosis and 50 controls (healthy controls and acute dermatophytosis controls) were included. Relevant risk factors for recurrence were analyzed and serum IgE levels were estimated. Flow cytometry evaluation of immune cells in peripheral blood and intradermal trichophytin skin test was done. Dermatophyte pathogens were isolated and antifungal susceptibility was performed.

Results: *Trichophyton mentagrophytes* complex (95.84%) and *T. rubrum* (4.16%) was isolated in culture. Serum IgE was elevated in 83.15 % cases (**P= 0.01**). IFN- γ ⁺ cells (**P=0.0501, P=0.0001, P=0.0014**), Th1 cells (P=0.1197, **P=0.0024, P=0.0169**), IL-17⁺ cells (**P=0.0127, P=0.0006, P=0.0007**), Th17 cells (P=0.0634, **P=0.0001, P=0.0054**) were reduced and IL-4⁺ cells (**P=0.0108, P=0.0175, P=0.0018**) were increased in cases. Intradermal test demonstrated negative DTH response in all cases (**P<0.001, P<0.001, P<0.001**), strongly positive IH response in 6%, and borderline positive IH response in 85% cases (**P=0.018, P<0.001, P<0.001**). Topical corticosteroids application, undergarment types (tight fit), poor frequency of washing clothes, family history of tinea, sharing of towels were significant risk factors for recurrent dermatophytosis.

Conclusions: Reduced IFN- γ +, Th1, IL-17+, and Th17 cells population along with impaired DTH response by the intradermal test was observed in patients with recurrent dermatophytosis.

Introduction

Dermatophytosis is the most common superficial fungal infection in humans. The cell-mediated immune (CMI) response through Th1 and Th17 cell-dependent mechanism is responsible for clearance of lesion from the skin in acute dermatophytosis while in chronic dermatophytosis the immunity is shifted towards Th2 response with the high level of IgE and IgG4.¹ Recurrent dermatophytosis have a protracted course with episodes of remission and exacerbation and had been considered a rare phenomenon until recent past.² The associated conditions which predispose to recurrent dermatophytosis include diabetes mellitus, atopy, intake of systemic corticosteroids, immunocompromised status, lymphoma, and HIV.³ The pathogenesis of chronic/recurrent dermatophytosis is not clearly understood. The complex interaction between host, environment, and agent associated with selective immune impairment is believed to be responsible for the development of recurrence and chronicity.⁴ Recurrent dermatophytosis is a fast-emerging issue of public health in the Indian subcontinent.⁵ Few studies have suggested the role of impaired delayed-type hypersensitivity (DTH) immune response and its association with an increase in immediate hypersensitivity (IH) immune response in patients with recurrent dermatophytosis.⁶ Inadequate clinical response to antifungal drugs is another problem that is gaining importance in the recent past. Indiscriminate use of antifungal agents, a gene mutation in drug binding sites present in dermatophytes, biofilm production by fungi are being proposed for

the emerging drug resistance to common dermatophytes pathogen.² Though most of the recent reports have observed an increase in MICs of the antifungals to the dermatophytes, it is not clear whether this increase in MIC is the only factor that can explain the resistance *in-vivo*. The present study was conducted to understand the contribution of host immune function in the form of DTH and IH immune response, risk factors in the host, and microbial factors such as antifungal drug susceptibility patterns causing recurrent dermatophytosis.

Methods

This was a single-center case-control study conducted in a tertiary care institute in India. All consecutive patients presenting with dermatophyte infections in the dermatology outpatient department were screened and 100 consecutive patients having recurrent dermatophytosis were included in the study. Case definition included a patient with 3 or more recurrences of cutaneous dermatophytosis in the last 6 months under our follow-up within few weeks of completion of antifungal treatment as per standard recommendation.⁷ The study was approved by the institute ethics committee and written informed consent was obtained from all patients. Patients with tinea unguium/capitis, pregnancy and lactation, patients on systemic immunosuppressive drugs, immunocompromised patients such as HIV positive/ post-renal transplant, etc., and patients less than 12 years were excluded from the study.

A total of 50 controls were included among them twenty-five patients had recent onset of tinea corporis/cruris without episodes of cutaneous dermatophytosis in the last six months (acute dermatophytosis control) and 25 other patients with a dermatological disease like naevi, molluscum contagiosum, seborrheic keratosis, melasma, etc. with no systemic inflammatory component (healthy control).

Baseline characteristics, history, and findings of clinical examination were recorded. The disseminated disease was defined as the lesions involving more than two distant anatomical sites while localized disease involving ≤ 2 anatomical sites. Risk factors associated with recurrent dermatophytosis were assessed.

The microbiological investigation included microscopic examination and culture of skin scrapings. The culture was done on Sabouraud dextrose agar (HiMedia) containing chloramphenicol and cycloheximide and incubated at 28°C for up to four to eight weeks. Any growth observed was identified based on phenotypic (macroscopic and microscopic) and confirmed by sequencing the internal transcribed spacer (ITS) region of ribosomal DNA.

Genomic DNA was extracted by the manual phenol-chloroform-isoamyl method. Universal ITS primer pair ITS1 (5' TCC GTA GGT GAA CCT TGC GG 3') and ITS4 (TCC TCC GCT TAT TGA TAT GC 3') were used for sequencing by Big Dye Terminator Cycle sequencing kit version 3.1 (Applied Biosystems, Foster City, CA, USA). Analysis of all the sequencing reactions was done on ABI 3130 Genetic Analyzer (Applied Biosystems). Sequences were compared with the GenBank DNA database using blast tool (<https://blast.ncbi.nlm.nih.gov>), ISHAM ITS database (<http://its.mycologylab.org/BioloMICSSequences.aspx>) and CBS database (<http://www.westerdijkinstituut.nl/Collections/BioloMICSSequences.aspx>).⁸ Antifungal

susceptibility testing was performed against four (fluconazole, griseofulvin, itraconazole, and terbinafine) antifungal drugs according to micro-broth dilution technique as per Clinical Laboratory Standards Institute (CLSI) M38 protocol with minor modification as previously described.⁸ The final concentration of antifungal tested ranged between 0.0625-64 µg/ml for fluconazole; 0.25-128 µg/ml for griseofulvin; 0.0078-4 µg/ml for itraconazole and 0.0156-64 µg/ml for terbinafine. *T. mentagrophytes/ interdigitale* complex (NCCPF 800035), *Candida*

parapsilosis (ATCC 22019), *Candida krusei* (ATCC 6258), and *Aspergillus flavus* (ATCC 204304) were used as quality controls strains.⁹

Immunological investigations included quantification of IFN- γ ⁺ cells, IL-4⁺ cells, IL-17⁺ cells, CD4⁺ cells, and CD3⁺ cells population in peripheral blood by using flow cytometry, intradermal trichophytin skin test, and assessment of serum IgE levels.

Flow cytometry- Percentage of CD4⁺IFN- γ ⁺, CD3⁺IL-4⁺, and CD4⁺IL-17⁺ cells were evaluated in PBMCs from patients and controls. Briefly, 1×10^6 PBMCs were stimulated for 4 to 6 hours with 10ng/ml phorbol-12-myristate 13- acetate (PMA, Sigma Aldrich, USA) and 1 μ g/ml ionomycin (Sigma Aldrich, USA) in the presence of 1 μ g/ml Brefeldin- A (Sigma Aldrich, USA).

After cell surface staining with FITC conjugated anti-CD3 antibody and APC (Allophycocyanin) conjugated anti-CD4 antibody (BD Biosciences, USA), further cells were fixed, permeabilized, and stained with phycoerythrin (PE) conjugated anti-IL17A (BD Biosciences, USA), PE-Cy7 labeled anti-IFN- γ (BD Biosciences, USA) and BV421 labeled anti-IL 4 (BD Biosciences, USA). Flow cytometry studies were performed on BD FACS LSR Fortessa using BD FACS Diva software (BD Biosciences, USA). Th1 cells were estimated as the percentage of total IFN- γ ⁺CD4⁺T cells out of absolute lymphocyte count. Th2 cells were estimated as the percentage of total IL-4⁺CD3⁺T cells out of absolute lymphocyte count. Th17 cells were estimated as the percentage of total IL-17⁺CD4⁺ T cells out of absolute lymphocyte count.

Intradermal skin test- Trichophytin antigen derived from *T. rubrum* was procured commercially from ALCIT Pvt. Ltd., Delhi (India).¹⁰ Trichophytin extract in a concentration of 0.1 μ g in 0.1 ml normal saline was injected into the case and control group. IH response was assessed after 30 minutes in the form of weal and flare and DTH response in the form of induration after 48 hours. Weal greater than 10 mm was considered strongly positive, between 5 to 10 mm was considered

borderline positive and $<5\text{mm}$ was considered negative IH response. Induration greater than 5 mm was considered as positive and $\leq 5\text{ mm}$ was considered negative DTH response.^{11, 12}

Estimation of serum levels of total IgE was done by ImmunoCAPkit commercially purchased from Thermo Fisher. The test was performed as per the manufacturer's protocol (Thermo Fisher Phadia 100). Patient samples were run in single determinations. Volumes required for per determination of test were sample 40 μl , conjugate 50 μl , development solution 50 μl , stop solution 600 μl . Incubations were performed at 37 °C by Phadia 100 instrument. The standard curve range of the kit was 2 to 5000 kU/L.

Statistical analysis was carried out using Jamovi (version 1.6.18), GraphPad Prism (version 7), and Medcalctrial version 18.5. Normality of data was checked by measures of Kolmogorov Smirnov tests of Normality. ROC curve analysis was used to calculate the cut-off with sensitivity and specificity. Qualitative or categorical data were described as frequencies and proportions. Pearson's Chi-square test and Fisher's exact test were used for comparing categorical variables. All statistical tests were two-sided and performed at a significance level of $= 0.05$.

Result

Among the recurrent cases, 60% had localized disease, while in 40%, the disease was disseminated. Mixed clinical pattern (tinea cruris/ corporis/ faciei/ manuum/ pedis) was present in 41%, tinea cruris in 24% and tinea corporis in 35% patients. Classical lesions (Figure 1A) of tinea were present in 58%, eczematous (Figure 1B) in 20%, papulosquamous (Figure 1C) in 13%, pustular (Figure 1D) in 4%, pseudo-imbricata (Figure 1E) in 4% and lichenified lesion (Figure 1F) in 1% patients. The mean duration of symptoms at the time of initial presentation

was 5.1 months. Among the recurrent dermatophytosis cases, 52 patients had a history of previous antifungal exposure. Sixty-two (62%) patients provided a history of the application of corticosteroids (topical and systemic) in the past for varying duration (2 weeks to 3 months) for the treatment of tinea. Out of 62 patients, 54 (87.09 %) used topical corticosteroids alone or in combination with either antibacterial and antifungal agents, whereas eight (12.90%) patients also gave a history of use of systemic corticosteroids in addition. Further, fifty patients (80.64%) were still using topical steroids at the point of patient recruitment. Among the topical corticosteroids (n=54), the majority of patients (n= 45, 83.34%) had used potent topical corticosteroids such as clobetasol propionate. Of these, forty-six (85.18%) used topical steroid as self or chemist dispensed over the counter (OTC) preparation, and the remaining eight (14.81%) were prescribed by the general practitioners. The risk factors associated with recurrent dermatophytosis included type of undergarments (tight fit), poor frequency of washing clothes, sharing of towel/clothes, family history of tinea, and use of topical corticosteroid in the past (Table 1).

Microbiological investigation- a total of 48 (48%) cases yielded dermatophytes growth from the skin scrapping specimen. *T. mentagrophytes complex* was the most common dermatophyte pathogen identified (n= 46, 95.84%) followed by *T. rubrum* (n= 2, 4.16%). Among 25 acute dermatophytosis control group, fungal growth was present in 3 (12%) patients all of which were identified as *T. mentagrophytes complex*. Out of 48 isolates of *Trichophyton*, 26 (54.16%) exhibited griseofulvin MIC of $\geq 16 \mu\text{g/ml}$ and, the remaining 22 (45.83 %) had MIC of $< 8 \mu\text{g/ml}$. Six (12.5%) *T. mentagrophytes complex* isolates exhibited a high terbinafine MIC of $\geq 1 \mu\text{g/ml}$. Out of 6 isolates with MIC of $\geq 1 \mu\text{g/ml}$ for terbinafine, only 2 had a definite history of exposure to terbinafine in the past. Among azoles, fluconazole had poor *in vitro* activity with

MICs of ≥ 8 $\mu\text{g/ml}$ in 8 (16.66%), and the remaining 40 isolates (83.3%) showed MIC with ≤ 4 $\mu\text{g/ml}$. On the other hand, all the 48 (100%) isolates exhibited a low MIC of ≤ 0.5 $\mu\text{g/ml}$ for itraconazole. MIC range for four antifungal drugs against the 48 isolates is illustrated in Table 2.

Immunological Evaluation- For analysis of immunological parameter from the recurrent cases (n=100), a total of 95 patients were assessed for Th1/Th17 cells and 94 patients for Th2 cells population whereas in the control group (acute dermatophytosis control, n= 25; and healthy control; n=25) 50 samples were assessed. Flow cytometry density contour plots are illustrated in Figure 2. Th1 cells (CD4+IFN- γ + * absolute lymphocyte count), Th2 cells (CD3+ IL-4+ * absolute lymphocyte count), Th17 cells (CD4+IL-17 + * absolute lymphocyte count), IFN- γ + cells, IL-4+ cells, and IL-17+cells, the receiver operating characteristic curve (ROC) analysis, and the cutoff values were determined (Table 3, Figure 3). In recurrent dermatophytosis cases versus healthy control group, the cutoff value obtained for Th1 cells, Th2 cells, Th17 cells, IFN- γ + cells, IL-4+cells, and IL-17+ cells were ≤ 181.26 counts/ μl , ≤ 4.25 counts/ μl , ≤ 4.6 counts/ μl , >25.9 %, ≤ 0.5 %, and ≤ 0.4 % respectively. Further, in the recurrent cases versus acute dermatophytosis control group, the cutoff obtained for Th1 cells, Th2 cells, Th17 cells, IFN- γ + cells, IL-4+, and IL-17+ were ≤ 84.39 counts/ μl , ≤ 7.06 counts/ μl , ≤ 6.54 counts/ μl , ≤ 10.2 %, ≤ 0.3 %, and ≤ 0.8 % respectively. Apart from the individual control group, combined control versus cases were analyzed to determine the cutoff for Th1 cells, Th2 cells, Th17 cells, IFN- γ + cells, IL-4+ cells, and IL-17+ cells which were ≤ 102.61 counts/ μl , ≤ 13.42 counts/ μl , ≤ 4.6 counts/ μl , ≤ 15.9 %, ≤ 0.5 %, and ≤ 1 % respectively. The values obtained for Th1 cells, Th17 cells, IFN- γ + cells, and IL-17+ cells which were less than the cutoff values mentioned above were determined as reduced. Reduced Th1 population was observed in 67.3% (64/95) recurrent dermatophytosis cases versus 32% (8/25) acute dermatophytosis controls and 72.6% (69/95) recurrent

dermatophytosis cases versus 52% (26/50) combined acute dermatophytosis and healthy controls with a significant $P=0.0024$ and $P=0.0169$ value respectively. Similar to Th1 response, reduced Th17 cell population was also detected in 68.4% (65/95) recurrent dermatophytosis cases versus 24 % (6/25) acute dermatophytosis controls and 41% (39/95) recurrent dermatophytosis cases versus 18% (9/50) combined acute dermatophytosis and healthy controls with a significant $P=0.0001$ and $P=0.0054$ value respectively. In both Th1 and Th17 cells population, there was no significant difference observed between recurrent dermatophytosis and healthy controls. Further, IFN- γ + cells was reduced in 64.2% (61/95) recurrent dermatophytosis cases versus 20% (5/25) acute dermatophytosis controls, 6.31% (6/95) recurrent dermatophytosis cases versus 20% (5/25) healthy controls, and 83.1 % (79/95) recurrent dermatophytosis cases versus 58% (29/50) combined acute dermatophytosis and healthy controls with a significant P-value of 0.0001, 0.0501 and 0.0014 respectively. Similarly, IL-17+cells was also reduced in peripheral blood in 74.7% (71/95) recurrent dermatophytosis cases versus 36% (9/25) acute dermatophytosis controls, 32.6% (31/95) recurrent dermatophytosis cases versus 8% (2/25) healthy controls, and 86.3% (82/95) recurrent dermatophytosis cases versus 60%(30/50) combined acute dermatophytosis and healthy controls with a significant P-value of 0.0006, 0.0127, and 0.0007 respectively. On the other hand, for Th2 and IL-4+ cells, the values obtained which were more than the cutoff was considered as increased. IL-4+ cells was increased in 60.6% (57/94) recurrent dermatophytosis cases versus 90% (18/20) acute dermatophytosis controls, 43.6% (41/94) recurrent dermatophytosis cases versus 75% (18/24) healthy controls, 43.6% (41/94) recurrent dermatophytosis cases versus 72.7% (32/44) combined acute dermatophytosis and healthy controls with a significant P-value of 0.0175, 0.0108, 0.0018 respectively. But there was no significant difference in Th2 response in recurrent

dermatophytosis cases versus acute dermatophytosis controls, healthy controls, and combined acute dermatophytosis and healthy controls (Table 4).

Intradermal trichophytin test- Of 100 recurrent dermatophytosis patients tested, 85 (85%) had borderline positive IH reaction, 6 (6%) had strongly positive and 9 (9%) were negative. Among 25 healthy controls, 10 (40%) had a negative reaction, 11 (44%) had a borderline positive reaction and 4 (16%) had a strongly positive reaction while out of 25 acute dermatophytosis controls 6 (24%) had a negative reaction, 15 (60%) had a borderline positive reaction and 4 (16%) had a strongly positive reaction ($P=0.018$, $P<0.001$, $P<0.001$ in cases versus acute dermatophytosis controls, cases versus healthy controls and cases versus combined acute dermatophytosis and healthy controls respectively). DTH response was negative in all cases of recurrent dermatophytosis, whereas among 25 healthy controls, 9 (36%) had a positive reaction and 16 (64%) had a negative reaction similarly among 25 acute dermatophytosis controls, 9 (36%) had a positive reaction and 16 (64%) had a negative reaction, ($P<0.001$, $P<0.001$, $P<0.001$ in cases versus acute dermatophytosis controls, cases versus healthy controls and cases versus combined acute dermatophytosis and healthy controls respectively, Table 1, Figure 4 a, b).

Total serum IgE was assessed in recurrent dermatophytosis cases ($n=95$), healthy controls ($n=16$) and acute dermatophytosis controls ($n=24$). Mean serum IgE $\pm$ standard deviation was 1113 ± 1474 IU/ml in cases, 161 ± 145 IU/ml in healthy controls, and 678 ± 1232 IU/ml in acute dermatophytosis controls. Serum IgE was raised significantly in 79 patients (83.15%, $P=0.01$).

Discussion

There has been a changing trend of epidemiological patterns of dermatophytes in the Indian subcontinent with an increased number of recalcitrant/ chronic patients of dermatophytosis and many reported cases of treatment failure.¹³ Thus, there is a necessity to study all aspects related to treatment response to recurrent dermatophytosis like immunological aspects, associated risk factors in the host, and *in-vitro* susceptibility of etiological agents.

Previous studies reported the association of recurrent dermatophytosis with various risk factors such as occupation, family history of dermatophytosis, pre-existing comorbidity, excessive sweating, frequency of washing clothes, taking baths, and prior use of topical steroids.¹⁴ In the present study, type of undergarments (tight fit), poor frequency of washing clothes, sharing of a towel/clothes, family history of dermatophytosis, and history of use of topical corticosteroid in the past were significant risk factors. Improper drying of clothes and the persistence of spores in the household environment may significantly impact the disease's recurrence.^{15, 16} Corticosteroid use leads to local suppression of host immunity and may also cause deficient expression of Langerhans cell markers, which leads to typical lesions of tinea almost unrecognizable.¹⁷ Further, topical steroids also favor fungal growth and increase the virulence property of etiological agents which ultimately lead to recurrence and chronicity.¹⁸ As a result, haphazard use of over the counter (OTC) medications is one of the significant causes for atypical morphology and chronic/recurrent dermatophytosis.^{18,19} In the present study, more than half of the patients presenting with atypical lesions had used topical corticosteroids in the past, suggesting its role in the rising incidence of tinea with atypical manifestation.

Recent studies from India have also observed an epidemiological shift in the dermatophyte species, *T. mentagrophytes* complex has emerged as the main organism in recurrent dermatophytosis cases.^{8,14} Similar to the previous study, we also noted *T. mentagrophytes* complex (95.84%) as a predominant species.²⁰⁻²⁶ In the present study, all the isolates were susceptible to itraconazole with lower MIC value, and higher MIC was observed in the case of terbinafine, fluconazole, and griseofulvin in 12.5% (6/48), 16.6% (8/48), and 54.1% (26/48) respectively. Several studies on *in-vitro* antifungal susceptibility of *Trichophyton* spp. suggest the increasing trend of resistance to terbinafine.^{8, 14} Only a few reports highlight the role of drug efflux, stress adaptation, and mutation in salicylate monooxygenase but are not conclusively linked to the *in-vitro* susceptibility.²⁷ Recently, azole resistance in dermatophytes is found to be as high as 19% in some regions of the world.²⁸ In contrast, isolates in the present study exhibited higher susceptibility to itraconazole which may be due to the less frequent use of this drug among Indian patients over the past many years. Cross-resistance among fluconazole and itraconazole is reported to be common.²⁹ However, the present study did not reveal such cross-resistance among the azole group of drugs.

The host immunity also plays an important role in the clearing of infection, however, there are very few studies focusing on host immune response impairment in recurrent dermatophytosis. Flow cytometry analysis of peripheral blood in cases showed reduced Th1 (CD4+IFN- γ + cells * absolute lymphocyte count) and IFN- γ + cells population. Jone *et al.* observed that Th1 cells secrete IFN- γ and IL-2 which is responsible for DTH response and eradication of dermatophytes.³⁰ Waldman *et al.* demonstrated lower cytotoxic effects of CD4+ and CD8+ T cells against dermatophytes in patients with chronic dermatophytosis.⁴ Similarly, Shimamura *et al.* and Hay and Calderon *et al.* reported that trichophyton infections clearance requires T cell-

mediated immunity.^{31, 32} Dahl *et al.* proposed that protracted antigen exposure can induce anergy by activation of suppressor T cells by downregulating cell-mediated immune response³³. Thus, a reduced number of Th1 and IFN- γ + cells in cases with recurrent dermatophytosis indicate a lack of cell-mediated immune response required for the resolution of infection.

Besides the Th1 pathway, few studies also suggest that Th17 protection is required against dermatophytes and coordinative action of both Th1 and Th17 cells is responsible for clearance of dermatophytosis. Burstein *et al.* and Sakuragi *et al.* demonstrated the role of IL-17 immune response in dermatophyte infection.^{34, 35} Recently Rai *et al.* reported an increasing number of Th17 cell subpopulations and raised levels of IFN- γ with a low level of IL-10 cytokines in chronic dermatophytosis cases. Thus, they indicated that the persistence of infection and chronicity might be due to the Th17 pathway's activation.³⁶ But in contrast, we noted a reduced population of the Th17 cells (CD4+IL-17+ cells* absolute lymphocyte count) and IL-17+ cells in patients with recurrent dermatophytosis. Deficient Th17 cell population in patients of recurrent dermatophytosis suggests the role of the Th17 pathway in immunological defense against dermatophytosis.

Th2 cells are mainly considered with humoral response regulation by secretion of IL-4, IL-5, and IL-10 with increased production of immunoglobulins (IgE and IgG).¹² There has been an association between chronic dermatophytosis and allergic diseases like asthma, atopic dermatitis, and urticaria.³⁷ Also, it is seen that there is a change in the balance between Th1 and Th2 immune responses in diseases associated with chronic dermatophytosis like HIV and Diabetes.^{38,39} Therefore there have been speculations about the development of Th2 immune response which is responsible for the persistence of dermatophyte infection.⁴⁰ IL-4+ cells were

raised in our cases but there was no difference observed in Th2 cells (CD3+IL-4+cells * absolute lymphocyte count) between cases and controls. Lack of increase in the Th2 cells in cases may be explained by the fact that IL-5, IL-10, and other inflammatory cytokines may also contribute to the Th2 immune response which was not assessed in this study.

Similar to the observation reported by Khosravi et al., an elevated serum IgE was noted in the present study.⁴¹ IgE's role in suppressing CMI response through histamine explains the patient's predisposition for recurrent or chronic dermatophytosis. In the present study, although Th2 cells (CD3+IL-4+cells * absolute lymphocyte count secreting) were not raised, increased IL-4+ cells, IgE levels, and reduced DTH response indicate skewing towards Th2 immune response in recurrent dermatophytosis. However, a history of atopy, allergic rhinitis or asthma was not seen as the significant predisposing factor in our patients.

The intradermal test revealed a significant difference in positive IH immune response between cases and healthy controls, acute dermatophytosis controls and both healthy and acute dermatophytosis controls which was similar to the study by Khosravi et al. and Hay et al.^{41, 42} Similar to the previous result it was observed that IH response increased in patients with recurrent dermatophytosis.³⁷ DTH response was negative in all of our cases which was significant that suggests a lack of cell-mediated immune response in our cases. This result was in accordance to study by Leivobici et al. and Jones et al.^{12, 43}

The study's limitation was that the few patients in the present study were using topical steroids until the day of recruitment which is likely to affect the host immune response to the infection and become a significant confounding factor. Further, *in-vitro* lymphocyte stimulations with dermatophyte antigen, cytokine proliferation assay in peripheral blood, and trichophytin specific

IgE levels were not performed in the present study. The control group was heterogeneous and did not take a comparable number of cases and controls. Despite the *T. mentagrophytes* complex being the predominant species isolated in culture, the *T. rubrum* extract was used to perform the intradermal test. To understand the role of intradermal test there is a need to further evaluate this test with predominant etiology responsible for this ongoing epidemic condition.

Conclusion

Patients with recurrent dermatophytosis have reduced IFN- γ + cells, Th1 cells, IL-17+ cells, Th17 cells, raised IL-4+ cells, increased serum IgE levels, and diminished DTH response in the intradermal skin test. Along with dysregulated immunity, several factors like prior use of topical corticosteroids, higher MIC of standard antifungal drugs, sharing of fomites among family members, similar history in family members, use of tight fit undergarments, poor frequency of washing clothes are some predisposing factors for recurrent tinea. Further studies are required concerning the role of genetic factors, innate immunity, local immune response in recurrences of the disease, as well as exploring the role of immunotherapy along with antifungal agents for the treatment of recurrent dermatophytosis.

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Figure legends

Figure 1. Clinical photographs depicting typical and atypical morphologies in patients with recurrent dermatophytosis.

A- Classical tinea, B- Eczematous lesion in tinea, C- Papulosquamous lesions in tinea, D- Pustular lesion in tinea, E- Pseudoimbricata lesion in tinea, F- Lichenified lesion in tinea

Figure 2. Flow cytometric evaluation

FACS gating showing CD3⁺ T cells positive for IL-4 and CD4⁺ T cells positive for IL-17A and IFN- γ in A-recurrent dermatophytosis case, B- Healthy control, and C- Acute dermatophytosis control

Figure 3. Receiver operating characteristic curve (ROC) analysis for determining the immunological parameters cutoff in recurrent dermatophytosis cases and controls (healthy and acute dermatophytosis)

Figure 4. Intradermal trichophytin skin test. Clinical photograph depicting immediate hypersensitivity and delayed hypersensitivity immune response in the skin after trichophytin antigen stimulation test. A- immediate hypersensitivity response and B- delayed hypersensitivity response.

Table 1- Clinico-demographic features, risk factors and characteristics of intradermal trichophytin skin test in recurrent dermatophytosis cases and controls (acute dermatophytosis and healthy control)

Risk factors		Recurrent dermatophytosis cases (n=100)	Acute dermatophytosis cases (n=25)	Healthy control (n=25)	P-value		
					Recurrent vs. acute dermatophytosis control	Recurrent vs. healthy control	Recurrent vs. acute dermatophytosis control and healthy control
Age	Mean± SD	32.4±11.2	33.1±9.65	34.8±15.8	-	-	-
Sex	Male	65 (65%)	16 (64%)	15 (60%)	0.925	0.641	0.897
	Female	35 (35%)	9 (36%)	10 (40%)			
Occupation	Housewife	27 (27%)	5 (20%)	6 (24%)	0.086	0.826	0.258
	Office workers	26 (26%)	14 (56%)	7 (28%)			
	Manual workers	14 (14%)	1 (4%)	3 (12%)			
	Students	23 (23%)	4 (16%)	6 (24%)			

Bathing habit	Farmers	4 (4%)	1 (4%)	0 (0%)	0.145	0.252	0.176
	Others	6 (6%)	0 (0%)	3 (12%)			
	Daily	46 (46%)	15 (60%)	16 (64%)			
	Alternate days	36 (36%)	10 (40%)	8 (32%)			
	Twice a week	13 (13%)	0 (0%)	1 (4%)			
	Weekly	5 (5%)	0 (0%)	0 (0%)			
Frequency of washing clothes	Daily	21 (21%)	7 (28%)	6 (24%)	0.452	<0.001	<0.001
	Alternate day	69 (69%)	14 (56%)	7 (28%)			
	Weekly	10 (10%)	4 (16%)	12 (48%)			
Types of undergarments	Tight	36 (36%)	13 (52%)	17 (68%)	0.143	0.004*	0.011*
	Loose fitting	64 (64%)	12 (48%)	8 (32%)			
History of atopy	Present	15 (15%)	1 (4%)	3 (12%)	0.141	0.702	0.333
	Absent	85 (85%)	24 (96%)	22 (88%)			
History of animal contacts	Present	9 (9%)	1 (4%)	1 (4%)	0.410	0.410	0.542
	Absent	91 (91%)	24 (96%)	24 (96%)			
Sharing of towel	Present	78 (78%)	10 (40%)	12 (48%)	<0.001	0.003	<0.001
	Absent	22 (22%)	15 (60%)	13 (52 %)			

Co-morbid illness	Diabetes	4 (4%)	0 (0%)	0 (0%)	0.506	0.472	0.475
	Autoimmune disease	5 (5%)	0 (0%)	1 (4%)			
	Malignancy	1 (1%)	0 (0%)	0 (0%)			
	Hypertension	2 (2%)	0 (0%)	2 (8%)			
	Absent	88 (88%)	25 (100%)	22 (88%)			
History of tinea in family members	Present	58 (58%)	4 (16%)	3 (12%)	<0.001	<0.001	<0.001
	Absent	42 (42%)	21 (84%)	22 (88%)			
Past history of corticosteroid use	Present	62 (62%)	0 (0%)	0 (0%)	<0.001	<0.001	<0.001
	Absent	38 (38%)	25 (100%)	25 (100%)			
Trichophytin skin test	<5mm	9 (9%)	6 (24%)	10 (40%)	0.018	<0.001	<0.001
Immediate hypersensitivity							

(IH)

5-10 mm 85 (85%) 15 (60%) 11 (44%)

>10 mm 6 (6%) 4 (16%) 4 (16%)

Trichophyton Present 0 (0%) 9 (36%) 9 (36%) <0.001 <0.001 <0.001
skin test

Delayed Type
hypersensitivity

(DTH)

Absent 100 (100%) 16 (64%) 16 (64%)

SD- Standard Deviation; *Atopy was defined based on Hanifin and Rajka's⁴⁴

Table 2- *In-vitro* activities of four antifungal agents against 48 strains of *Trichophyton* spp.

Antifungal agent	MIC range (µg/ml)	MIC 50 (µg/ml)	MIC 90(µg/ml)	Geometric Mean	Isolates with high MIC value	Percentage(%)
Griseofulvin	0.25-128	16	32	9.79	26	54.16
Fluconazole	0.015-32	2	8	2.37	8	16.66
Terbinafine	0.015-8	0.03	1	0.06	6	12.5
Itraconazole	0.015-0.5	0.06	0.25	0.08	0	0

MIC- Minimum inhibitory concentration

Table 3-Receiver operating characteristic curve (ROC) analysis for determining the predictive value of immunological parameters in recurrent dermatophytosis cases vs. healthy controls, recurrent dermatophytosis cases vs. acute dermatophytosis controls and recurrent dermatophytosis cases vs. combined acute dermatophytosis and healthy controls

		AUC	95% C.I of AUC	Z-statistics	Criterion counts	Sensitivity	Specificity	P-value
Case vs. Healthy control	Th1	0.512	0.420- 0.605	0.184	≤181.26 (counts/μl)	87.37	100	0.8542
	Th2	0.554	0.459- 0.645	0.839	≤4.25 (counts/μl)	35.11	100	0.4016
	Th17	0.622	0.529- 0.709	2.120	≤4.6 (counts/μl)	47.37	100	0.0340*
	IFN- γ + cells	0.503	0.410- 0.595	0.0375	>25.9 %	5.26	4.00	0.9701
	IL-4 + cells	0.639	0.545- 0.725	2.133	≤0.5 %	56.38	66.32	0.0329*
	IL-17 + cells	0.634	0.541- 0.720	2.201	≤0.4 %	32.63	60.00	0.0277
	Th1	0.707	0.617- 0.787	3.594	≤84.39 (counts/μl)	67.37	100	0.0003*

Case vs. Acute Dermatophytosis control	Th2	0.536	0.441- 0.630	0.521	≤7.06 (counts/μl)	53.19	99.17	0.6027
	Th17	0.724	0.635- 0.801	3.915	≤6.54 (counts/μl)	68.42	100	0.0001*
	IFN- γ+ cells	0.758	0.671- 0.831	4.989	≤10.2 %	64.21	100	<0.0001*
	IL-4+ cells	0.654	0.559- 0.741	2.603	≤0.3 %	39.36	62.50	0.0092*
	IL-17+ cells	0.750	0.663- 0.824	4.519	≤0.8 %	74.74	68	<0.0001*
Case vs. Healthy and Acute Dermatophytosis control	Th1	0.610	0.525- 0.690	2.192	≤102.61 (counts/μl)	72.63	100	0.0284*
	Th2	0.546	0.459- 0.631	0.882	≤13.42 (counts/μl)	80.85	98.59	0.3780
	Th17	0.673	0.590- 0.748	3.767	≤4.6 (counts/μl)	47.37	99.75	0.0002*
	IFN- γ+ cells	0.628	0.543- 0.706	2.506	≤15.9 %	83.16	98	0.0122*
	IL-4+ cells	0.646	0.560- 0.725	2.944	≤0.5 %	56.38	64.46	0.0032*
	IL-17+ cells	0.692	0.610- 0.766	4.196	≤1 %	86.32	65.20	<0.0001*

*Significant P-value

Table 4- Characteristics of recurrent dermatophytosis cases, healthy controls and acute dermatophytosis controls in respect to the cut-off value obtained from ROC curve analysis for immunological parameters

Variable	Case vs. Healthy control				Case vs. Acute dermatophytosis control				Case vs. Healthy and acute dermatophytosis control			
	Case description	Normal (n,%)	Reduced (n,%)	P-value		Normal (n,%)	Reduced (n,%)	P-value	Case description	Normal (n,%)	Reduced (n,%)	P-value
Th1 (counts/μl)	Case (n=95)	12 (12.6%)	83 (87.3%)	0.1197	Case (n=95)	31 (32.6%)	64 (67.3%)	0.0024*	Case (n=95)	26 (27.3%)	69 (72.6%)	0.0169*
	Healthy Control (n=25)	7 (28%)	18 (72%)		Acute dermatophytosis Control (n=25)	17 (68%)	8 (32%)		#Control (n=50)	24 (48%)	26 (52%)	
Th17 cells (counts/μl)	Case (n=95)	56 (59%)	39 (41%)	0.0634	Case (n=95)	30 (31.6%)	65 (68.4%)	0.0001*	Case (n=95)	56 (58.9%)	39 (41.0%)	0.0054*
	Healthy Control (n=25)	20 (80%)	5 (20%)		Acute dermatophytosis Control (n=25)	19 (76%)	6 (24%)		#Control (n=50)	41 (82%)	9 (18%)	

29 (58%)

0.0007*

30 (60%)

P- value

0.0018*

Healthy
Control

(n=24)

**Th2 cells
(counts/ μ l)**

Case (n=94)	33 (35.1%)	61 (64.8%)	0.2260
Healthy Control (n=24)	5 (20.8%)	19 (79.1%)	

Case (n=94)

50 (53.1%)
44
(46.8%)

0.3307

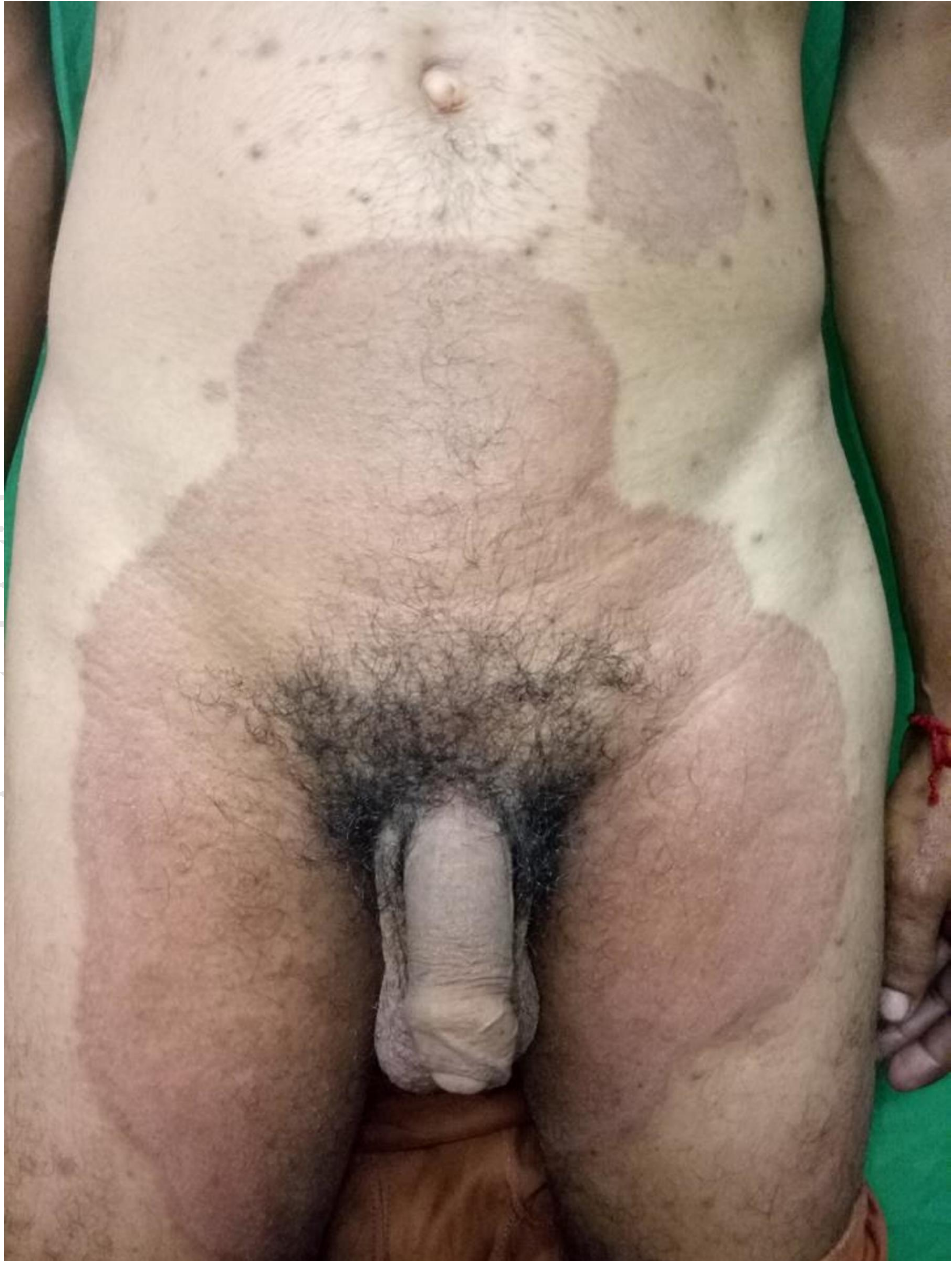
Case (n=94)

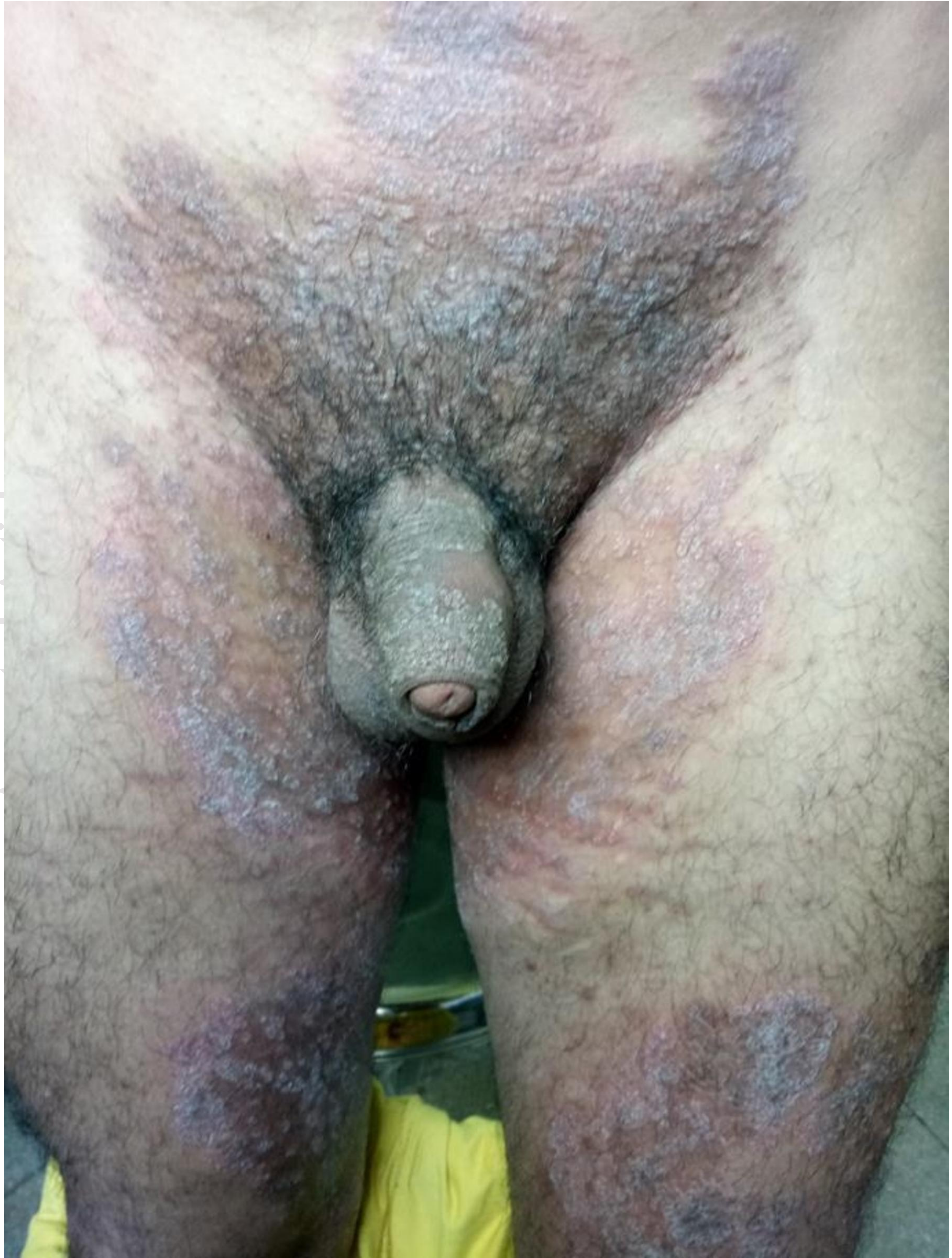
76 (80.8%)
18
(19.2%)

0.1297

Acute
dermatophytosis
Control (n=20)8 (40%)
12
(60%)#Control
(n=44)30 (68.1%)
14
(31.9%)

*Significant P-value





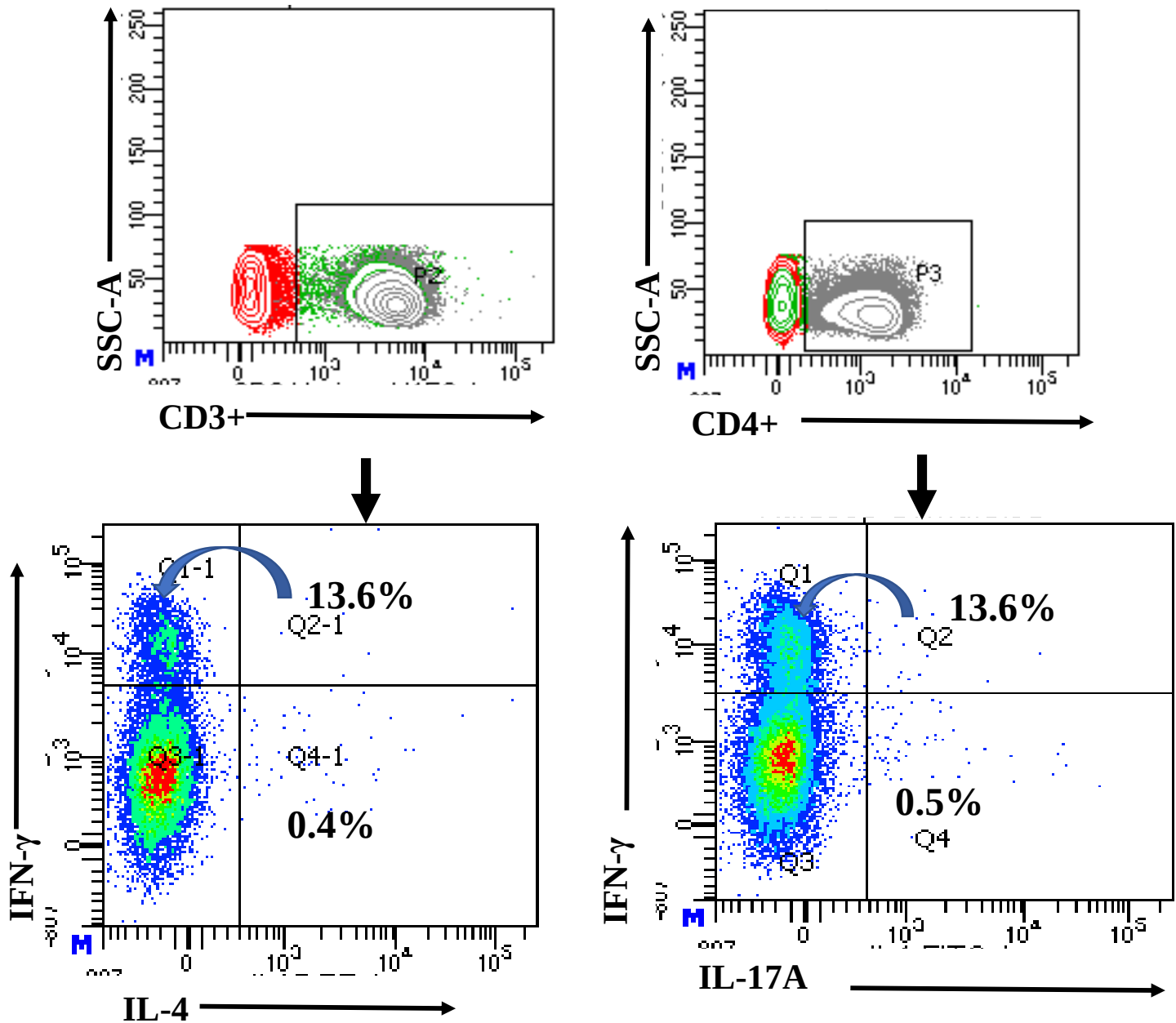






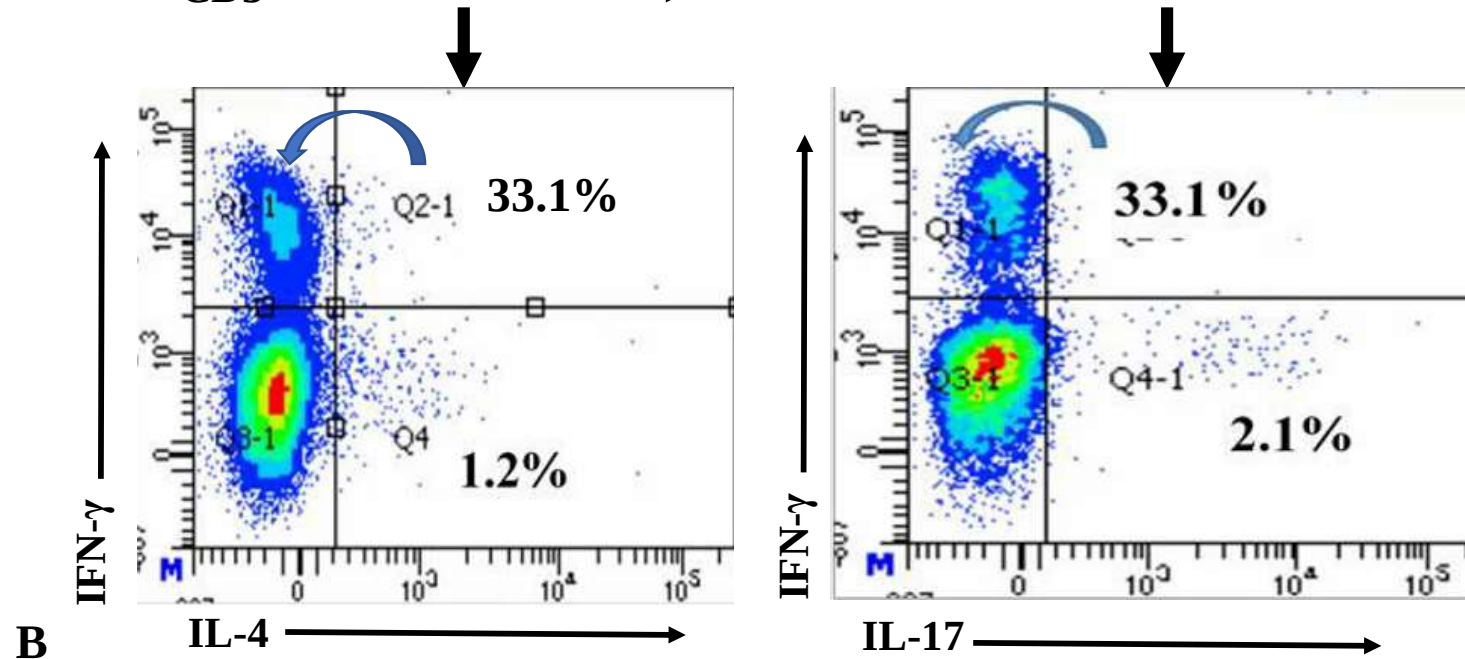
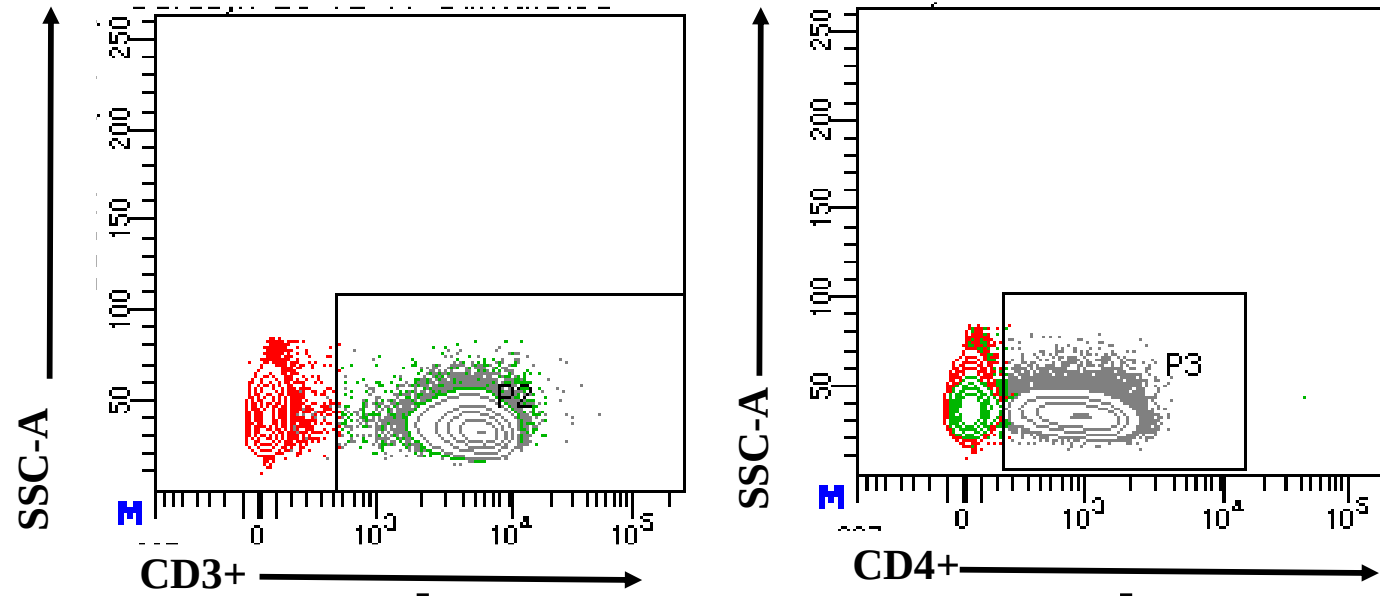


Recurrent Dermatophytosis cases

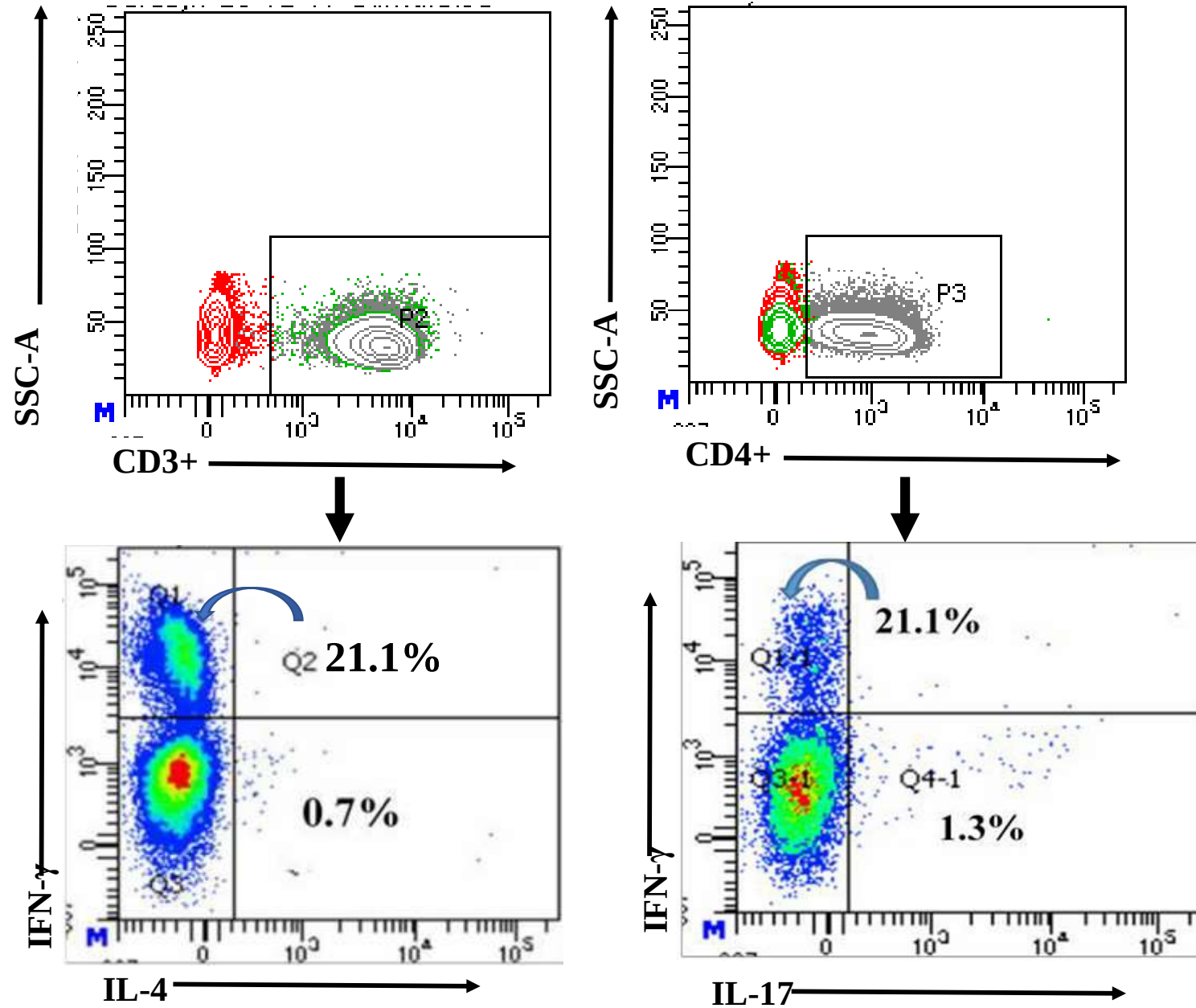


A

Healthy controls



Acute Dermato­ph­ty­sis con­trols

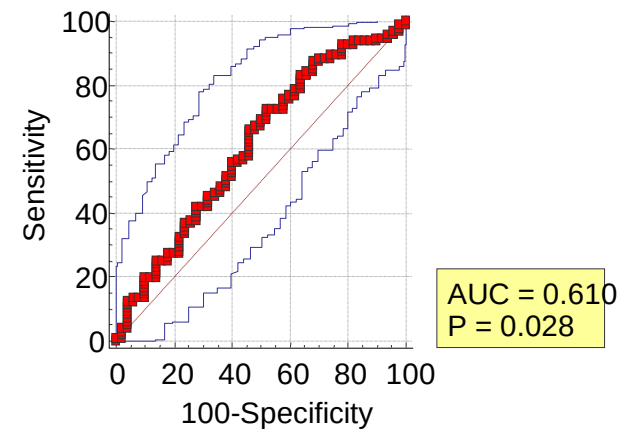
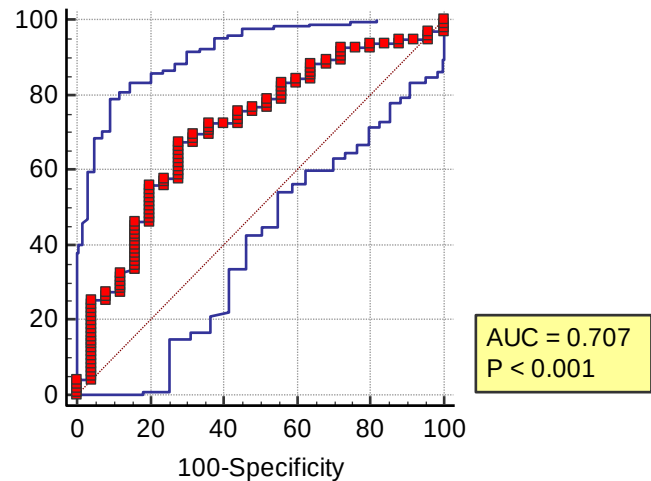
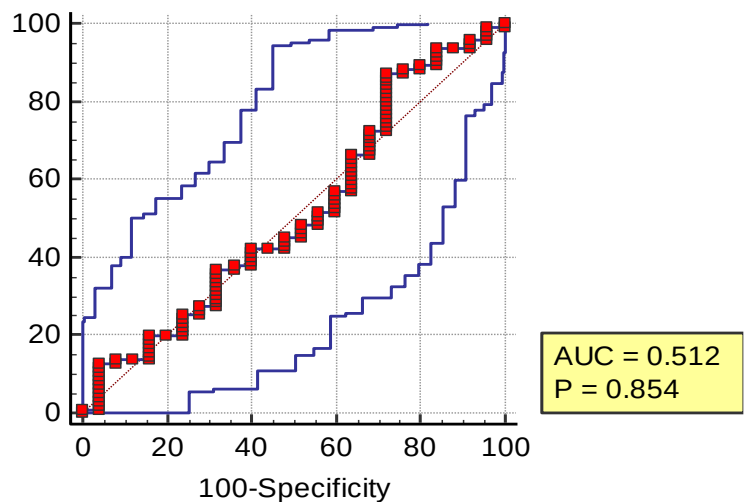


Case vs. Healthy control

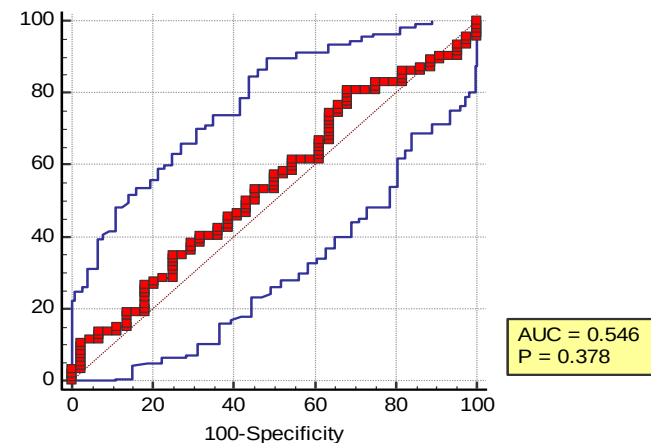
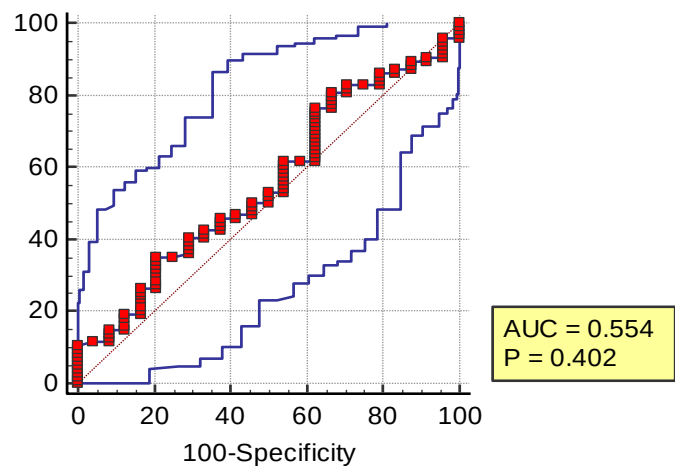
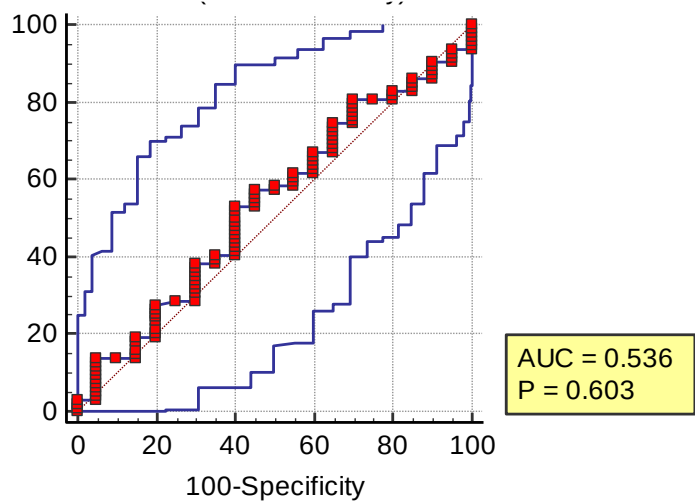
Case vs. Acute Dermatophytosis control

Case vs. Control (Healthy and Acute Dermatophytosis)

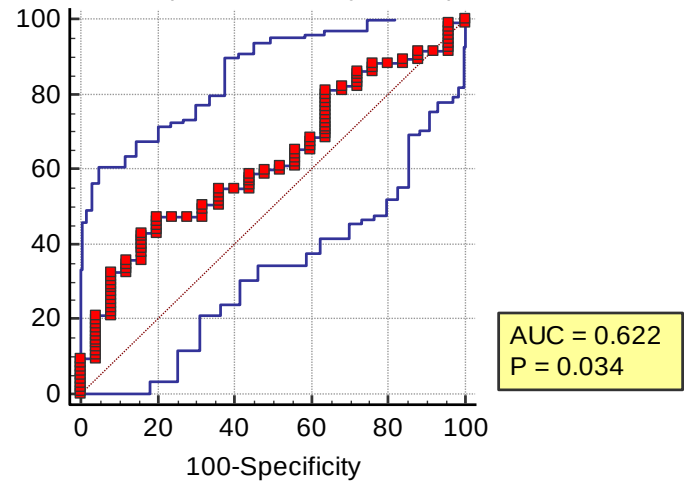
Th1



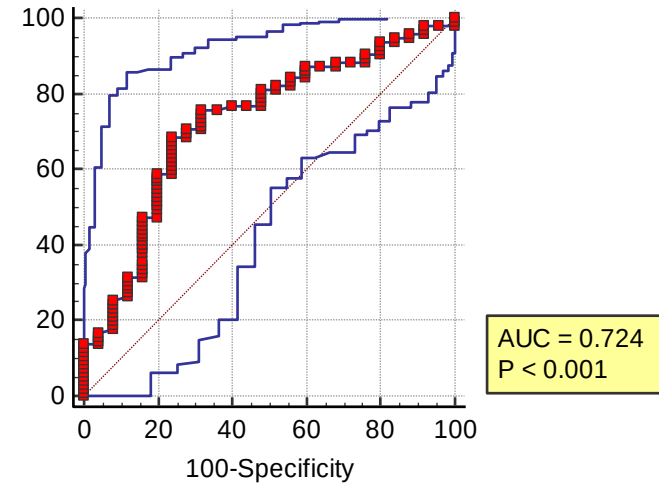
Th2



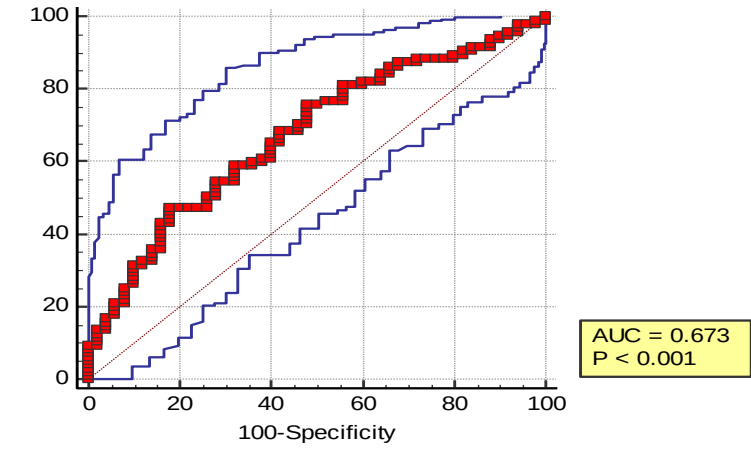
Case vs. Healthy control



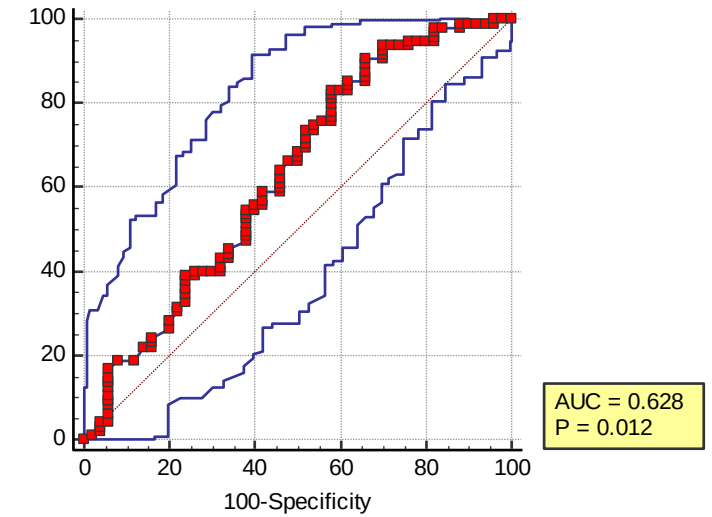
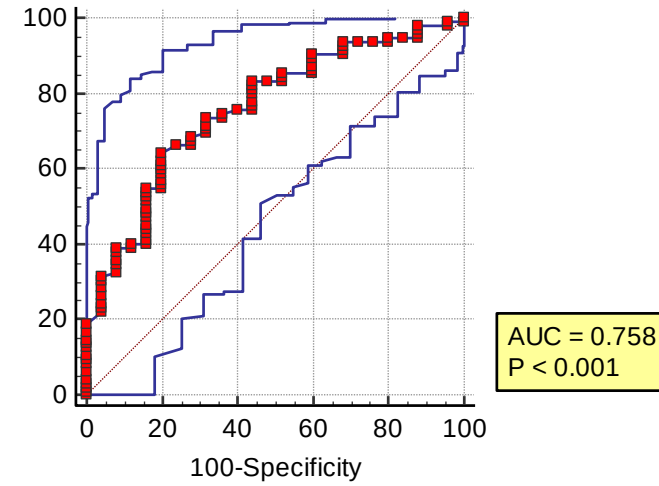
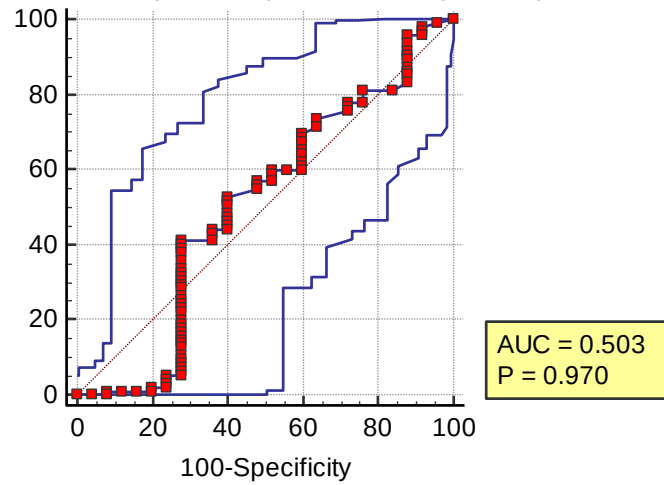
**Case vs. Acute
Dermatophytosis control**



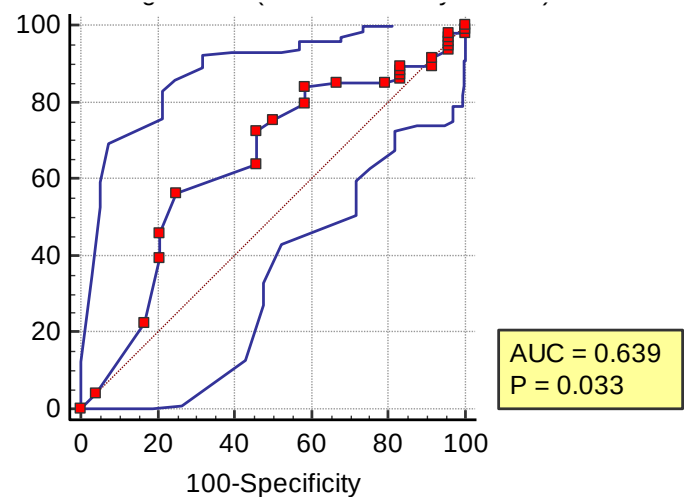
**Case vs. Control (Healthy and Acute
Dermatophytosis)**



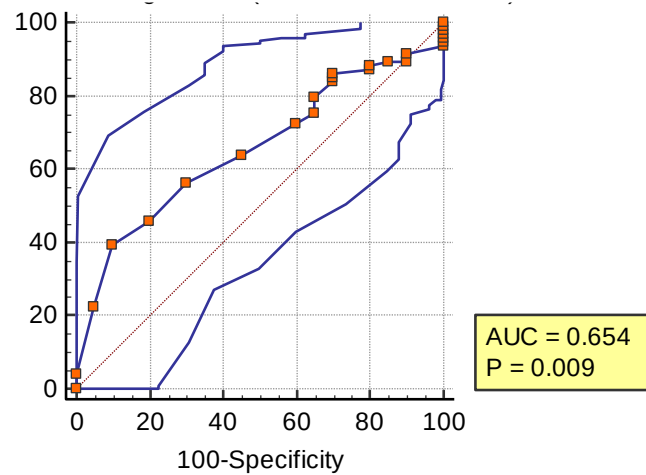
IFN- γ



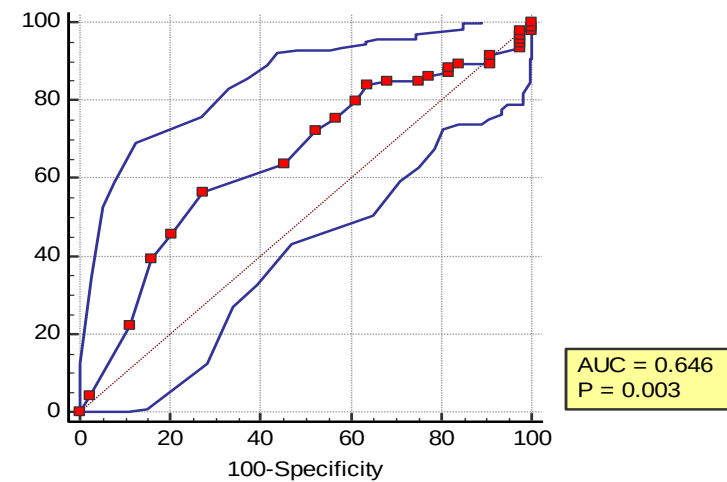
Case vs. Healthy control



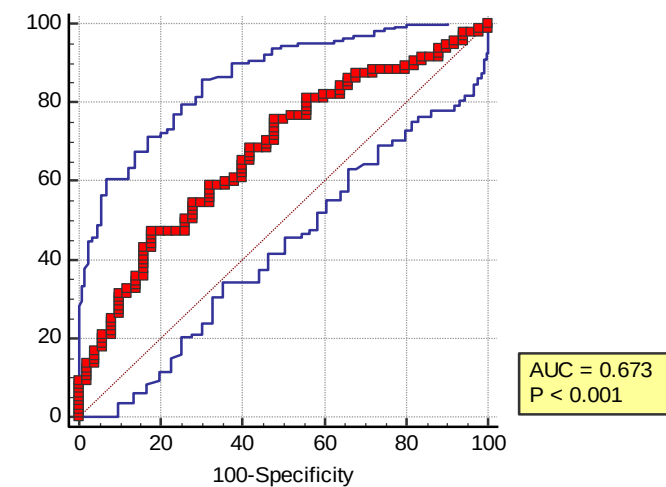
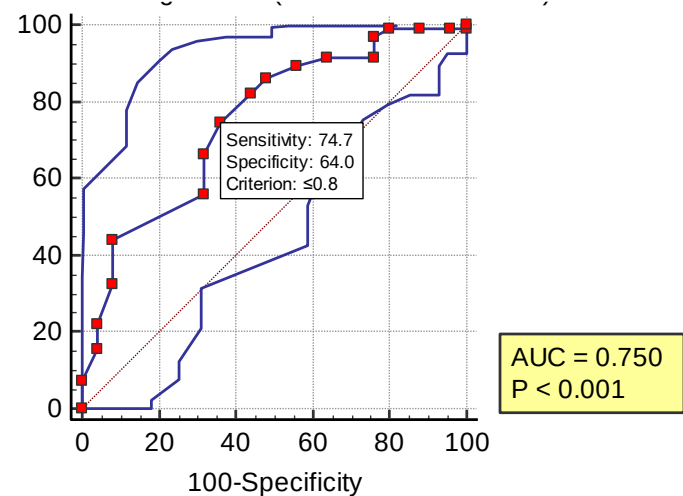
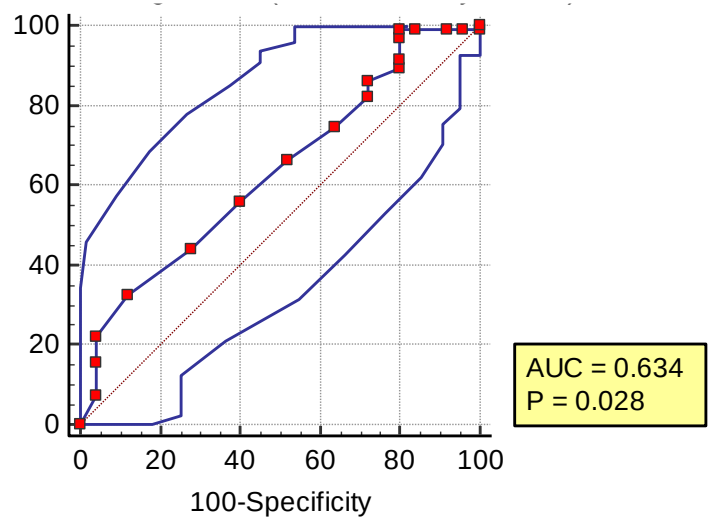
**Case vs. Acute
Dermatophytosis control**



**Case vs. Control (Healthy and Acute
Dermatophytosis)**



IL-4



IL-17



