

**Pityrosporum ovale in Healthy Children,  
Infantile Seborrhoeic Dermatitis and  
Atopic Dermatitis**

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#### ABSTRACT

The occurrence of *Pityrosporum ovale* was studied in healthy children, children with infantile seborrhoeic dermatitis (ISD) and in patients with atopic dermatitis (AD).

Twenty children with ISD and twenty healthy infants were subjected to culture for *P. ovale*. Positive cultures were found in 18 of 20 infants with ISD, compared with 4 of 20 controls. The same culture medium containing olive oil as one of the lipids was used to evaluate the frequency of positive *P. ovale* cultures in 60 patients with AD, 40 patients with rhinoconjunctivitis and/or asthma (RA) and 40 children and young adults with no atopic history (HC). The results of the quantitative cultures from the forehead did not differ between the groups. *P. ovale* cultures were positive in 0-20% of children aged 0-10 years and in 60-90% of the 11-20-year-old subjects.

Positive *P. ovale* cultures were found in 87 % of 138 healthy children aged 2 months to 15 years when cultures were performed on a medium containing whole fat cows' milk as one lipid source. The largest number of colonies was found among children aged 2-23 months and among children older than 9 years.

The occurrence of specific IgE antibodies to *P. ovale* was evaluated with a skin prick test (SPT) and RAST and compared in 3 groups (AD, RA, HC) of patients aged 0-20 years. Specific IgE were found most often in patients with AD. In patients with AD on different parts of the body, 15 % had a positive SPT to *P. ovale*. In another group of patients, aged 14-53 years, with AD localised mainly to the head and neck area, the SPT was positive in 55% of the patients.

Sera from 13 patients with positive SPT to *P. ovale* were further analysed with IgE immunoblotting using both *P. ovale* and *C. albicans* antigens. Simultaneous IgE-binding to both these yeasts was found in 5 sera and these were analysed with RAST-inhibition. Cross-reacting IgE antibodies to *P. ovale* and *C. albicans* were found in two of these sera. Cross-reacting sera were pooled and used as an IgE probe in crossed radioimmuno-electrophoresis and Tandem-crossed immunoelectrophoresis. Cross-reacting epitopes were suggested to be located in the mannan polysaccharide of *C. albicans* and in a high molecular weight fraction of *P. ovale*.

To evaluate the effect of topical antimycotic treatment in patients with AD affecting the head and neck area, 53 patients aged 28 years (range 14-53 years) were included in a double-blind study for 6 weeks. In addition to oral antibiotic treatment, patients in one group were given miconazole-hydrocortisone cream and ketoconazole shampoo whereas patients in the other group were given hydrocortisone cream and placebo shampoo. After 4 weeks' treatment, there was a decrease in *P. ovale* colonisation in the group given the antimycotics but not in the placebo group. The decrease in eczema score did not differ between the groups.

In conclusion, we found that patients with AD harboured *P. ovale* on the skin in the same frequency as patients with or without other atopic manifestations. Sensitisation to this yeast was found most often in AD but although topical antimycotic treatment was effective in decreasing the amount of *P. ovale* on the skin no additional therapeutic effect was noted.

**Key words:** Children, culture, *Staphylococcus aureus*, comparative study, rhinoconjunctivitis, asthma, IgE antibodies, skin prick test, RAST, antigen, mannan, healthy children, double-blind study.

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- II. Broberg A, Faergemann J, Johansson S, Johansson S.G.O, Strannegård I-L, Svejgaard E. *Pityrosporum ovale* and atopic dermatitis in children and young adults. Acta Derm Venereol (Stockh) 1992;72:187-192.
- III. Savolainen J, Broberg A. Crossreacting IgE antibodies to *Pityrosporum ovale* and *Candida albicans* in atopic children. Clin Exp Allergy 1992;22:469-474.
- IV. Bergbrant I-M, Broberg A. *Pityrosporum ovale* culture from the forehead of healthy children. Acta Derm Venereol (Stockh) 1994;74:260-261.
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# CONTENTS

ABBREVIATIONS .....	6
INTRODUCTION .....	7
<i>Pityrosporum ovale</i> .....	8
Historical background .....	8
Culture medium .....	9
Morphology and serology .....	10
Saprophyte and pathogen .....	11
Atopic dermatitis .....	11
AD and <i>P. ovale</i> .....	12
Infantile seborrhoeic dermatitis .....	13
ISD and <i>P. ovale</i> .....	13
AIMS OF THE STUDY .....	14
MATERIALS AND METHODS .....	15
Patients and control subjects .....	15
ISD and <i>P. ovale</i> (I) .....	15
<i>P. ovale</i> and AD in children and young adults (II) .....	15
Crossreacting IgE antibodies to <i>P. ovale</i> and <i>C. albicans</i> in atopic children (III) .....	15
<i>P. ovale</i> culture from the forehead of healthy children (IV) .....	16
Topical antimycotic treatment of AD in the head and neck area (V) .....	16
<i>P. ovale</i> culture .....	16
Papers I and II .....	16
Papers IV and V .....	16
Bacterial culture (I,V) .....	17
Immunological investigations .....	17
SPT (II,V) .....	17
Total serum IgE (II,V) .....	17
RAST (II) .....	17
ELISA (II) .....	17
Antigens (III) .....	18
SDS-PAGE and IgE-immunoblotting (III) .....	18
RAST-inhibition (III) .....	18

MATERIAL AND METHODS contd	
CIE, CRIE and Tandem-CRIE (III) .....	18
Experimental design (V) .....	19
Scoring of AD (V) .....	19
Statistical methods (I,II,V) .....	20
RESULTS .....	21
<i>P. ovale</i> culture .....	21
In children with ISD (I) .....	21
In children and young adults with AD (II) .....	21
In healthy children (IV) .....	22
In AD with head and neck dermatitis (V) .....	22
Bacterial culture .....	23
In children with ISD (I) .....	23
In AD with head and neck dermatitis (V) .....	23
SPT with <i>P. ovale</i> extract .....	23
In children and young adults with AD (II) .....	23
In AD with head and neck dermatitis (V) .....	24
Total serum IgE .....	24
In children and young adults with AD (II) .....	24
In AD with head and neck dermatitis (V) .....	24
RAST to <i>P. ovale</i> , <i>C. albicans</i> and <i>C. herbarum</i> (II) .....	24
RAST and SPT to <i>P. ovale</i> (II) .....	25
IgG antibodies to <i>P. ovale</i> (ELISA) (II) .....	25
Crossreacting IgE antibodies to <i>P. ovale</i> and <i>C. albicans</i> in atopic children (III) .....	25
SDS-PAGE and IgE-immunoblotting .....	25
RAST-inhibition .....	27
CIE, CRIE and Tandem-CRIE .....	27
Topical antimycotic treatment of AD in the head/neck area (V) .....	27
MAIN FINDINGS .....	29
DISCUSSION .....	30
ACKNOWLEDGEMENTS .....	37
REFERENCES .....	38

# ABBREVIATIONS

AD	Atopic dermatitis
C. albicans	Candida albicans
C. herbarum	Cladosporium herbarum
CIE	Crossed immuno-electrophoresis
CRIE	Crossed radio-immuno-electrophoresis
ELISA	Enzyme-linked immunosorbent assay
HND	Head and neck dermatitis
ISD	Infantile seborrhoeic dermatitis
P. ovale	Pityrosporum ovale
RAST	Radio-allergosorbent test
S. aureus	Staphylococcus aureus
SCORAD	Scoring of atopic dermatitis
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamid gel electrophoresis
SPT	Skin prick test

## INTRODUCTION

In research it is often necessary to try to isolate problems which in reality are part of a highly complex whole. Results must be related to the whole picture if the conclusion is to be relevant to natural conditions. In this thesis, one factor has been isolated from the complex picture of atopic dermatitis. *Pityrosporum ovale* belongs to the normal microflora of the human skin and has been shown to trigger the immune system in healthy persons. In patients with atopic dermatitis specific IgE antibodies to *P. ovale* have been found. Environmental factors in relation to AD have become more important to study with further elucidation of the immunological mechanisms in AD.

Our knowledge of atopic disease has increased enormously since 1921, when Prausnitz (1) showed that fish allergy could be transferred by serum from an allergic patient to a healthy person. The propensity to produce reaginic antibodies in patients with AD was well known even before further characterisation of this immunoglobulin was achieved. The significance of the wheal and flare reaction for the development of the spongiotic dermatitis has not been clear. The word "atopic", coined by Arthur Coca in 1922, and meaning strange, is still justified (2).

In 1963 Frankland (3) wrote: *"that man when atopic does have peculiar antibodies present. These reaginic antibodies are present in all the tissues and in blood serum and give the characteristic wheal and flare reaction of the "immediate" type and can be transferred to normal non-sensitized man."*

Reaginic antibodies were isolated by Ishizaka et al. in 1966 and called IgE (4). In 1968 Johansson et al. were able to characterise this immunoglobulin further when they found a patient with an atypical myeloma protein with similar properties to the reagins found in patients with ragweed sensitivity (5). Although more knowledge was gained about IgE and this immunoglobulin was found in high concentrations in the majority of patients with AD, its role in the pathogenesis of the eczema remained unclear. In 1986 the presence of IgE on epidermal Langerhans' cells from patients with AD was discovered, a finding that theoretically could link type I and type IV reactions (6). The recent findings relating to the pathogenesis of AD have been summarised by Cooper (7) (Fig. 1). This complex figure illustrates the T-lymphocyte activation, hyperstimulatory Langerhans' cell and B-cell IgE overproduction, clearly indicating that Rostenberg (8), in 1955, was right when he questioned that the union of antigen and antibody was the whole story for the development of AD.

Not only have these historical discoveries given us a better understanding of the mechanisms of the spongiotic dermatitis, they have also opened up new perspectives concerning triggering factors and therapies in AD.



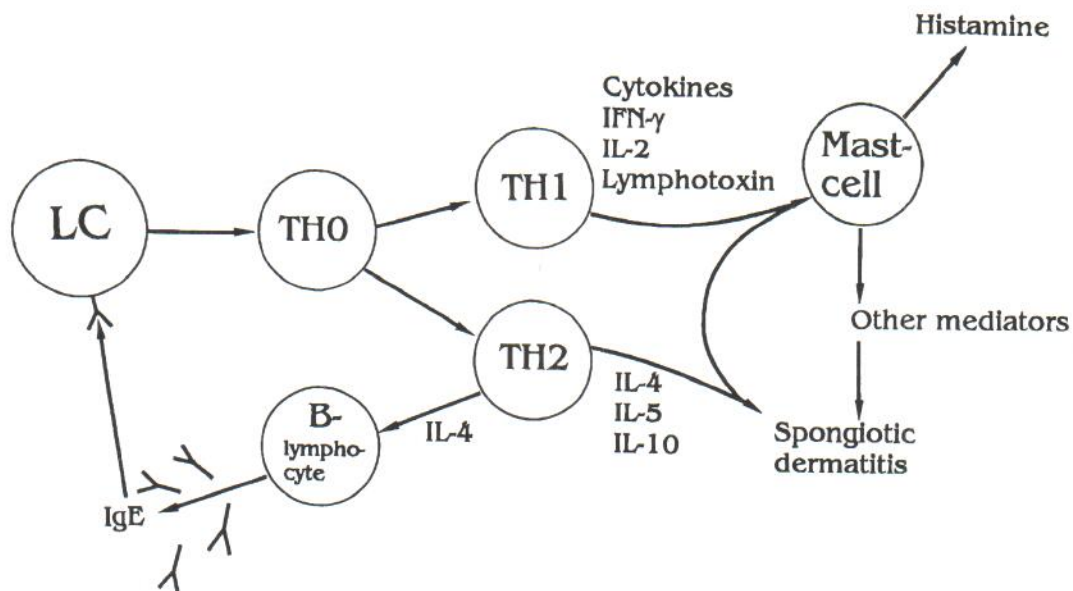


Fig. 1. Schematic illustration of suggested interacting inflammatory mechanisms in AD. Hyperstimulatory Langerhans cells (LC) present antigens to T cells (TH0) and induce T cell activation (TH1 or TH2), cytokine production and spongiotic dermatitis (7).

## *Pityrosporum ovale*

### Historical background

Early descriptions of the yeast *P. ovale* were limited due to failure to culture this fastidious organism, which requires lipids in the medium for growth. Micromorphological descriptions from direct microscopy were presented by Rivolta in 1873 (9) and Malassez in 1874 (10). The yeast-like cells in the stratum corneum were named *Malassezia furfur* although many synonyms have been used in the literature (11) (Table I). In 1913, Castellani and Chalmers cultured the organism and named the yeast *Pityrosporum ovale* (12). The yeast may present as round or spherical and this has been interpreted as reflecting different cell cycles (13). With improved methods for isolation, a variety of colony types have been noted (14). It is not clear whether these subgroups of *P. ovale* represent stable genotypic heterogeneity or less stable phenotypic subgroups due to different environments. *P. ovale* with different micromorphology have been shown to be very similar genetically (15). Cunningham et al. differentiated 3 types of *P. ovale* based on morphology and physiology and serological studies demonstrated different surface antigens (16). In a recently published paper, the authors could not find any association between different subgroups of *P. ovale* and pityriasis versicolor or seborrhoeic dermatitis (17).

Table I. Various names used for *Pityrosporum ovale*\*.

<i>Microsporon furfur</i>	<i>Dermatophyton malassezi</i>
<i>Cryptococcus psoriasis</i>	<i>Pityrosporum ovale</i>
<i>Saccharomyces ovalis</i>	<i>Pityrosporum orbiculare</i>
<i>Saccharomyces sphaericus</i>	<i>Cryptococcus malassezia</i>
<i>Malassezia ovalis</i>	<i>Malassezia.furfur</i>
<i>Malassezia tropica</i>	<i>Microsporon macfadyeni</i>
<i>Pityrosporum malassezii</i>	<i>Monilia furfur</i>
<i>Microsporon tropica</i>	<i>Pityrosporum cantlieni</i>

\*Adapted from Ingham and Cunningham (11)

### Culture medium

The genus *Pityrosporum* (spore of the scale) includes the lipophilic yeast *P. ovale* seen as a saprophyte on the normal skin of most healthy persons and the non-lipophilic *P. pachydermatis* which is normally found in animals (18). *P. ovale* needs the addition of fatty acids of C<sub>12</sub> or higher chain lengths for growth since a block in the capacity to synthesise this fatty acids has been demonstrated (19) (Table II). Coconut oil and olive oil were initially used as a covering

Table II. Media used for cultivation of *P. ovale*. Antibiotics, to prevent bacterial overgrowth, and cycloheximide, to retard the development of mould spores, were added to all three media.

#### Dixon's media (20)

Malt extract agar 6%  
Ox bile desiccated 2%  
Tween 40 1%  
Glycerol monooleate 0.25 %

#### Faergemann (21)

Bacto agar 18 g/litre  
Glucose 40 g/litre  
Neopeptone 10 g/litre  
Yeast extract 0.1 g/litre  
Glycerol monostearate 2.5 g/litre  
Tween 80 2 ml/litre  
Olive oil 20 ml/litre

#### Leeming & Notman (14)

Agar 12 g/litre  
Glucose 5 g/litre  
Bacterological peptone 10 g/litre  
Yeast extract 0.1 g/litre  
Ox bile, desiccated 4 g/litre  
Glycerol monostearate 0.5 g/litre  
Glycerol 1 ml/litre  
Tween 60 0.5 ml/litre  
Whole-fat cows' milk 10 ml/litre

layer on the basal nutrient medium with the disadvantage that the colonies tended to coalesce. Van Abbe (1964) used Dixon's medium containing ox bile, Tween 40 and glycerol monooleate as lipids (20). Faergemann recommended the following lipids; glycerol monostearate, Tween 80 and olive oil (21). As is evident from an overview of media used for cultivation of *P. ovale* by Korting et al., olive oil has been widely used (22). In 1987 Leeming and Notman tested several media and found the most suitable to be a medium containing whole fat cows' milk, ox bile, glycerol, glycerol monostearate and Tween 60 (14). This medium has also been tested with the same result by Korting et al. (22).

### Morphology and serology

*P. ovale* grows under aerobic conditions in the temperature range 28-37°C and colonies are 3-6 mm in diameter and white to creamy in colour (21) (Fig. 2). The yeast is 2-7 µm in diameter with a varying micromorphology (Fig. 3). The yeast-mycelial transformation is influenced by the culture medium (23). Electron microscopy has shown a thick cell wall with indentations arranged in a spiral (24,25). The cell wall extract of one analysed strain of *P. ovale* contained 72% polysaccharide, 18.3% lipid and 7.5% protein (26).

IgG and IgM antibodies to *P. ovale* have been found in healthy individuals and in patients with *P. ovale* associated skin diseases (27,28,29). Specific IgE antibodies have been found in patients with AD (30,31,32). Immunocompromised patients are often more severely affected by seborrhoeic dermatitis and pityriasis versicolor and this favours the theory that cell-mediated immunity is involved in the defence against *P. ovale* (33). *P. ovale* can also activate the alternative complement pathway (34).

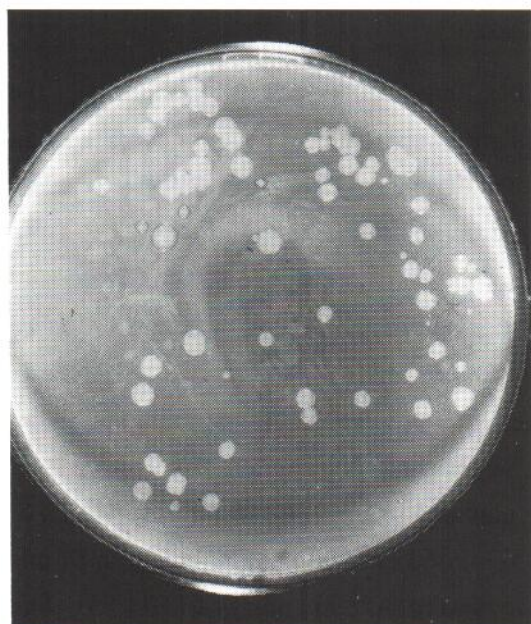


Fig. 2. Colonies are 3-6 mm in diameter and white to creamy in colour.



Fig. 3. The yeast is 2-7 µm in diameter with a varying micromorphology.

### **Saprophyte and pathogen**

In adults, *P. ovale* belongs to the normal microflora limited to the most superficial part of the skin and hair follicles, with regional variations probably due to the lipid content of the skin (35,36,37). In children, the carrier rate is less clear and results in the literature vary (38,39,40). Differences in results may be due to different experimental designs with different age-groups studied, culture media used and body sites investigated. In a group of children from newborn to 15 years of age, Faergemann found *P. ovale* only in children from 5 years of age and older when cultures were taken from the back (38). Leeming, however, cultured the organism from the scalp, ears and back of children in different age-groups (40). In babies up to 1 year of age, high counts of *P. ovale* were found from the ears compared with low numbers in the age-group 1-10 years when cultures were taken from the back.

*P. ovale* can, under the influence of predisposing factors, act as a pathogen and be associated with cutaneous and systemic diseases including pityriasis versicolor, seborrhoeic dermatitis, folliculitis and various systemic infections (sepsis, pneumonitis, peritonitis (11) and sinusitis (41)). Predisposing factors such as high temperature, high relative humidity, hyperhidrosis and systemic corticosteroids may be triggering factors in those diseases.

### **Atopic dermatitis**

AD is usually the first manifestation of atopic disease, with onset within the first year of life in the majority of patients (42). Diagnosis is based on the clinical picture and diagnostic criteria have been proposed (43,44). AD is divided into 3 phases from the infantile to the adult type. Atopic hand eczema, infra-gluteal eczema and head and neck dermatitis are other patterns of AD. In babies infantile seborrhoeic dermatitis (ISD) is by many regarded as an early phase of AD.

The genetic predisposition for atopic disease has long been known (45). Studies of the genetics of atopy indicate that environmental factors are likely to affect penetrance of the trait (46,47). There is now clear evidence that AD is steadily increasing (48). Among Swedish children aged 7-14 years investigated in 1979/80, the cumulative incidence was 12 % (49). Schultz Larsen et al. found it unlikely that genetic factors are responsible for the increase and speculated that environmental factors that change from one generation to the other might be the reason for the increase (47).

AD is a multifactorial skin disease and in genetically predisposed individuals triggering factors can be nonspecific (e.g. irritant substances, stress) or specific allergenic factors (e.g. foods, aeroallergens). Many patients with AD have elevated serum IgE but the role of this in the pathogenesis of the eczema is not clear (50,51,52). Food is often the first allergen of importance, preceding aeroallergens, and is related to severe AD in infants (53,54,55). The incidence and clinical implications of food allergy are still the subject of controversy (56,57).

Since Bruynzeel-Koomen et al. demonstrated the presence of IgE on epidermal mononu-

clear cells (6), the role of cell-mediated immunity in AD has been further investigated and a model of interacting inflammatory mechanisms and trigger factors has recently been presented by Cooper (7).

Exposure to house-dust mite correlates with frequency of AD (58) but reducing mites in the home setting has not been successful in healing eczema. Natamycin and vacuum-cleaning, alone or in combination, could not reduce mite numbers sufficiently to provide clinical benefit (59). Theoretically, both *Staphylococcus aureus* and *P. ovale* may be able to maintain the chronic skin inflammation of AD (7). Patients with AD are colonised with *S. aureus* in 74-100% (60, 61,62). Children with AD more often carry *S. aureus* in the nose (20%) compared with healthy children (10%) (62). However, the role of *S. aureus* in AD is still not clear. This microorganism may act as an aggravating factor by causing clinical infection or it may act as an allergen (63). IgE antibodies to staphylococcal toxins have recently been found (64).

*Candida albicans* does not belong to the normal microflora of the skin. The possible role of *C. albicans* in atopic allergy has been attributed to its common colonisation of the gastrointestinal tract. Gumowski et al. demonstrated that challenge with *C. albicans* in patients with asthma and rhinitis gave an immediate reaction in many patients (65) and positive RAST to *C. albicans* has also been found among AD patients (66).

#### **AD and *P. ovale***

Patients with AD are a heterogeneous group regarding clinical appearance and distribution. Triggering factors may differ in subgroups of AD patients. Patients with atopic head and neck dermatitis (HND) and positive skin prick tests (SPT) to *P. ovale* responded significantly better than patients with AD of other body regions when treated with ketoconazole tablets (67). This was the first clinical indication that *P. ovale* may play a role in atopic HND.

Positive SPT to *P. ovale* was first reported in 1958 among a group of atopic patients (68) and in subsequent papers has been reported in 28-79% of patients with AD with the highest frequency among patients with HND (69,32). Waersted and Hjorth investigated 741 consecutive patients and found skin prick test (SPT) positive to *P. ovale* only in atopic patients with active dermatitis and most frequently among patients with HND (28%) (69). Kiefer et al. reported 79% of patients with HND to be positive in SPT, compared with 45% of patients with dermatitis on other locations (32). Rokugo found the frequency of positive SPT to vary according to age (70). Among AD patients younger than 10 years, 39% were positive in SPTs, while 64% of patients older than 10 years were positive.

Specific IgE antibodies to *P. ovale* have also been investigated with RAST (30,31). In children aged 7-18 years, a strong correlation between atopic dermatitis and the occurrence of IgE antibodies to *P. ovale* was found (30).

In one report, patch tests with *P. ovale* extracts were positive in 64% of AD patients irrespective of the distribution of the AD lesions (70).

## **Infantile seborrhoeic dermatitis**

Even though ISD is presented as a separate entity and diagnostic features are described in dermatological textbooks (71,72), uniform criteria like those presented in AD (43) are lacking for ISD. The condition may reflect a variety of different skin disorders with a similar reaction pattern in the young infant's skin. Based on clinical features, Menni et al. describe three forms of ISD; true seborrhoeic dermatitis, psoriasiform seborrhoeic dermatitis and erythrodermic seborrhoeic dermatitis (73). The true incidence of ISD is uncertain and geographic variations have been reported (72).

The eruption usually starts before 2 months of age with well-defined areas of erythema and a greasy yellowish-brown scaling on the scalp and face, in skinfolds, and in the napkin area. On the trunk, a typical predilection site is around the umbilicus but involvement of most of the trunk can also be seen. Even with very extensive involvement, the child is usually unaffected by itch and feeding and sleep disturbances are seldom a problem.

Factors discussed as triggering ISD are influences from maternal and placental hormones (74), an altered essential fatty acid pattern (75), biotin deficiency (76), *C. albicans* (77) and *P. ovale* (78).

The differential diagnosis comprises AD, psoriasis, Langerhans cell histiocytosis and primary immunodeficiency. Different opinions exist as to whether ISD and AD are separate entities or if ISD is the first manifestation of AD (79,80,81). Yates et al. found a difference according to total and specific IgE and prognosis in ISD compared with children with AD (82,83). Podmore et al., however, in a retrospective study, demonstrated an increased incidence of atopic manifestations in children with ISD (84).

Topical treatment with weak corticosteroid preparations, sometimes in combination with anti-candidal and antibacterial agents, is recommended. The prognosis of ISD is often favourable, with healing within a few months.

### **ISD and *P. ovale***

Interest in microorganisms in ISD has mainly been focused on the role of *C. albicans* (77). ISD is presented by Ive et al. as a "variant" of adult seborrhoeic dermatitis but the authors also state that although the clinical appearance of ISD is similar to adult seborrhoeic dermatitis, the relationship between the conditions is not clear (85). Adult seborrhoeic dermatitis is associated with *P. ovale* and treatment with topical ketoconazole has proved to be effective (86). Since the first report in 1985 by Devred et al. (78) on the finding of *P. ovale* in ISD, this has become a field of interest.

## AIMS OF THE STUDY

To investigate the role of *P. ovale* in AD and more specifically;

- I. to compare *P. ovale* cultures taken from the forehead of healthy children and children with ISD.
- II. to compare *P. ovale* cultures taken from the forehead and lesional skin in infants, children and young adults who had AD, rhinitis, conjunctivitis and/or asthma or were healthy.  
to compare the occurrence of IgE antibodies to *P. ovale* in the same patients groups.
- III. to analyse the possible crossreacting IgE antibodies and their target antigens in *P. ovale* and *C. albicans*.
- IV. to determine the prevalence and density of *P. ovale* on forehead skin in healthy children of different ages using a culture medium with whole fat cows' milk as the lipid source.
- V. to compare the efficacy of topical treatment with miconazole-hydrocortisone cream and ketoconazole shampoo with that of hydrocortisone cream and placebo shampoo alone in the treatment of AD in the head and neck area.

# MATERIALS AND METHODS

## Patients and control subjects

### ISD and *P. ovale* (I)

Twenty patients with ISD were included in this study, 9 females and 11 males with a mean age of 9 weeks referred to the Department of Dermatology with a clinical picture of ISD with erythematous squamous scaly eczema involving the scalp, face and napkin area. The mean age at onset was 4 weeks. Twenty healthy children were recruited from the well-baby clinic, 8 females and 12 males with a mean age of 7 weeks.

### *P. ovale* and AD in children and young adults (II)

Children attending the outpatient Allergy Clinic at the Department of Pediatrics, Östra Hospital or the Department of Dermatology at Sahlgrenska Hospital Göteborg or the Department of Dermatology, Rigshospital, Copenhagen were investigated. Children from the surgical ward of the Department of Pediatrics, Östra Hospital served as healthy controls. The patients and controls were divided in to the following 3 groups:

**Patients with AD (Group AD).** This group consisted of 60 children and young adults aged 7 months to 21 years (mean 10 years 6 months). The diagnosis was based on the criteria of Hanifin & Rajka (43). Six children had concomitant asthma, 8 had rhinoconjunctivitis and 6 had asthma and rhinoconjunctivitis.

**Patients with rhinoconjunctivitis and/or asthma (Group RA).** This group consisted of 40 children and young adults aged 2 to 20 years (mean 10 years 6 months) with asthma and/or rhinoconjunctivitis but no ongoing AD, although one of them had AD in early infancy. Twenty-one of the children had asthma, 8 had rhinoconjunctivitis and 11 had combined asthma and rhinoconjunctivitis.

**Healthy controls with no atopic history (Group HC).** This group consisted of 40 children and young adults aged 1.5 to 21 years (mean 11 years). Children aged 1 to 10 years were mostly inpatients from the surgical ward, scheduled for operations like hernia and strabismus.

### Crossreacting IgE antibodies to *P. ovale* and *C. albicans* in atopic children (III)

Sera from 28 children investigated in Paper II were included. Thirteen children with positive SPT, aged 3-21 years (median 14 years), were included. Two were males and 11 females. Twelve patients had AD, 3 young adults had the typical head and neck distribution and one child was non-atopic. Fifteen children with negative SPT, aged 4-20 years (median 13 years),



were included. Five were males and 10 females. Of these children, seven had AD, four asthma and/or rhinoconjunctivitis and 4 were healthy.

#### ***P. ovale* culture from the forehead of healthy children (IV)**

One hundred and thirty healthy children, 59 boys and 79 girls aged 2 months to 15 years were recruited from a well-baby clinic and from schools after obtaining parental permission.

#### **Topical antimycotic treatment of AD in the head and neck area (V)**

Sixty patients, 36 females and 24 males, aged 14-53 years (median 28 years), were included. All had atopic dermatitis with the typical clinical picture of head and neck dermatitis. They were all outpatients at the Department of Dermatology, Sahlgrenska University Hospital, Göteborg.

### ***P. ovale* culture**

#### **Papers I and II**

All samples taken from the babies in Paper I were taken from the forehead or temporal area. In Paper II samples were taken from Group AD from the forehead, eczematous skin and uninvolved skin close to the eczema. In Group RA and Group HC samples were taken from the forehead and the antecubital fossa.

Qualitative cultures for *P. ovale* were taken with a curette and transferred to a medium containing neopeptone, bacto agar, glucose and yeast extract. Lipid supplements were glycerol monostearate, Tween 80 and olive oil. Quantitative culture for *P. ovale* was performed using contact plates (PDM *Pityrosporum* Contact Plates AB, Biodisk, Solna, Sweden) (87) containing the same medium as was used in the qualitative cultures. The contact plate was pressed against the skin area for 15 s, incubated in a Bio-Bag SFJ (Marion Laboratories, Kansas City, USA), which contains 5-10% oxygen and 8-10% carbon dioxide, at 37° and read after 6 days.

#### **Papers IV and V**

Samples were taken from the forehead using contact plates (*P. ovale* Maxiplate, Max Lab Diagnostic HB, Källered, Sweden) (88). This contact plate contains peptone, bacto agar, glucose and yeast extract. Lipid supplements were glycerol, glycerol monostearate, Tween 60, whole-fat cows' milk and ox bile with the antimicrobial supplements chloramphenicol and cycloheximide. The contact plate was pressed against the forehead skin for 15 s, incubated in a plastic bag at 37°C and read after 6 days. The results were expressed as number of colony forming units.

## **Bacterial culture (I,V)**

Material for bacterial culture was taken with a cotton swab and transferred to blood agar and read after 24 hours.

## **Immunological investigations**

### **Skin prick test (SPT) (II,V)**

SPT was performed on the volar aspect of the forearm with a water-soluble extract of *P. ovale*, protein concentration 5 mg/ml (ALK Laboratories, Denmark). The results were evaluated in relation to a histamine reference equivalent to histamine hydrochloride 10 mg/ml and regarded as 3+ positive if the wheal was equal to the histamine skin reaction (89).

### **Total serum IgE (II,V)**

Total serum IgE were determined by radioimmunoassay (RIA) (Pharmacia IgE RIA 100, AB, Uppsala, Sweden), following the recommendations of the manufacturers. The results are given in kU/l.

### **Radio-allergosorbent test (RAST) (II)**

IgE antibodies to *P. ovale*, *C. albicans* and *Cladosporium herbarum* were measured by RAST (90,91) using commercially available reagents (Pharmacia AB) with the exception of the *P. ovale* discs, which were produced as described earlier (92). Briefly, an isolate of *P. ovale* (no. 42132) from the American Type Culture Collection (ATCC) was grown on a selective solid culture medium. The yeast cells were harvested, freeze-dried, sonicated in phosphate buffered saline (PBS), extracted overnight and finally coupled to cyanogen bromide-activated paper discs. The sera were tested in duplicate and the results expressed in Phadebas RAST Units (PRU) /ml or RAST classes. A positive RAST means  $\geq 0.35$  PRU/ml. In children 0 to 5 years of age, IgE antibodies to common inhalant or food allergens were determined by RIA (Phadiatop® Paediatric RIA, Pharmacia) while in the other three age-groups IgE antibodies to inhalant allergens were measured by another RIA (Phadiatop® RIA, Pharmacia). The sera were investigated in duplicate and results were expressed as positive or negative. A positive test was taken as an indication of atopy. The commercial reagents were used following the recommendations of the manufacturer.

### **Enzyme-linked immunosorbent assay (ELISA) (II)**

IgG antibodies to *P. ovale* were measured by ELISA (93). The protein extract used as antigen was the same as the one used for SPT. The wells of Titertec polyvinyl chloride (PVC) microplates (Flow labs, Herts, England) were coated by incubation with antigen solution (10  $\mu$ g/ml

in PBS, pH 7.2) for 5 hours at room temperature. The wells were then washed 3 times with PBS-Tween 20 (PBS-Tw). Serum was diluted in PBS with 0.1% bovine serum albumin (BSA), in two-fold steps, starting with a 1/400 dilution, and each dilution was tested in triplicate. After incubation over night at 4°C, the plates were washed 3 times in PBS-Tw. Alkaline-phosphatase-conjugated rabbit antihuman IgG (Dakopatts, Copenhagen, Denmark), diluted 1/1500 in PBS-Tw, was added and allowed to incubate for 4 hours at 30 °C. The plates were washed 3 times in PBS-Tw. Finally, 4-nitrophenyl phosphate substrate (Sigma, USA), 1mg/ml in diethanolamine buffer pH 9.8 (Merck, Germany), was added and the optical density was read at 405 nm after 50 min with a Titertec photometer (Flow). As a control serum, we used pooled positive sera from 30 healthy adults. The absorbance readings were compared with those of the reference serum and the per cent activity calculated.

### **Antigens (III)**

*P. ovale* antigen was purchased in lyophilised form from ALK, Horsholm, Denmark. Crude cytoplasmic antigen of *C. albicans*, CBS 1894, was prepared as previously described (94).

### **Sodium dodecyl sulphate-polyacrylamid gel electrophoresis (SDS-PAGE) and IgE-immunoblotting (III)**

Immunoblotting was performed essentially according to Bengtson et al. as previously described (95,96). *P. ovale* and *C. albicans* extracts, corresponding to 2 mg protein per large gel, and molecular weight markers (LMW Calibration Kit, Pharmacia LKB Biotechnology, Uppsala, Sweden) were separated with SDS-PAGE and transferred to nitrocellulose sheets. The specific IgE-binding to allergens on nitrocellulose was probed with beta-galactosidase-labelled anti-IgE antibodies ( Phadezym RAST, Pharmacia Diagnostics, Uppsala, Sweden). The dilution of individual patient sera as well as the conjugate was 1:4. Two millilitres of diluted serum was used for each strip.

### **RAST-inhibition (III)**

RAST-inhibition was performed with selected atopic and non-atopic control sera according to Yman et al. except that nitrocellulose discs were used instead of CnBr-activated paper discs (97). The solid phase allergen was a *P. ovale* extract coupled to nitrocellulose discs by the method of Walsh et al. (98). One millilitre of a 10mg/ml dilution of the extract was used per 25 nitrocellulose discs, based on uptake titration curves according to Walsh et al. (98). *C. albicans* extract was used as the inhibiting antigen in tenfold dilutions from 40 mg/ml.

### **Crossed immunoelectrophoresis (CIE), Crossed radio immuno-electrophoresis (CRIE) and Tandem-CRIE (III)**

CRIE and Tandem-CRIE were performed according to the basic principles originally presented by Weeke and Loewenstein and Kroell as modified by Uhlin and Einarsson (99,100,101). CIE

was performed with the gel moulded on a Gel Bond plastic sheet (Pharmacia, Uppsala, Sweden) which could be bent along the inside of a cylinder for roller incubation to reduce the volume of serum and conjugate needed (102). A 1% agarose gel (Litex, Glostrup, Denmark) and a Tris-Barbital buffer pH 8.6 were used. *P. ovale* and *C.albicans* extracts, corresponding to 20-30 µg protein per hole, were applied for the first dimension electrophoresis in two holes each, one for the single CRIE and one for the Tandem-CRIE. The second dimension run was performed with the anti-*C. albicans* antiserum (DAKO immuno-globulins, Denmark), corresponding to 9 µl per cm<sup>2</sup>, absorbed in a filter paper and laid on the gel according to Kuusi (103). The first dimension was performed at 40 V/cm for 35 min and the second dimension at 10 V/cm for 20 h. The plate was washed, pressed and dried at room temperature. For CRIE the plate was incubated for 16 h at room temperature in the cylinder with 4 millilitres of pooled allergic sera undiluted. The plate was washed three times and then incubated for 2 h on a roller at 37° C with 125 I-labeled anti-IgE antibodies (Phadebas RAST, Pharmacia, Uppsala, Sweden) diluted 1:10. After incubation with the conjugate, the plate was washed three times with PBS-Tween and twice with water. The washed CRIE-plate was dried and laid on an X-ray film (Trimax) with intensifying screens (Trimax) and kept at -70° C for autoradiography.

## Experimental design (V)

In a double-blind controlled study, patients with AD in the head and neck area were allocated to one of two treatments (for the affected area head, neck and upper back). Patients in group A were treated with miconazole-hydrocortisone cream (Daktacort<sup>®</sup>) and ketoconazole shampoo (Fungoral shampoo<sup>®</sup>), patients in group B with hydrocortisone cream and a placebo shampoo (Fungoral shampoo base). The cream was applied twice daily and the shampoo was used twice weekly. Emollients were permitted throughout the study. The duration of the study was 6 weeks, with follow-up visits in week 4 and 6. Healed patients stopped the treatment after 4 weeks and patients who deteriorated after 4 weeks were considered treatment failures. During the first 2 weeks of the study, all patients were treated with flucloxacillin tablets 750 mg twice daily, or, in case of allergy, with erythromycin 500 mg twice daily. If eczema on other parts of the body was severe, this was treated as before the study (mild to potent corticosteroids).

## Scoring of AD (SCORAD) (V)

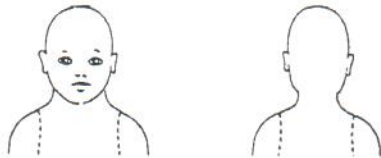
All evaluations of patients in Paper V were made by the same observer (A.B.) at the start of the study, after 4 weeks and after 6 weeks. The SCORAD (scoring atopic dermatitis) index for assessment of AD was used (104). However, the protocol was modified due to the design of the study, in which only the head, neck and upper part of the trunk were studied (Fig. 4).

The extent was evaluated as 100% if the above-mentioned area was covered and proportionally less with decreasing area involved. With this design, the weight given to each item remained 20% for extent, 60% for intensity and 20% for subjective symptoms, which is the design of the composite score in the SCORAD index. Instead of the item sleep loss, patients evaluated overall condition (dryness, burning sensation) in the affected area.

## Statistical methods (I,II,V)

Mann-Whitney's U-test for differences between groups, Wilcoxon's test for differences within groups and Spearman rank correlation were used. For qualitative data, the chi-square test was used.

A: EXTENT (0-100%)



100%=head, neck and upper part of the trunk

B: INTENSITY

CRITERIA	INTENSITY	MEANS OF CALCULATION
Erythema		INTENSITY ITEMS (average representative area) 0= absence 1= mild 2= moderate 3= severe
Edema/Papulation		
Oozing/crust		
Excoriation		
Lichenification		
Dryness*		

\*Dryness is evaluated on uninvolved areas

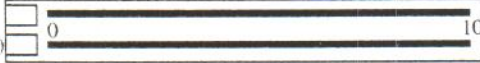
C: SUBJECTIVE SYMPTOMS

(for the area head, neck and upper trunk)

VISUAL ANALOG SCALE  
(AVERAGE FOR THE LAST 3 DAYS OR NIGHTS)

PRURITUS (0-10)

OVER ALL CONDITION (0-10)



SCORAD:  $A/5+7B/2+C$

Fig. 4. SCORAD record used in Paper V, modified after the SCORAD index as presented by the European Task Force on Atopic Dermatitis (104).

# RESULTS

## *P. ovale* culture

### Children with ISD (I)

*P. ovale* was cultured significantly more often ( $p < 0.01$ ) in children with ISD, 18 out of 20 (90%) as compared with 4 out of 20 (20%) healthy babies. (Tables III and IV).

Table III. Results of cultures from lesional skin in 20 children with ISD

	<i>P. ovale</i> microscopy	Qual. culture	Quant. culture
1	few	+	0
2	many	+	10
3	few	+	1
4	few	+++	11
5	neg	++	23
6	many	++	4
7	few	++	24
8	few	++	8
9	few	+	6
10	few	+	1
11	neg	++	8
12	few	++	47
13	few	++	12
14	many	+++	0
15	many	++	0
16	few	+	16
17	few	+	9
18	few	0	0
19	neg	0	0
20	neg	+	2

\* + = < 5 col, ++ = 5-10 col, +++ = > 10 col.

Table IV. Results of skin cultures in 20 healthy control children

	<i>P. ovale</i> microscopy	Qual. culture	Quant. culture
1	neg	+	8
2	neg	0	0
3	many	+	1
4	few	+	3
5	neg	0	0
6	neg	0	0
7	neg	0	0
8	neg	0	0
9	neg	0	0
10	neg	0	0
11	neg	0	0
12	neg	0	0
13	neg	0	0
14	few	+	0
15	neg	0	0
16	neg	0	0
17	few	0	0
18	neg	0	0
19	few	0	0
20	neg	0	0

\* + = < 5 col, ++ = 5-10 col, +++ = > 10 col.

### Children and young adults with AD (II)

Cultures were taken from 3 groups of patients: AD, RA and HC. Results of the qualitative and quantitative cultures did not differ between the groups (Table V). There was, however, a difference between the age-groups, as would be expected from earlier reports. *P. ovale* cultures

were positive in 0-20% of children aged 0-10 years and in 60-90 % of 11-21-years-olds.

The occurrence of *P. ovale* in different skin areas in patients with AD is shown in table VI. Skin affected with eczema did not have an increase of *P. ovale* compared with uninvolved skin.

Table V. Occurrence of *P. ovale* (+) on the skin in children with atopic dermatitis (AD), rhinoconjunctivitis and/or asthma(RA) and healthy controls (HC).

Age (years)	AD		RA		HC	
	+	%	+	%	+	%
0- 5	2	13	1	10	0	0
6-10	2	13	2	20	1	10
11-15	10	67	6	60	9	90
16-21	11	73	7	70	9	90
Total	25/60 (42%)		16/40 (40%)		19/40 (48%)	

Table VI. The occurrence of *P. ovale* in different skin areas in AD patients.

Forehead	Eczema	Uninvolved skin
33%	22%	22%

#### Healthy children (IV)

Samples were taken from the forehead with a contact plate and cultured on a medium containing whole fat cows' milk as one component of the lipid source. Eightyseven per cent of children aged 2 months to 15 years had a positive culture. The largest number of *P. ovale* was found among children aged 2-23 months, among whom 11 out of 17 (65%) had more than 10 CFU/plate, and children older than 9 years of whom 39 out of 54 (72%) had more than 10 CFU/plate (Fig. 5).

#### AD with head and neck dermatitis (V)

*P. ovale* cultures were positive in 83 % of all patients (aged 14-53 years).

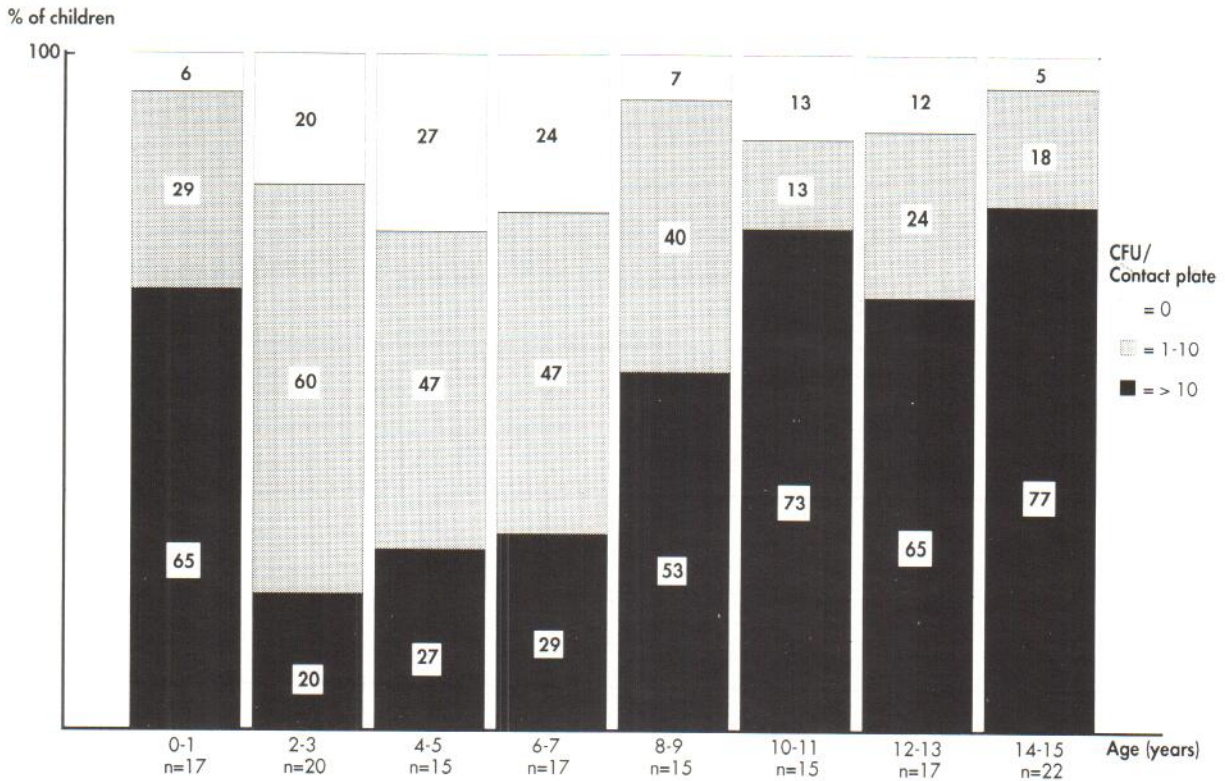


Fig. 5. *Pityrosporum ovale* in healthy children, quantitative cultures. Number of colony forming units (CFU)/contact plate.

## Bacterial culture

### Children with ISD (I)

*S. aureus* were cultured in significantly higher numbers from children with ISD compared with controls ( $p < 0.01$ ), 14 out of 20 (70%) in ISD and 1 out of 20 (5%) healthy babies. In one child with ISD *Streptococcus pyogenicus* group A was cultured from the eczema of the face and perianal region. The mother had tonsillitis caused by the same organism.

### AD with head and neck dermatitis (V)

Bacterial culture for *S. aureus* was positive in 89% of all patients.

### SPT with *P. ovale* extract

#### Children and young adults with AD (II)

In this paper even weak "one plus reactions" are shown (Table VII). If these weak reactions are excluded, 15% of our AD-patients are still SPT positive for *P. ovale*. These patients were all in the age-group 11-21 years.



Table VII. Results of skin prick tests with a *Pityrosporum* extract in children with atopic dermatitis (AD), rhinoconjunctivitis and/or asthma(RA) and healthy controls (HC).

Age (years)	AD		RA		HC	
	+	%	+	%	+	%
0- 5	1	7	0	0	3	30
6-10	3	20	0	0	0	0
11-15	4	27	0	0	0	0
16-21	6	40	0	0	0	0
Total	14 ( 23% )		0 (0)		3 ( 8% )	

#### AD with head and neck dermatitis (V)

Among the 53 patients selected for this study, positive SPTs were found in 55%, 14 patients having ++ reactions and 15 patients +++ reactions.

#### Total serum IgE

##### Children and young adults with AD (II)

The median concentration of IgE was in Group AD 77 kU/l (n=47, range 2-6100 kU/l), in Group RA 165 kU/l (n=30, range 2-7200 kU/l) and in Group HC 11 kU/l (n=34, range 2-290 kU/l).

##### AD with head and neck dermatitis (V)

The median concentration of IgE was 750 kU/l (range= <2-19300 kU/l).

##### RAST to *P. ovale*, *C. albicans* and *C. herbarum* (II)

In patients with AD, 10-16% of the patients were positive in RAST for one or more of the fungi, with no differences found between the 3 fungal species. In Group RA, two patients were positive to one or more fungi and all individuals in Group HC were negative (Table VIII).

Table VIII. Number of patients positive in RASTs for *P. ovale*, *C. herbarum* and *C. albicans*. Atopic dermatitis (AD), rhinoconjunctivitis and/or asthma(RA) and healthy controls (HC).

Age (years)	RAST <i>P. ovale</i>			RAST <i>C. herbarum</i>			RAST <i>C. albicans</i>		
	AD	RA	HC	AD	RA	HC	AD	RA	HC
Total	8/59 (14%)	1/38 (3%)	0/35 (0%)	5/51 (10%)	2/30 (7%)	0/33 (0%)	9/57 (16%)	1/34 (3%)	0/35 (0%)

### RAST and SPT to *P. ovale* (II)

Fourteen patients with positive SPT belonged to Group AD and 7 were positive in the RAST for *P. ovale*, compared with only one of 45 SPT-negative children in Group AD.

In all children, the correlations between SPT and IgE and SPT and RAST for *P. ovale* and IgE and RAST for *P. ovale* were measured with Spearman's correlation coefficient. SPT and RAST for *P. ovale* ( $r=0.55$ ) showed the strongest correlation ( $p<0.001$ ).

There were no significant differences between children with a head and neck distribution compared with all other children in the AD group according to the SPT and RAST for *P. ovale*. The group of children with this distribution was in this study very small, only five.

### IgG antibodies to *P. ovale* (ELISA) (II)

A significant difference between subjects with eczema (median per cent activity  $336.5 \pm 188.0$  (SD)) and healthy subjects (median per cent activity  $158.0 \pm 96.8$  (SD)) was seen in the age-group 16-21 years ( $p<0.05$ ).

### Crossreacting IgE antibodies to *P. ovale* and *C. albicans* in AD children (III)

#### SDS-PAGE and IgE-immunoblotting

Of the 15 SPT negative children, 14 exhibited no bands in immunoblotting. One asthmatic control (strip N) showed a strong IgE-response to both yeasts even though she failed to respond in the skin prick test. The immunoblotting results were in agreement with her RAST-results. The results of IgE-immunoblotting in 13 children with positive SPTs (A-M) and patient N are shown in table IX.

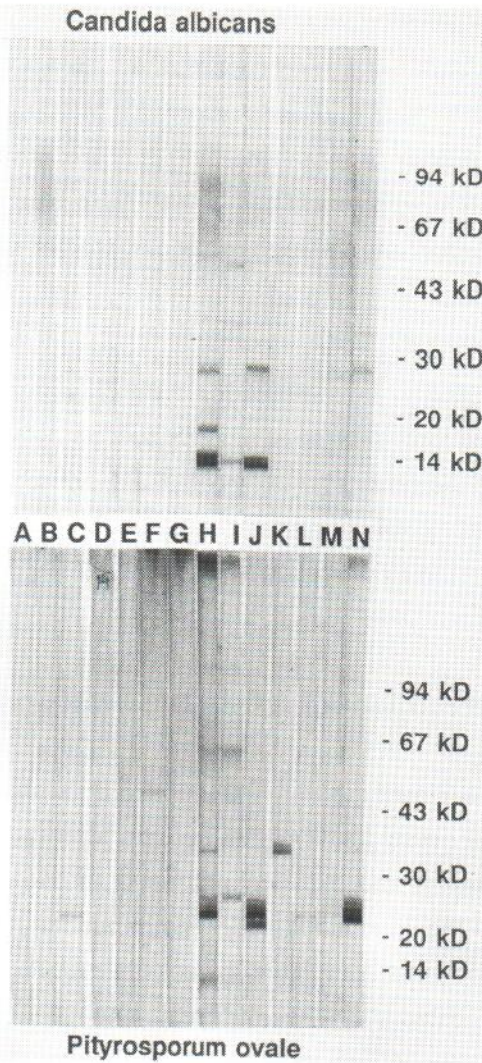


Table IX. Results of IgE-immunoblotting.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Age (years)	3	10	10	14	15	12	14	16	21	20	17	19	4	16
Group *	AD	AD	AD	AD	AD	AD	HND	AD	HND	HND	AD	AD	HC	RA
<i>P. ovale</i> culture*	+					+	+		+	+	+	+	+	
<i>P. ovale</i> RAST class*			2				3	3	2	2	2	2		1
SPT to <i>P. ovale</i> *	1+	1+	1+	1+	1+	2+	3+	2+	2+	3+	2+	3+	1+	
<i>C. albicans</i> * RAST class		2					2	2	2					2
<i>P. ovale</i> bands (kD)			23			34		10, 23 26, 34 dHm	10, 26 dHm	21, 23 26	34	23		23 dHm
<i>C. albicans</i> bands (kD)		Man	27					Man 13, 18 21, 27 55	13, 37 50	13, 27				Man 27, 34

\* Results from paper II

### **RAST inhibition**

Sera C, H, I, J and N were chosen for RAST-inhibition. The RAST-inhibition curves revealed inhibition with sera H and N. Sera C, I and J showed inhibition, still remaining below 50%, only at very high concentrations of inhibiting antigen. This inhibition was regarded as non-specific. The RAST-inhibition with sera H and N was interpreted as being due to crossreacting epitopes in *C. albicans* mannan and the high molecular weight stain of *P. ovale*.

### **CIE, CRIE and Tandem-CRIE**

Pooled IgE-positive sera (H,J,N) in combination with *C. albicans*-specific rabbit antibodies visualised four IgE-stained precipitates of *C. albicans* and one IgE-stained precipitate of *P. ovale*. In Tandem-CRIE the fuzzy *P. ovale*-precipitate is fused with the fuzzy precipitate of *C. albicans*. The crossreacting structures were most likely due to mannan in *C. albicans* and to a polysaccharide component in the cell wall of *P. ovale*.

## **Topical antimycotic treatment of AD in the head and neck area (V)**

Of the 60 patients enrolled, 7 patients were excluded from the study. Two patients were lost to follow-up. One patient deteriorated severely some days after the first visit. One patient never started the antibiotic treatment and 3 patients stopped antibiotic treatment after some days because of side effects. Of the 7 excluded patients, 4 belonged to group A and 3 to group B.

All the 53 evaluable patients completed 4 weeks' treatment, and 43 completed 6 weeks' treatment. Seven patients healed after 4 weeks' treatment and continued only with emollients. Two patients (one from Group A and one from Group B) deteriorated and stopped the treatment after 4 weeks, and one patient from Group B was unable to come back for the last visit.

During the study, 15 patients healed, 7 after 4 weeks and 8 after 6 weeks of treatment. Of the patients who healed, 7 belonged to Group A and 8 to Group B. After 4 weeks' treatment, the reduction of the eczema score was significant ( $p < 0.001$ ) in both groups. The decrease in eczema score did not differ between Group A and Group B. A further decrease of the eczema score was seen in both groups at the end of the study, but there was no difference between the groups (Table X).

Except for *C. albicans* cultured from 2 patients, no other fungi were found among the patients.

*P. ovale* culture at the start of the study was positive in 83% of all 53 patients, with no difference between the groups. After 4 weeks' treatment, positive cultures were found in 50% in Group A and in 93% in Group B. The decrease in number of colonies was significant in Group A ( $p < 0.001$ ) but not in Group B (Fig. 6). In Group A, 10 patients became negative for *P. ovale* at the end of the study, whereas in Group B only 3 patients became negative. Among the 10 patients in Group A who became negative, 3 healed. There was no correlation between

Table X. Number (n) of patients healed, eczema score, median and (range), in the two groups.  
Group A, n=26. Group B, n=27.

SCORAD	Group A	Group B
Baseline	58.60 (36.70-84.30)	60.10 (37.40-92.30)
After 4 weeks	33.20 (0-71.20)	22.90 (1.40-90.20)
End of study	18.75 (0-71.20)	16.45 (0-90.00)
Healed at 4 weeks	n=5	n=2
Healed at 6 weeks	n=2	n=6

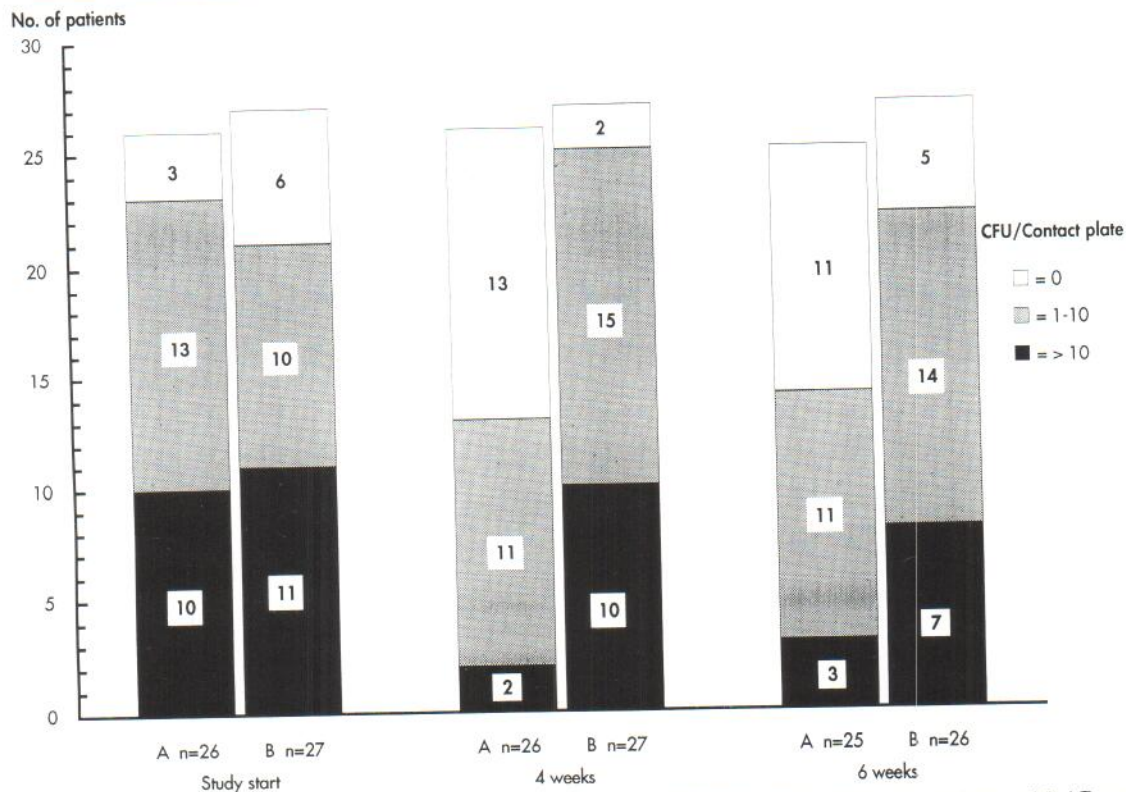


Fig. 6. *Pityrosporum ovale* culture at the start of the study and after 4 and 6 weeks in patients with (Group A) and without (Group B) topical antimycotic treatment. Number of colony forming units (CFU)/contact plate.

the number of *P. ovale* at the start of the study or the decrease in number during the study and the improvement of eczema in Group A or Group B.

Total serum IgE correlated with the SPT result ( $r=0.35$ ,  $p<0.05$ ). There was no correlation between the SPT result and the decrease in eczema score.

One patient from Group A, whose AD cleared during the study, developed an acneiform eruption, probably as a side-effect of the treatment. Two patients, one from Group A and one from Group B, deteriorated during the first 4 weeks of treatment, and further epicutaneous tests will evaluate a possible connection with the topical treatment given.

## MAIN FINDINGS

- A majority of healthy children of all ages were found to harbour *P. ovale* on forehead skin although low numbers of colonies were found between 2 and 9 years.
- Children with infantile seborrhoeic dermatitis were more frequently colonised with *P. ovale* on the forehead skin than were age-matched healthy children.
- *P. ovale* could be cultured with about the same frequency in children and young adults with atopic dermatitis and age-matched children with or without other atopic manifestations.
- In spite of similar colonisation, IgE antibodies to *P. ovale* were found only in patients with atopic disease and especially in patients after puberty with head and neck dermatitis.
- Topical antifungal treatment of adults with head and neck dermatitis was not found to be more effective than only topical steroid and oral antibiotic treatment, although anti-mycotics decreased skin colonisation of *P. ovale*.
- Crossreacting epitopes are suggested to be located in the mannan polysaccharide of *C. albicans* and high molecular weight diffuse stain of *P. ovale*.

## DISCUSSION

Although more than 100 years have passed since *P. ovale* was first described by Rivolta, real interest in this lipophilic yeast as a pathogen has a shorter history (9). Quantification of *P. ovale* on the skin has been hampered by the difficulty in culturing the yeast due to the characteristic unique feature of *P. ovale* of requiring lipids for optimal growth (19). *P. ovale* is the only yeast found as a member of the normal microflora of adult skin, preferentially colonising areas with high sebum excretion rates, i.e. the scalp, face and upper trunk. The normal ecology in children has been less clear and varying prevalences have been reported in different studies.

The role of *P. ovale* as a pathogen in pityriasis versicolor and adult seborrhoeic dermatitis is now accepted and the use of antimycotics in these skin diseases is a well-established treatment (105). Systemic infections caused by *P. ovale* have been reported in predisposed neonates and have been described as a cause of i.v. catheter-related sepsis (106,107). In recent years *P. ovale* has been proposed to be of importance in AD and ISD. The reported favourable effect of oral ketoconazole in adult patients with AD distributed to the head and neck area draws attention to the possible role of *P. ovale* as an allergen in this subgroup of AD patients (67). The association with the yeast in ISD was first reported by a French group in 1985 (78). However, in both AD and ISD the role of *P. ovale* as a pathogen is a controversial issue.

This study was performed in order to evaluate a) the prevalence of *P. ovale* on the skin in healthy children, in children with ISD and in patients with AD, b) the immunological response to the yeast in patients with AD, c) the effect of antimycotic treatment in the subgroup of AD patients with HND and d) the possible crossreactivity between reaginic antibodies to *P. ovale* and *C. albicans*.

***P. ovale* -microbiology:** Direct microscopy was performed with cellophane tape, stained with methylene blue and mounted on a microslide. The result of the method was reported but we found it difficult and unreliable to use and direct microscopy has not been used in further studies.

Since *P. ovale* needs lipids for optimal growth, any culture medium must supply a suitable lipid source. In Papers I and II we used a culture medium containing glycerol monostearate, Tween 80 and olive oil as lipids, further described by Faergeman (21). When we started our investigation in 1987, this medium was used for *P. ovale* culture in our laboratory.

In Papers IV and V we used a medium with ox bile, glycerol, glycerol monostearate, Tween 60 and whole-fat cows' milk, a medium that was reported by Leeming and Notman to be superior to other tested substrates for growth of *P. ovale* (14). They found that the incorporation of cows' milk gave better results than a variety of other lipid sources, a result also found

by Korting et al. 1991 (22). The medium used in Papers IV and V containing cows' milk as one lipid source was tested in our laboratory by Bergbrant et al. and found to give high counts of colonies which were easy to identify (88).

Reports on *P. ovale* colonisation in children gave varying results, possibly due not only to different media used to culture *P. ovale* but also to variations in experimental designs, with different age-groups studied and body sites investigated. Geographic and seasonal variations may also influence the results because of effects on the yeast of temperature, humidity and UV-light (108,109,110).

***P. ovale* in healthy children:** In Paper IV 87% of healthy children were found to harbour *P. ovale* on the forehead skin. The largest number of colonies was found among children aged 2-23 months and among children older than 9 years. These results are in contrast to those of Faergemann, who, when he performed cultures from the back of healthy children, found *P. ovale* only in children older than 5 years, but the differences in results could be explained by the fact that he used another culture medium and investigated another skin area (38).

The results in our study are comparable with the results presented by Leeming et al. in Göteborg in 1989 (40). They also used a medium with cows' milk for *P. ovale* culture and investigated children aged 0-18 years with cultures performed from the back, the scalp and the ears. Clear variations between body sites were found. Cultures from the back were with few exceptions negative in children aged 1-10 years, scalp cultures showed increasing numbers of *P. ovale* from 6 years of age and ears became colonised increasingly and a high count was seen in children from 1-9 months as well as in children older than 10 years. In a report from Mexico, healthy infants aged 1-8 months were evaluated with both direct microscopy and cultures from various skin areas and based on these methods 53% were found to harbour *P. ovale* (111).

It thus seems that *P. ovale* belongs to the normal microflora in a majority of children of all ages although the density is low between 2 and 9 years and there is a clear variation according to the body area investigated.

***P. ovale* and Infantile Seborrhoeic Dermatitis:** During the period October 1987 to May 1988 pediatricians at outpatient clinics near Sahlgrenska University Hospital, Göteborg were asked to send patients with ISD to the outpatient clinic at the Department of Dermatology. This patient group is otherwise most often seen and treated by the pediatricians. The majority of the referred patients had extensive skin disease and all children selected for the study showed the diagnostic features described in the dermatological textbooks as typical of ISD (71,72). General health was unaffected. In two cases a clinical picture of bacterial infection in the skin lesions was obvious as an aggravating factor.

Parents of children taking part in our study in 1987/88 (Paper I) were contacted by letter or, if no answer was received, by telephone during the spring of 1994 to evaluate the frequency



of atopic manifestations (AD, asthma, rhinoconjunctivitis or food allergy) in the child. All control children were reached and 18 of the 20 children who belonged to the group with ISD during their first months of life. Seven of the 20 control children (35%) and 12 of the 18 children with ISD (66%) were affected by at least one atopic manifestation. Food allergy was reported in 3 of the children with ISD but in no control child. In the control group, half of the families had a history of atopic disease, which may explain the high incidence of atopic manifestations in this group. Although a careful selection was performed, based on the clinical picture, to find children with "pure" ISD, a high percentage of the children developed atopic manifestations, which is in accordance with earlier studies and may favour the view that ISD is part of the spectrum of AD (84). As has been discussed by Atherton, the diagnosis ISD is perhaps only possible to make retrospectively (80).

At the time when we started our study, only one report, from 1985, showed the presence of *P. ovale* in a group of children with ISD (78). In Paper I we used a culture medium containing olive oil, Tween 80 and glycerol monostearate and found a significant difference in culture results between the two groups of children. In children with ISD 90% had a positive culture for *P. ovale* when samples were taken from the forehead, as compared with 20% of healthy infants, a difference which probably would not have been found if we had used a more efficient growth medium which also reveals *P. ovale* in a high proportion of healthy children (40). The difference found in our investigation between the two groups of children may be due to differences in the density of colonising yeasts on the skin. Powell et al. identified some risk factors for colonisation with *P. ovale* in infants hospitalised in an intensive care unit (109). The overall colonisation rate correlated with days spent in an incubator, close skin contact with lambswool and use of occlusive dressings in combination with low birth weight and long hospital stay. In our study, no such risk factor as low birth weight or treatment in an incubator or long hospital stay could be identified as an explanation for the high recovery rate of *P. ovale* in the children with ISD.

Since our study was completed, Ruiz-Maldonado and his group reported *P. ovale* in infants with ISD in a higher frequency as compared with children with AD or other skin dermatoses or healthy children (111). The results were based on direct microscopy and/or cultures from various skin areas and they found *P. ovale* in 73% of infants with seborrhoeic dermatitis, 33% of those with AD, 33% of those with other dermatoses and 53% of healthy infants.

Although *P. ovale* is found on the skin in ISD more often than in healthy children, the importance of *P. ovale* as a pathogen is unclear. One way to evaluate the role of *P. ovale* is to perform treatment studies. Only 2 reports are published in which 2% ketoconazole cream has been used in ISD. Ruiz-Maldonado treated infants with ISD in an open study with 2% ketoconazole cream twice daily for 3 weeks with a reported favourable effect (111). In 11 of 15 children negative mycology after treatment corresponded to healing. Taieb et al. treated 19 children with ISD in an open study with 2% ketoconazole cream once daily for 10 days and found a good response rate in 15 of 19 children (112). Three patients with a psoriasiform

histology and one patient with probable AD were non-responders or poor responders. Since ketoconazole also has an anti-inflammatory effect and ISD has a good prognosis with healing often within a few weeks to a few months, a double-blind placebo study with antimycotic preparations is necessary before any conclusions can be drawn about the role of *P. ovale* in ISD (113).

***P. ovale* and atopic dermatitis:** Environmental factors are of utmost importance for the occurrence of AD in the genetically susceptible person and the increase of AD noted in many industrialised countries is more likely to be explained by the influence of exogenous factors than by genetic alterations (47,48,114). The favourable effect of allergy prevention programmes on the prevalence of atopic manifestations in risk-group infants indicates that exposure to potent allergens is of crucial importance in inducing allergies that may be of importance for the development of eczema (115-118).

Even though the atopic patient often has a propensity to specific IgE responses to different allergens, there are variations within the group and according to the age of the patient (119,120). IgE antibodies to foods often develop during the first 8 months of life while IgE antibodies to inhaled allergens such as pollen and mites occur later in life (121). These age-related differences probably have various explanations. However, exposure to an antigen is a prerequisite for sensitisation (122). When current knowledge of immunopathogenesis in AD is presented, the dysregulation of IgE and the abnormal cytokine secretion pattern by T-cells in the skin are emphasised as important findings with implications not only for new therapies but also for possible environmental trigger factors (7,123-126).

There is no ideal treatment for AD and in most cases we can only mitigate the symptoms while "nature" heals. However, one important part of the treatment is elimination of obvious trigger factors. In young infants, allergy to hens' egg and cows' milk can be responsible for severe eczema and improvement can be achieved if the child is given an adequate elimination diet (127). In atopic hand eczema trigger factors like nickel, rubber or perfumes are often looked for in epicutaneous tests. Topically applied cortisone preparations can also cause sensitisation and have recently been reported as a cause of recalcitrant atopic eczema (128).

The favourable effect on "head and neck dermatitis" of oral ketoconazole reported by Clemmensen & Hjorth 1983 prompted the discussion about the role of *P. ovale* as a trigger factor in AD (67). The authors of this pioneer report carefully declared their selection of patients included in the study, namely patients with "head and neck dermatitis comprising eczema at one or more of the following sites; face, neck, and upper back, and with serious itching for at least 6 days per month". In addition to these clinically based criteria, all patients had a positive SPT to *P. ovale*.

Since patients with AD comprise a heterogeneous group as regards age, clinical picture, propensity to produce IgE antibodies and specific IgE antibodies and response to treatment, results drawn from a selected patient group cannot be assumed to be valid for "all" patients

with AD before further studies have been performed.

When we started our study (Paper II), little was known about *P. ovale* colonisation in patients with AD. Theoretically, such factors as lipid composition and frequent applications of greasy emollients could influence the colony rate and density. In Paper II, *P. ovale* colonisation in AD patients aged 0 to 20 years was compared with colonisation in patients with atopic disease but no eczema and with that in healthy children. We found that *P. ovale* could be cultured from the skin with about the same frequency in children and young adults with AD and in age-matched subjects with or without other atopic manifestations. Eczematous skin did not show an increased frequency of *P. ovale* compared with perilesional skin.

With an improved medium for growth of *P. ovale*, cultures have shown that the majority of children of all ages include *P. ovale* in their normal microflora and we can only speculate that this may also be true for the atopic child although studies in this group should be performed before this can be stated with certainty.

Since Nordvall et al. reported a strong correlation between AD and the occurrence of IgE antibodies to *P. ovale* (RAST) in atopic children aged 7-18 years, we found it interesting to evaluate the occurrence of IgE-antibodies in patients included in Paper II with AD and compare these findings with those in age-matched patients with other atopic manifestations and healthy controls (129). We measured the occurrence of specific IgE to *P. ovale* by SPT and RAST. Our data suggest that in spite of similar colonisation rates, IgE antibodies to *P. ovale* occur only in atopy and more frequently in patients with AD than in those with other types of atopic disease. Positive SPT to *P. ovale* was found in 9 patients (15%) but only in the age range 11-21 years.

IgE antibodies to *P. ovale* in AD have been measured by SPT, RAST and ELISA by other groups but results are difficult to compare since age and eczema distribution are not always reported. Positive SPT has been found in 28 to 79% of patients with typical HND (69,32) and with lower prevalence in other types of AD. Reports on the prevalence of positive SPT to *P. ovale* in young children are few. Wessels included patients from 2 years of age although the mean age in the study was 23.9 years (130). Rokugo included a group of patients with a mean age of 9 years and found SPT to be less prevalent (39%) in children younger than 10 years than in older children (64%) (70). They included no data about the youngest patients. In the study by Nordvall et al. children included were 7 to 18 years old although the age of the patients with AD and positive RAST was not stated (129). Other studies include only adults (31,32).

In Paper II, children were enrolled based on their age and ongoing AD as defined according to Hanifin's & Rajka's criteria but with no selection for eczema distribution (43). Only 5 of 60 patients had a typical HND. These 5 patients were all in the oldest age-group. Three of them were positive in SPT and RAST for *P. ovale*. Even though this group is small, the results are in agreement with the finding in other studies that specific IgE for *P. ovale* is found mainly in young adults with HND and in our study children with AD with another distribution seldom had IgE to *P. ovale*.

To evaluate the effect of topical antimycotic treatment in patients with HND, which has not been reported before, patients in Paper V were selected mainly from the same outpatient clinic as patients in Paper II but enrolled in the study only if their AD was a typical head and neck dermatitis. Among these patients aged 14 to 53 years, SPT to *P. ovale* were seen in 55%. Patients with negative SPT were also included because there have been reports of positive patch tests to *P. ovale*, in one report without correlation to the SPT result (70).

Topical antimycotic treatment in HND is commonly used in everyday practice even though no controlled studies have been performed. If *P. ovale* is of importance in patients with involvement of an area where this yeast is often found, a topical antimycotic agent would be expected to be an important part of the treatment. The majority of our patients were colonised with *S. aureus*, which is in accordance with other studies (60-62). Antibacterial treatment often has a good effect in AD, indicating that *S. aureus* is involved in the pathogenesis of AD (131). We tried to eliminate the possible effect of *S. aureus* with oral antibiotic treatment in both groups. Since ketoconazole has been shown to have not only an antimycotic but also an anti-inflammatory action (113), we treated all patients in the study with a mild steroid cream.

In this study, Daktacort<sup>®</sup> and Fungoral shampoo<sup>®</sup> were effective in reducing the numbers of *P. ovale* on the forehead. In the control group, treated with hydrocortisone and placebo shampoo, the decrease in *P. ovale* was not significant. Eczema improved significantly in both groups. However, neither the number of patients who healed (7 in group A and 8 in group B), nor the decrease in eczema score, after 4 weeks or at the end of the study, differed between the groups. Even in patients whose *P. ovale* cultures became negative during the study, eczema scores were not significantly lowered when compared with the decrease of eczema scores in patients with positive *P. ovale* culture after treatment. Patients with positive SPT did not respond differently to treatment compared with SPT-negative patients. Findings of specific serum IgE antibodies are not always of clinical significance. This is clearly illustrated by the difficulty in interpreting the clinical relevance of results of RAST or SPT to foods in children with AD.

The result of our study may mean that *P. ovale* is not involved in the pathogenesis of HND. Specific IgE antibodies to *P. ovale* merely reflect the fact that the damaged skin in this region enables sensitisation to the normal microflora to occur, without further clinical significance. However, the interpretation of the results are difficult for several reasons.

1. The material comprised only 26 index subjects and 27 controls. Sixty patients in the group were planned to be included based on the assumption that 80 % of the patients given the antimycotic treatment would improve, compared with only 40% in the other group. However, the majority of patients in both groups improved, and the number of patients enrolled should therefore have been much higher in order to show a significant difference. This result was unexpected since it is our experience that patients with HND are difficult to heal with standard treatment for AD.

2. Topical antimycotic treatment may be insufficient to reduce *P. ovale* in the hair follicles.

3. The design of the study did not enable us to determine if topical antimycotics can act prophylactically, as has been shown in seborrhoeic dermatitis (132).

4. Crossreacting antigens have been found in *C. albicans* and *P. ovale*. Gastro-intestinal colonisation of *C. albicans* may still persist as a trigger for the eczema, in spite of topical antimycotic treatment (133).

Further studies are needed to evaluate the role of antifungal therapy in HND. Some of the above-mentioned objections can be met if the study includes more patients, if newer generations of antimycotics, topical or systemic, are used and if longer treatment and observation periods are planned.

**Crossreacting IgE antibodies to *Pityrosporum ovale* and *Candida albicans*:** A positive correlation between IgE antibodies to these two yeasts was found by Nordvall et al. (129). Since 5 of 14 patients in our study (Paper III) had positive IgE-immunoblotting to *P. ovale* and *C. albicans* simultaneously, this question was possible to investigate further. RAST-inhibition and Tandem-CRIE were performed with the purpose of investigating possible crossreacting IgE antibodies and their target antigens in *P. ovale* and *C. albicans*.

Serum from SPT-positive patients with AD and from one atopic patient with asthma and rhinoconjunctivitis were analysed. IgE-immunoblotting revealed IgE binding to various fractions in *P. ovale* when a lyophilised *P. ovale* extract was used as an antigen. Only a few reports have been available concerning the antigenic structures on *P. ovale*. In 1983, Bruneau, based on CIE studies, reported the genus *Pityrosporum* to have a complex antigenic structure (134). IgE binding components were later found to be located in the molecular weight range of 14 to 94 kD (92). Differences in reported antigens in *P. ovale* may be due to analysis of small populations, use of different growth phases of the yeast or technical interlaboratory variations in SDS-PAGE. Characterisation of the antigenic components of *P. ovale* is of importance for the preparation of standardised extracts for research and clinical use.

Sera with simultaneous IgE binding to both *P. ovale* and *C. albicans* were investigated with RAST-inhibition. Crossreacting epitopes were suggested to be located in the mannan polysaccharide fraction of *C. albicans* and a high molecular weight region of *P. ovale* based on the IgE-immunoblotting and RAST-inhibition patterns of the studied sera.

As the next step, 3 sera were pooled and used as an IgE probe in CRIE and Tandem-CRIE. These experiments revealed a fused precipitin line indicating presence of a common structure on *P. ovale* and *C. albicans*, most likely a polysaccharide precipitate.

However, our conclusions can only be tentative. To confirm these findings, results have to be based on more patients and RAST-inhibition studies with purified allergens.

Since we completed this study, our findings have been confirmed by Doekes et al., who found that *P. ovale* antibodies in many AD sera crossreacted with extracts of *C. albicans* measured by a sensitive ELISA method and inhibition ELISA (135). The IgE-binding components in *P. ovale* extracts were shown to be partially sensitive to pronase or trypsin treat-

ment, whereas periodate oxidation resulted in complete loss of IgE-binding capacity, thus suggesting the involvement of carbohydrate structures (136).

Because of the significance as an antigenic determinant, mannan in *C. albicans* has been subjected to a variety of studies (137-140). *C. albicans* mannan is supposed to be an important allergen, consisting of branching mannose. *C. albicans* and AD have been further studied by Savolainen et al. and their findings suggest continuous exposure and induction of IgE antibodies by *C. albicans* in AD patients (133). Further clinical studies are needed to evaluate the clinical significance of the suggested allergenic crossreactivity of *P. ovale* and *C. albicans*.

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