STUDIES ON COLONIZATION AND INFECTION WITH

STAPHYLOCOCCUS AUREUS AND OTHER MICROBES IN SKIN DISEASE

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UNIVERSITY OF GOTHENBURG´

To my family

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ABSTRACT

The skin is colonized with a wide range of microbes. Some offer vital protection from colonization and infection with pathogenic strains while others have the capacity to cause or exacerbate disease. The aim of this thesis was to investigate the role and management of microbes found on skin affected by three disorders; balanoposthitis, impetigo and atopic dermatitis (AD).

Paper I investigates the frequency and distribution of bacteria, *Candida* and *Malassezia* species in balanoposthitis, a common inflammatory and/or infectious disorder of the prepuce and glans penis. Patients with balanoposthitis were colonized with microbes more often than a control group. Specifically, *S. aureus* was found in 19% of patients with balanoposthitis and not at all in the control group. There was no significant increase of *Candida* species in balanoposthitis. Different clinical manifestations did not predict the presence of specific microbes. There was no association with seborrhoeic dermatitis or psorisasis.

Paper II describes the bacterial spectrum and proportion of fusidic acid-resistant *S. aureus* (FRSA) in cultures from lesional skin in impetigo and secondarily infected AD. *S. aureus* was the most frequent finding (76-93%) and fusidic acid-resistance was found in 75%, 32% and 6.1% of *S. aureus* isolates from patients with bullous impetigo, non-bullous impetigo and secondarily infected AD, respectively.

In paper III the *in vitro* antimicrobial activity of topical skin pharmaceuticals was tested against *S. aureus*, *S. epidermidis*, *Streptococcus pyogenes*, *Escherichia coli* and *Candida albicans*. Formulations with clioquinol, halquinol and hydrogen peroxide had a broad antimicrobial effect. The azole class of antifungal formulations had an anti-staphylococcal effect.

Paper IV describes the variations in *S. aureus* colonization in relation to the severity of AD (assessed with SCORAD) in adult patients during a 5-month follow-up. High density of *S. aureus* on lesional skin, colonization of multiple body sites and persistent colonization with one strain was associated with more severe disease.

Conclusion: Balanoposthitis was associated with increased colonization with potentially pathogenic microbes. The primary therapeutic target in mild to moderate cases without overt signs of infection should be to decrease inflammation and microbial load with a topical corticosteroid-antimicrobial combination. FRSA were a common cause of impetigo but remained relatively infrequent in secondarily infected AD. Use of topical fusidic promotes the spread of resistant strains and should be avoided. Topical non-resistance-promoting antiseptic formulations could be useful in the management of superficial skin infections and help reduce the use of systemic antibiotic treatment. Detailed investigation of different aspects of *S. aureus* colonization in relation to AD severity can increase understanding of the complex *S. aureus*-AD interaction and the possible value of anti-staphylococcal interventions in clinically non-infected AD.

Keywords: balanoposthitis; microbes; *Staphylococcus aureus*; *Candida*; *Malassezia*; fusidic acid; fusidic acid-resistant *S. aureus*; impetigo; atopic dermatitis; azoles; clioquinol; halquinol; hydrogen peroxide; skin infection; SCORAD

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Mikael Alsterholm, Ingela Flytström, Ragna Leifsdottir, Jan Faergemann and Ing-Marie Bergbrant
Frequency of Bacteria, *Candida* and *Malassezia* Species in Balanoposthitis
Acta Derm Venereol 2008; 88: 331–336

II. Mikael Alsterholm, Ingela Flytström, Ing-Marie Bergbrant and Jan Faergemann
Fusidic Acid-resistant *Staphylococcus aureus* in Impetigo Contagiosa and Secondarily Infected Atopic Dermatitis
Acta Derm Venereol 2010; 90: 52–57

III. Mikael Alsterholm, Nahid Karami and Jan Faergemann
Antimicrobial Activity of Topical Skin Pharmaceuticals – An *In vitro* Study
Acta Derm Venereol 2010; 90: 239–245

IV. Mikael Alsterholm, Louise Strömbeck, Annika Ljung, Nahid Karami, Johan Widjestam, Martin Gillstedt , Christina Åhren and Jan Faergemann
Variations in *Staphylococcus aureus* Colonization and Disease Severity in Adults with Atopic Dermatitis during a 5-month Follow-up
In manuscript

CONTENTS

ABBREVIATIONS

AD	Atopic dermatitis
C. albicans	*Candida albicans*
CA-MRSA	Community-associated methicillin-resistant *S. aureus*
CFU	Colony-forming unit
Clioquinol	5-chloro-7-iodo-8-quinolinol
CoNS	Coagulase-negative staphylococci
DMF	Dimethylformamide
E. coli	*Escherichia coli*
EEFIC	Epidemic European Fusidic acid-resistant Impetigo Clone
EF-G	Elongation factor G
FLG	Filaggrin
FRSA	Fusidic acid-resistant *S. aureus*
Halquinol	5,7-dichloro-, 5-chloro- and 7-chloro-8-quinolinol mixture
MIC	Minimal inhibitory concentration
MRSA	Methicillin-resistant *S. aureus*
PBP	Penicillin-binding protein
PBS	Phospate-buffered saline
PFGE	Pulsed-field gel electrophoresis
PVL	Panton-Valentine leukocidin
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
S. pyogenes	*Streptococcus pyogenes*
SCORAD	Severity SCORing of Atopic Dermatitis
SSSS	Staphylococcal scalded skin syndrome
SSTI	Skin and soft tissue infection
TSST-1	Toxic shock syndrome toxin-1

INTRODUCTION

Microbes are everywhere. We tend to think of them as causes of disease and decay and have, in vain, invented numerous ways to abolish them from our lives. In reality, humans live in harmony with many microbes, are struck with disease by some and benefit from others. For instance, the commensals of the human nasopharynx, skin and gut are crucial for bacterial homeostasis, preventing colonization and infection with pathogenic strains. *Staphylococcus aureus*, a potentially pathogenic bacteria which will be discussed in further detail in this thesis, can cause a wide range of different diseases, such as impetigo, other skin and soft tissue infections (SSTIs), staphylococcal scalded skin syndrome (SSSS), endocarditis and food poisoning [1]. Bacterial fermentation produces the desired consistency and flavour of yoghurt and cheese [2, 3]. The carbon dioxide produced by fungi makes dough rise in breadmaking and their metabolic activity on the juice of grapes provide the unique taste of wine [4].

The work presented in this thesis was aimed at investigating the role and management of microbes found on skin affected by three different skin disorders; balanoposthitis, impetigo and atopic dermatitis (AD).

The study of balanoposthitis presented in paper I was prompted by the lack of a comprehensive description of the spectrum of microbes found in this particular skin disease. Specifically, there were no reports of the frequency and distribution of *Malassezia* species in balanoposthitis. The *Malassezia* yeasts cause seborrhoeic dermatitis, a skin disease that, not unlike balanoposthitis, is associated with erythema, papules and scales in areas where sebaceous glands are abundant.

A Scandinavian outbreak of bullous impetigo caused by a strain of *S. aureus* resistant to the topical fusidic acid preparations which were widely used at the time inspired the next study. The frequency of fusidic acid-resistant *S. aureus* (FRSA) in patients with impetigo attending the Department of Dermatology at Sahlgrenska University Hospital in 2004-2008 was investigated. In addition, the frequency of FRSA in patients with atopic dermatitis (AD), a group of patients who are highly prone to colonization and infection with *S. aureus*, was recorded during the same time period. The results are presented in paper II.

As demonstrated by the emergence of FRSA it is vital for each clinician to use antibiotics sensibly. Antibiotic resistance is a rapidly increasing global health care problem. SSTIs are common conditions and treatment with topical antiseptic preparations can sometimes be an alternative to oral antibiotics. In paper III the *in vitro* antimicrobial properties of currently available topical skin pharmaceuticals were tested with the agar dilution assay.

Clinical experience suggests that there is a positive correlation between skin colonization with *S.*

aureus and disease severity in patients with AD. Patients with severe AD experience improvement when treated with antibiotics, even in the absence of flare-ups. Despite this there is no convincing scientific data demonstrating an effect of anti-staphylococcal treatment in clinically non-infected AD [5]. One way to investigate a possible connection between *S. aureus* colonization and severity of clinically non-infected AD is to monitor the properties of colonization and disease intensity over time. In paper IV the variation in AD severity in adult patients during a 5-month follow-up was described and related to changes in *S. aureus* colonization.

The introduction of a thesis should give a theoretical back-drop for the topics which are about to be discussed. Therefore, the introductory part of this body of work is focused on describing the key players in the microbial drama that is played out on, and in, the skin. The leading character is the multi-talented *S. aureus*. However, without supporting actors there would be no plot so the *Malassezia* species, *Candida albicans*, *S. epidermidis* and *Streptococcus pyogenes* are also presented in some detail.

The epidermal stage is vast and complex and also deserves some mentioning. Sometimes the floor boards cannot be trusted, fragile due to mutations of structural proteins such as filaggrin in AD. The plot and manuscript of our plays, and by that we mean the pathogenesis and current treatment guidelines for balanoposthitis, impetigo and AD, will be reviewed. The mode of action for fusidic acid and selected antiseptics will also be covered before we move on to discuss papers I-IV.

So please, take a seat! Curtain up! Enjoy!

Mikael Alsterholm

Gothenburg, 17th of October 2012

Photo: Mikael Alsterholm

THE MICROBES

COLONIZATION AND INFECTION

Colonization

Colonization is characterized by the presence of microbes in a host where those microbes do not disrupt the normal body functions of that host. Colonization usually occurs on surfaces in contact with the surrounding environment, i. e. the skin, the mucous membranes of the nasal and oral cavities, urogenitalia, and the gastrointestinal mucosa [6]. Colonizing microbes can be commensals as well as symbionts. Commensals live closely together with their host and benefit by the relationship whereas the host neither benefits nor is harmed. Symbionts are organisms that live together and where the relationship is of mutual advantage [4]. The term commensal is, somewhat incorrectly, often used as a synonym to colonizing microbial flora. When the term is used in this thesis it will be in this referred meaning.

Infection

Infection is defined as an invasion of the host by microbes that can cause pathological conditions or diseases [7].

STAPHYLOCOCCUS AUREUS

Physiology and structure

The members of the genus *Staphylococcus* are spherical, gram-positive bacteria which can be found on the skin and mucous membranes of humans, other mammals and birds [4]. They are facultative anaerobes, meaning that they have the ability to generate energy by aerobic as well as anaerobic respiration, the latter yielding lactic acid. The name *Staphylococcus* is derived from the Greek term staphylé which means "a bunch of grapes" and refers to the growth pattern of the staphylococci [4]. Currently, the genus *Staphylococcus* consists of approximately 40 species [8].

S. aureus is the most virulent of the staphylococci. It is a highly versatile and adaptable spherical bacteria with a diameter of about one µm [1]. The colonies of *S. aureus* are golden because of carotenoid pigments that form during growth [9]. Staphylococci produce catalase, an enzyme which catalyzes the conversion of hydrogen peroxide to water and oxygen. This ability is used to differentiate them from gram-positive, catalase-negative cocci such as the streptococci. *S. aureus*

also produces coagulase which interacts with prothrombin leading to blood clotting by the conversion of fibrinogen to fibrine. The coagulase test is used to distinguish between *S. aureus* and other staphylococci in clinical laboratories since *S. aureus* is the only *Staphylococcus* species found in humans that produces coagulase [4]. In other words, *S. aureus* can promote the conversion of fibrinogen to fibrin in a test tube whereas other clinical *Staphylococcus* isolates cannot. Therefore, all other members of the *Staphyloccocus* species found in humans (for instance *S. epidermidis* and *S. saprophyticus*) are collectively referred to as coagulase-negative staphylococci (CoNS).

The cytoplasm of *S. aureus* is encapsulated by a cytoplasmic membrane comprised of proteins and lipids, a cell wall and sometimes a polysaccharide slime layer. The latter is believed to inhibit chemotaxis and phagocytosis by polymorphonuclear leukocytes as well as help *S. aureus* to adhere to synthetic materials. Not all clinical isolates of *S. aureus* are able to produce a slime layer, reports range from 45-70% [10, 11]. Bacterial populations enclosed in a polysaccharide slime layer are referred to as biofilm.

The principal component of the rigid *S. aureus* cell wall is peptidoglycan, which consists of peptide cross-linked glycan chains. Teichoic acid is attached to the peptidoglycan of the cell wall. Teichoic acid aids the binding of *S. aureus* to fibronectin of mucosal surfaces. Another component of the *S. aureus* cell wall is bound coagulase, discussed above. In addition, several virulence factors such as adhesins are linked to the cell wall. These and other virulence factors are discussed below.

S. aureus can cause a wide range of diseases and is one of the most studied bacterial pathogens. At the same time it can also reside on the skin and mucous membranes of humans as a commensal. The anterior nares is the most frequent carriage site for *S. aureus*. Longitudinal studies show that about 20% of individuals are persistent *S aureus* carriers in the anterior nares, 30% are intermittent carriers and 50% non-carriers [12-16]. The frequency of carriage on different body sites ranges from 10-20% in the general population and from 40-90% in nasal carriers. Nasal carriage increases the risk of developing a *S. aureus* infection [13, 17].

Virulence factors

S. aureus produces a plethora of virulence factors making it a very powerful pathogen. It can cause infections of the blood, skin, soft tissue, bone and lower respiratory tract. The virulence factors produced by *S. aureus* include adhesins, exotoxins and superantigen toxins.

The complete genome of two methicillin-resistant strains of *S. aureus* (MRSA) was first sequenced in 2001 [18]. In addition to coding segments for previously known virulence factors the sequencing revealed an array of genes coding for other possible toxins and adhesins, meaning that the

pathogenic potential of *S. aureus* is still not realized in full.

Another extremely important aspect of the "success" of *S. aureus* as a pathogen is a remarkable ability to become resistant to antibiotics.

Adhesins

S. aureus expresses several microbial surface components recognizing adhesive matrix molecules (MSCRAMMS). The function of the MSCRAMMS is to help *S. aureus* bind to the tissue of its host.

Fibronectin binding proteins A and B mediate the attachment of *S. aureus* to fibronectin, an extra-cellular matrix component. This mode of binding is believed to be important for the colonization of atopic skin where fibronectin is redistributed to the cornified layer [19]. Collagen binding protein, (Cna) is needed for the attachment of *S. aureus* to collagen and cartilage [20]. Clumping factors A and B (ClfA and ClfB) mediate, as revealed by their names, clumping but also help *S. aureus* adhere to fibrinogen when fibronectin is present. This mechanism is thought to be of importance in wound infections [21].

Protein A is covalently linked to the peptidoglycan layer of the cell wall of most *S. aureus* strains but not to the cell wall of the CoNS. It binds the Fc-receptor of IgG1, IgG2 and IgG4, thereby preventing opsonization and phagocytosis of *S. aureus* by the cells of the immune system [1, 4]. Further protection from the immune system as well as from antibiotics and antiseptics can be provided by the formation of the polysaccharide slime layer and biofilm, a mechanism which can be considered an adhesin-related virulence factor.

Exotoxins

S. aureus can secrete numerous toxins which form pores in the cytoplasmic membranes of host cells and cause subsequent cytolysis. Alpha-, beta- and gamma-hemolysins, leukocidin and Panton-Valentine leukocidin (PVL) are all examples of exotoxins. PVL is a virulence factor of community-associated MRSA (CA-MRSA) and consists of two proteins (LukF-PV and LukS-PV) that hetero-oligomerize in the host´s cytoplasmic membrane to form a pore. The main target of PVL is leukocytes which in part explains the ability of CA-MRSA to cause necrotizing pneumonia and skin infections [22].

The exfoliative toxins of *S. aureus* (exfoliative toxins A (*etaA*) and B (e*taB*)) are serine proteases that cause the epidermal blistering of bullous impetigo and SSSS. The toxin cleaves desmoglein 1, a structural protein of the desmosome which holds the keratinocytes in junction [23]. In bullous impetigo the toxin is produced locally at the site of infection whereas in SSSS the toxin circulates and produces blisters and desquamation at multiple body sites.

Superantigen toxins

While adhesins help *S. aureus* to colonize and infect cells and exotoxins target host cells and immune cells with different affinity, the superantigen toxins have a different mode of action. Superantigen toxins crosslink MHC class II molecules on antigen-presenting cells with T-cell receptors. This connection induces antigen-independent T-cell proliferation and cytokine release which in turn causes capillary leak and hypotension. Examples of superantigen toxins are toxic shock syndrome toxin-1 (TSST-1) and the enterotoxins A, B, C, D, E, G and Q. TSST-1 causes toxic shock syndrome, a potentially fatal condition characterized by fever, hypotension, macular rash and desquamation [24]. The enterotoxins, which cannot be hydrolysed by intestinal enzymes and are stable at 100°C for up to 30 minutes, cause staphylococcal foodborne disease [4].

In paper IV we have studied the pattern of colonization with *S. aureus*, a powerful pathogen as well as a commensal, in relation to disease severity in adult patients with AD during a 5-month follow-up.

Resistance to antibiotics

During the antibiotic area *S. aureus* has proven to be highly skilled at developing resistance. In the mid 1940s, just a few years after the introduction of penicillin in clinical practice, penicillin-resistant *S. aureus* strains were found in hospitals [25, 26]. These strains produced a penicillinase which hydrolysed the β-lactam ring of penicillin. Within a decade penicillin-resistant *S. aureus* strains were pandemic [27]. In the late 1950s methicillin, a new β-lactam antibiotic, was introduced and in 1961 the first report of methicillin-resistant *S. aureus* (MRSA) was published [28]. Methicillin-resistance is broad and confers resistance to all β-lactam antibiotics (penicillins, cephalosporins and carbapenems). The mode of action for β-lactam antibiotics is to bind to penicillin-binding proteins (PBPs) in the bacterial cell wall and inhibit transpeptidation of glycan chains. The genetic basis for methicillin-resistance is the large mobile genetic element staphylococcal cassette chromosome *mec* (SCC*mec*). The SCC*mec* contains a coding sequence for an alternative penicillin-binding protein called PBP2a. PBP2a has low affinity for all β-lactam antibiotics. Therefore, β-lactam antibiotics cannot bind PBP2a and inhibit the transpeptidation of glycan chains. The origin of SCC*mec* is unknown and so is its mode of transmission.

Until the late 1970s reports of MRSA strains remained relatively rare and confined to hospitals in Europe and the United States [27]. The last years of that decade and the first half of the 1980s gave rise to a pandemic of MRSA in hospitals. The only remaining antibiotic effective against these strains was vancomycin. Predictably, the increased use of vancomycin resulted in vancomycin-resistant *S. aureus* strains (VRSA) [29]. Since the mid 1990s MRSA have become widespread in the

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community on a global scale, the so-called CA-MRSA. CA-MRSA can cause fatal systemic infections but are also a frequent cause of necrotic SSTIs in dermatology out-patients [30-32]. Further testament to the ability of *S. aureus* to develop resistance to antibiotics is the novel clone of fusidic acid-resistant *S. aureus* (FRSA) which was discovered in epidemics of bullous impetigo in Sweden and Norway at the turn of the millenium. In paper II we have investigated the frequency of FRSA in impetigo and secondarily infected AD and we discuss the future implications of our findings.

Figure 1. S. aureus on blood agar plate.
Photo: Mikael Alsterholm

STAPHYLOCOCCUS EPIDERMIDIS AND OTHER COAGULASE-NEGATIVE STAPHYLOCOCCI

Physiology and structure

S. epidermidis colonizes all humans and is the most common coccus on human skin. It can be found on all body sites, especially in intertriginous areas. *S. epidermidis* is a member of the normal skin flora together with *S. aureus*, *S. saprophyticus*, *Micrococcus luteus*, *M. roseus*, *M. vaians* (all aerobic cocci), *Corynebacterium minutissimum*, *C. lipophilicus*, *C. xerosis*, *C. jeikeium*, *Brevibacterium epidermidis* (aerobic coryneform bacteria), *Propionibacterium acnes*, *P. granulosum*, *P. avidum* (anaerobic coryneform bacteria), *Acinetobacter spp.* (gram-negative bacteria) and *Malassezia* species [33]. *S. epidermidis* and *S. saprophyticus* belong to the CoNS. Another 16 species within the CoNS subset have been isolated from humans [8]. Colonization with *S. epidermidis* and other CoNS is believed to play an important role for microbial homeostasis on the skin, competing with *S. aureus* and other potentially harmful microbes [34].

In addition to being commensals on human skin, the CoNS are important nosocomial pathogens,

often multidrug-resistant and have become disseminated worldwide. *S. epidermidis* is the best studied species of the CoNS with regards to antibiotic resistance, virulence factors and pattern of dissemination. In contrast with the "aggressive" *S. aureus* which produces numerous cytolytic exotoxins and superantigen toxins, *S. epidermidis* has a much less confrontational approach. Once it passes a disrupted skin barrier (for instance a surgical incision) it evades the immune system, primarily by biofilm formation [34].

S. epidermidis has long been known to cause endocarditis and infections related to synthetic implantable devices such as prosthetic heart valves and joints, vascular grafts and intravascular catheters [8]. *S. saprophyticus*, frequently isolated from the perineal region causes urinary tract infections in women [35]. *S. epidermidis* and other CoNS are, second only to *S. aureus*, the most common cause of surgical site infections [36]. Many of the isolates of *S. epidermidis* from hospital-acquired infections are methicillin-resistant and have the capacity to produce biofilm. Taken together, *S. epidermidis* infections present a therapeutic challenge since the site of infection is often remote and associated with a synthetic object where these frequently multidrug-resistant microbes form biofilm.

S. epidermidis and skin disease

On the surface, *S. epidermidis* lives in harmony with its host, balances the epithelial microbial flora and rarely causes skin disease. However, if the epithelial barrier is passed, the pathogenic potential can be revealed as just exemplified [37].

In paper III we have tested the antimicrobial activity of topical skin pharmaceuticals against a panel of microbes which are commonly found on the skin either as commensals, pathogens or both. *S. epidermidis* was part of that panel.

MALASSEZIA SPECIES

Physiology and structure

The *Malassezia* yeast is a unicellular eukaryotic organism belonging to the microbial flora of the skin. It ranges in size from 1-8 µm in diameter and has a thick multilayered cell wall mainly consisting of carbohydrates, proteins and lipids. The yeast cells reproduce by unilateral budding and can assume various shapes such as bottle-shape, globose, ovoid or cylindrical [38]. They colonize the skin of humans and animals, including hair follicles, with a particular preference for sebum-containing skin sites.

The taxonomy and identification methods for *Malassezia* species are continuously being revised as new species are discovered. A revision and enlargement of the genus was performed in 1996, by then including seven species; *M. furfur*, *M. pachydermatis*, *M. sympodialis*, *M. globosa*, *M. obtusa*, *M. restricta* and *M. slooffiae* [39].

In recent years, the knowledge of the genus *Malassezia* has expanded further and, to date, includes an additional seven species that have been isolated from healthy and diseased human and animal skin (*M. dermatis*, *M. japonica*, *M. nana*, *M. yamatoensis*, *M. equina*, *M. caprae* and *M. cuniculi*) [40].

With the exception of *M. pachydermatis*, the *Malassezia* species need lipids to grow *in vitro*. The differentiation of *Malassezia* species in clinical settings and in epidemiological studies has traditionally been based on morphology in combination with physiological properties (page 49) [38, 41, 42]. Now, an option in epidemiological studies is the identification and quantification of *Malassezia* DNA from skin specimens.

Malassezia and skin disease

Malassezia species are associated with several common skin diseases such as pityriasis versicolor, seborrhoeic dermatitis and dandruff.

Pityriasis versicolor is characterized by small hypo- or hyperpigmented scaly plaques, primarily located to seborrhoeic areas of the skin such as the chest, back and neck. High temperature and humidity, genetic predisposition and poor immune status are factors contributing to the disease. In Sweden, prevalence rates are 0.5% for males and 0.3% for females [43]. Microscopy of scales from pityriasis versicolor lesions reveals the presence of abundant *Malassezia* spores and hyphae whereas *Malassezia* are much more rarely found on unaffected skin or on the skin of healthy controls [44, 45]. In addition, the hyphal state appears to play an important role in the pathogenesis of pityriasis versicolor, since hyphae are found in lesions irrespective of which *Malassezia* species is isolated [40]. *M. globosa* is the species that is most often isolated from pityriasis versicolor. However, while the association between pityriasis versicolor and *Malassezia* is considered confirmed, current epidemiological data does not permit any definitive conclusion as to which of the *Malassezia* species are implicated in this disease [40].

Seborrhoeic dermatitis is a relapsing skin disease with erythema and scaling in the seborrhoeic areas of the skin. The disease has a predilection for the scalp, eyebrows and paranasal folds but can also affect chest, back, axillae and genitals. Prevalence rates are reported to be 2.6% for men and 3.0% for women [46]. Seborrhoeic dermatitis is believed to be caused by a nonspecific immune response to *Malassezia* but the exact mechanism remains obscure [40, 47]. Dandruff is the mildest manifestation of seborrhoeic dermatitis.

In paper I we have investigated a possible role for *Malassezia* in balanoposthitis, a common form of dermatitis affecting the area of the prepuce and the glans penis where several infectious agents have been implicated. The frequency and distribution of *Malassezia* species in the male genital region was investigated previously in both uncircumcised and circumcised men but not in relation to balanoposthitis. It was concluded that *Malassezia* species belong to the resident microflora of the male genital region. Isolation rates were lower in circumcised men [48, 49]. The involvement of *Malassezia* species in skin diseases affecting the male genital region such as balanoposthitis, seborrhoeic dermatitis and psoriasis was suggested but not further investigated [49].

CANDIDA ALBICANS

Physiology and structure

Like *S. aureus* and the *Malassezia* species, the ubiquitous yeasts of the *Candida* species can be human pathogens as well as commensals. *C. albicans* is the most important member of its genus. The cytosol contains a nucleus, ribosomes, mitochondria and an endoplasmatic reticulum. The cell membrane consists of phospholipids, glycoproteins and, importantly, ergosterol. Ergosterol is the target of many antifungals because human cells contain cholesterol instead of ergosterol. Exterior to the cellmembrane is a cell wall containing chitin.

C. albicans is part of the normal flora of the oral cavity, gastrointestinal tract and external genitalia [4, 50]. It is likely that most individuals experience at least one episode of candidal disease in their lifetime. Finding a *Candida* species in a swab sample doesn´t immediately qualify it as a relevant pathogen. The clinical context has to be carefully evaluated. Erythema, pustules or other signs of inflammation at the sample site makes clinical relevance of the finding more likely.

C. albicans and skin disease

C. albicans is favoured by the warm and moist microenvironment which arises at intertriginous sites in obesity, under diapers, due to incontinence, drooling and improperly fitted dentures. Conditions and illnesses which predispose for candidiasis include immunosuppression and diabetes mellitus. Treatment with systemic antibiotics can alter the normal flora of the skin and mucous membranes and thereby permit an overgrowth of *Candida* species.

In dermatology, *C. albicans* is encountered as a pathogen in oral candidiasis, genital candidiasis, intertrigo, candidal paronychia and in diaper candidiasis [33]. The first consideration in treatment is to, whenever possible, reduce moisture and limit predisposing factors. Topical treatment

alternatives with azoles, nystatin or amphotericin B are available in several different vehicles which are chosen depending on body site [33, 50]. For oral candidiasis gel or mouth wash is a good option, genital candidiasis can be treated with a vaginal tablet or vaginal cream and for cutaneous candidiasis a cream is usually the best choice. Systemic fluconazole treatment is an important alternative for oral candidiasis responding inadequately to local treatment and for therapy-resistant or frequently recurring genital candidiasis.

C. albicans can be a clinically relevant pathogen in the male genital region. *C. albicans* is found in swab samples from the area of the prepuce and glans penis in patients with balanoposthitis and has been considered the principal causing microbe of this condition [51-54].

In paper I we have studied the frequency of *C. albicans* in balanoposthitis in relation to *Malassezia* species and bacteria. It was also included in the panel of microbes tested in paper III.

STREPTOCOCCUS PYOGENES

Physiology and structure

The genus *Streptococcus* include a wide variety of different gram-positive cocci which appear arranged in pairs or chains. In contrast with the staphylococci the streptococci are catalase-negative. There are three different, not mutually exclusive, schemes for differentiation of streptococci; serological properties (Lancefield groupings A-H and K-V), haemolytic patterns and biochemical properties. Haemolysis can be complete (β-hemolysis), incomplete (α-hemolysis) or absent (γ-hemolysis) [4].

S. pyogenes, also called Group A *Streptococcus* (GAS), are 0.5-1 µm spherical cocci that form short chains. *In vitro* they grow on enriched blood agar media. A large zone of β-hemolysis is observed around colonies. *S. pyogenes* has several virulence factors including the surface-anchored M-protein which forms the basis for modern serological differentiation of *S. pyogenes* strains. The M-protein mediates adhesion to host cells and resistance to opsonization and phagocytosis. Invasive *S. pyogenes* isolates are surrounded by a hyaluronic acid polysaccharide capsule which protects from opsonization and phagocytosis and is almost identical to human polysaccharides, further facilitating the evasion of the host immune response [55].

S. pyogenes and skin disease

S. pyogenes is a common cause of pharyngitis, sometimes complicated by scarlet fever [50]. A couple of days after the first symptoms of pharyngitis patients develop a diffuse erythematous rash

on the upper chest and extremities. Circumoral pallor (sparing around the mouth) and sparing of the palms and soles is a common finding. The tounge is initially covered with a yellowish white membrane which is later shed revealing the red strawberry tongue.

S. pyogenes is also the cause of several different SSTIs, ranging from superficial to deep [33]. Nowadays *S. pyogenes* is a rare cause of impetigo but remains a frequent cause of echtyma which penetrates further into the dermis. Erysipelas is by some authors (and particularly in the U.S.) by definition caused by *S. pyogenes*, whereas European authors tend to recognize *S. aureus* as well [50, 56]. The list of pathogens causing cellulitis is more diverse but *S. pyogenes* is the most common. It is likely that health care professionals worldwide will have to re-evaluate the management of SSTIs in the years to come due to the CA-MRSA epidemic. CA-MRSA cause echtyma-like skin infections and in recent years outbreaks have been reported among military trainees, prisoners and athletes [32, 57].

In paper III we have tested the antimicrobial activity of topical skin pharmaceuticals against a panel of microbes which are commonly found on the skin either as commensals, pathogens or both. *S. pyogenes* was part of that panel.

THE SKIN DISEASES

BALANOPOSTHITIS

Balanoposthitis is defined as inflammation of the skin of the glans penis (balanitis) or the glans penis and prepuce combined (balanoposthitis) [58, 59]. Balanoposthitis usually presents as pruritic or sore diffuse rubrosquamous macules. Symptoms can also be more pronounced with glazed erythema, pustules, erosions or even ulcers.

 It is described that approximately 10% of men attending genitourinary medicine clinics in the U.K. suffer from balanoposthitis [60]. The main cause of balanoposthitis is considered to be infection, with particular attention given to yeasts, but bacteria have also been implicated [51-54]. A risk factor for developing *Candida* balanoposthitis is diabetes mellitus. In fact, a symptomatic infection with *Candida* in the area of the prepuce and glans penis or acquired phimosis (sometimes a consequence of balanoposthitis) can be an early cutaneous marker for diabetes mellitus [61, 62]. Interestingly, no specific microbial aetiological factor can be detected in 31-41% of patients with balanoposthitis [51-54]. Irritant dermatitis caused by traumitteration is one cause of balanoposthitis which probably explains a large proportion of those cases [60]. In fact, balanoposthitis has been

referred to as a combination of a biomechanical and infectious disorder where abrasive trauma from frequent retraction of the prepuce and genital washing creates a beneficial milieu for microbes [62]. Balanoposthitis seems to mainly, but not exclusively, affect men who are not circumcised [58, 63]. There are several important differentials to the diagnosis of balanoposthitis as it is defined above. AD, seborrhoeic dermatitis and, perhaps more commonly, psoriasis can affect the male genitalia, causing a similar clinical picture. Genital involvement of pityriasis versicolor is rare but has been described [64, 65]. Plasma cell balanitis, sometimes referred to as Zoon balanitis, is a distinct clinical entity usually presenting as a solitary sharply circumscribed red plaque on the glans penis of middle-aged and older men. Histopathologically, plasma cells are seen. The aetiology is unknown [66]. Erythroplasia of Queyrat is the clinical appearance of Bowen´s disease (carcinoma *in situ* of squamous cells) of the prepuce and glans penis and can sometimes be mistaken for balanoposthitis of other aetiology. The condition is associated with human papilloma virus (HPV) and has a relapsing course with a risk of progression to squamous cell carcinoma [67].

The role of microbes

Studies on the role of microbes as causative agents of balanoposthitis have shown differing results. Some authors have published data supporting the role of *C. albicans* (10-60% of cases) whereas others have detected a high frequency of group B streptococci (4-28% of cases) [51-54]. Among those studies, only two have included a control group to investigate the frequency and distribution of microbes on the glans penis and in the preputial sac of men without balanoposthitis in the same clinical setting [51, 52]. The carriage rate of *C. albicans* in the sulcus coronarius is reported to be 14-20% in healthy men and does not seem to be influenced by circumcision [49, 68, 69].

Several other microbes such as *Trichomonas vaginalis*, *Gardnerella vaginalis*, anaerobic bacteria, mycobacteria, *Entamoeba histolytica*, *Treponema pallidum*, herpes simplex virus and HPV, are infrequently associated with balanoposthitis [70, 71].

Despite being a common skin pathogen, *S. aureus* has only been associated with balanoposthitis on rare occasion [52]. A possible role for *Malassezia* species in the pathogenesis of balanoposthitis has been suggested but not examined further [49].

Diagnosis and management

In most mild to moderate cases the clinical presentation together with the patient´s history allows the clinician to diagnose balanoposthitis without an elaborate work-up. However, balanoposthitis is a descriptive term which covers inflammatory, infectious, pre-malignant and malignant disorders so every case should be carefully considered.

There are no Swedish guidelines for the management of balanoposthitis. In 2008 the Clinical

Effectiveness Group of the British Association for Sexual Health and HIV published an update of The U.K. National Guideline on the Management of Balanoposthitis [71]. Selected investigations from that guideline, adapted to Swedish conditions and intended to aid diagnosis in cases of uncertainty are summarized in the following.

- A skin biopsy should always be secured if there is any suspicion of pre-malignant or malignant disease.
- Whenever there is diagnostic uncertainty a sub-preputial swab for *Candida* species and bacterial culture should be undertaken.
- Urinalysis for glucose – especially if candidal infection is suspected.
- Polymerase chain reaction (PCR) analysis for herpes simplex virus (HSV) when appropriate.
- PCR-analysis for *Treponema pallidum* in case of ulcerations.
- Screening for sexually transmitted infections (STIs) such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Mycoplasma genitalium* infection.
- In chronic cases (especially if unresponsive to therapy) consider dermatoses such as psoriasis, AD or contact dermatitis.

The aims of the investigation and treatment are to diagnose and treat STIs, to minimize sexual and urinary dysfunction and to detect and treat pre-malignant and malignant conditions.

The patients are instructed to avoid frequent genital washing while inflammation is present. Genital washing should be restricted to once daily using only water and possibly a mild non-scented soap. Sexual intercourse can cause abrasive trauma to the prepuce and glans penis and should initially be avoided.

Topical treatment with a hydrocortisone imidazole cream is generally effective in mild to moderate cases of irritant balanoposthitis and *Candida* balanoposthitis. The treatment of balanoposthitis will be discussed further in relation to the results in paper I (page 65-66). Balanoposthtitis is often relapsing and patients should be informed of this in order to improve compliance and obtain a good treatment result.

On rare occasion, the presentation is dramatic with pronounced oedema, pain, oozing and crusts. This clinical picture has been associated with streptococcal or HSV infection and warrants oral antibiotic or antiviral treatment [72, 73]. Metronidazole should be chosen if there is suspicion of an anaerobic infection (foul smelling discharge, oedema and inflamed glands) [71].

In paper I we have investigated the frequency and distribution of bacteria and yeasts in balanoposthitis. A possible association between different clinical presentations and microbes and

with seborrhoeic dermatitis was also explored.

IMPETIGO CONTAGIOSA

Impetigo contagiosa (in the following referred to as impetigo) is a localized superficial skin infection which affects both children and adults but is more common in children. Impetigo exists in two clinical forms; non-bullous impetigo and bullous impetigo. Non-bullous impetigo accounts for 70% of impetigo cases. It is characterized by erythematous macules rapidly evolving into short-lived vesicles or pustules which are replaced by yellowish (honey-coloured) crusts and superficial erosions. Predilection sites are the face and extremities. Bullous impetigo affects the face and the extremities but also the trunk, the buttocks and the perineum. Lesions are initially small vesicles which later enlarge into superficial bullae ranging from 1 to 5 centimetres in diameter [33, 50]. Differentials for impetigo include herpes simplex, secondarily infected AD, fungal infections, eczema herpeticum and, importantly, early stages of SSSS. In fact, the exfoliation seen in SSSS is explained by systemic dissemination of the exfoliative toxin which causes bullous impetigo. Children under the age of six years and immunocompromised adults can be affected by SSSS.

The role of microbes

In the vast majority of cases, non-bullous and bullous impetigo are caused by *S. aureus.* Occasionally non-bullous impetigo can be caused by *S. pyogenes*.

General predisposing factors for impetigo are a warm climate and high humidity. In Sweden, the incidence of impetigo peaks during the warmer months of the year (July-September). Predisposing factors for individuals are skin trauma and atopy.

As revealed by its full name, the infection is highly contagious and minor epidemics often occur in day care centers and schools. Impetigo also spreads rapidly within households.

Despite being consistently cited as a very common infection in medical textbooks and scientific publications (even the most common skin infection in children globally) incidence rates are hard to appreciate. The most exact incidence numbers probably come from a series of meticulous Norwegian studies of impetigo in an island community during the years 2001-2009. Incidence rates ranged from 0.0057 to 0.0260 cases per person-year in a well-defined population of 4500 people [74].

Diagnosis and management

Typical skin lesions are usually present and the diagnosis impetigo is easily made. Even though *S. aureus* is almost always the causative agent a swab sample for bacterial culture should still be undertaken to rule out *S. pyogenes* and to obtain an antibiotic resistance profile for the *S. aureus* isolate at hand.

The Swedish Medical Products Agency (Läkemedelsverket) regularly issues evidence-based recommendations for the treatment of impetigo and other bacterial SSTIs. The latest update was published in 2009 and the key points are summarized in the following [75]. Earlier this year (2012) an updated Cochrane review of interventions for impetigo was published and the result of that review is commented in relation to the Swedish guidelines [76].

- Mild cases of impetigo are usually self-healing with the aid of a careful hygiene regimen to promote quicker recovery and prevent further spreading. Crust should be removed daily and the skin washed with water and a mild soap. Repeated use of unwashed towels should be avoided. Hands should be washed frequently and hand disinfectant used. The Swedish Medical Products Agency states that there is some evidence to suggest that application of chlorhexidine on lesions can be beneficial. The Cochrane review found lack of support for disinfection measures to manage impetigo.

- Topical retapamulin applied twice daily for five days is recommended if symptoms persist despite the described hygiene regimen. Retapamulin was approved in the EU in 2007. It belongs to a new class of antibiotics, the pleuromutilins, and targets bacterial ribosomes in a novel way which it does not share with any other antibiotic [77]. To date, clinically relevant resistance to retapamulin or cross-resistance with other antibiotics has not been reported.

- Topical fusidic acid is <u>not</u> recommended due to resistance among S. *aureus* isolates. Similarly, topical mupirocin is <u>not</u> recommended due to increasing reports of resistance outside of Sweden. At present, topical mupirocin is used exclusively in the attempt to eradicate MRSA from Swedish carriers and holds no place in the treatment of impetigo. The Cochrane review found that topical fusidic acid and topical mupirocin were equally or more effective than oral antibiotic treatment but remarked on the growing resistance rates for commonly used antibiotics.

- Oral antibiotic treatment is recommended in widespread or progressing impetigo. The Cochrane review comments that there is a lack of studies in patients with extensive impetigo so it is unclear if oral antibiotics are superior to topical antibiotics in this group.

- For children cefadroxil 25-30 mg/kg and day divided into two doses daily or flucloxacillin

50-75 mg/kg and day divided into three doses daily is recommended. Clindamycin 15 mg/kg and day divided into three doses is recommended in case of allergy to penicillin. Seven days of treatment is suggested for cefadroxil, flucloxacillin and clindamycin.

- For adults flucloxacillin or dicloxacillin 750-1000 mg three times daily or cefadroxil 500-1000 mg twice daily is recommended. Clindamycin 150-300 mg three times daily is recommended in case of allergy to penicillin. Seven days of treatment is suggested for flucloxacillin, dicloxacillin, cefadroxil and clindamycin.

In paper II we have investigated and compared the bacterial spectrum in two mainly *S. aureus*-associated skin diseases, impetigo and secondarily infected AD, with special emphasis on fusidic acid-resistant *S. aureus* (FRSA).

ATOPIC DERMATITIS

AD is a chronic or relapsing inflammatory skin disease characterized by dry skin, erythematous macules or plaques and pruritus. Up to 20% of children and 1-3% of adults, with some regional variations, are affected throughout the world [78]. Children of parents with AD have increased risk of developing AD and there is 80% concordance in monozygous twins and 20% in heterozygous twins, demonstrating a strong hereditary influence. AD is strongly associated with other manifestations of atopy (allergic rhinoconjunctivitis and asthma).

The exact pathophysiology of AD is not known but alterations in immune reactivity, skin barrier function, allergens and microbial colonization are all considered important factors.

AD patients have an immune deviation towards Th2 and increased IgE production. Elevation of total or allergen-specific IgE levels is one of the most typical laboratory biomarkers for AD. However, this is controversial since not all patients with AD have raised IgE levels, a fact which further complicates the understanding of the pathophysiology of the disease. The terms extrinsic (IgE-associated) and intrinsic (non-IgE-associated) AD have been coined to separate two clinically distinct groups of patients. Patients with intrinsic AD do not have bronchial asthma or allergic rhinitis, show normal total serum IgE levels, no specific IgE, and negative skin-prick tests to aeroallergens or foods [79].

Genetic studies have not been able to detect candidate genes which are unique to AD but rather found genes which are also associated with other diseases of immune dysregulation such as asthma

and allergies. Examples of identified genes are Toll-like receptor 2 (TLR2), nucleotide-binding oligmerization domain I (NOD1) and CD14 (monocyte differentiation antigen), involved in antigen presentation and cell-mediated and humoral immune response pathways [80].

The impaired skin barrier function of AD has received much attention in recent years after the discovery of loss-of-function mutations in the epidermal barrier protein filaggrin (FLG) as a predisposing factor for AD [81]. Filaggrin functions in the ordered formation of the cornified envelope, the outermost layer of the epidermis. It does so by aggregating the cytoskeleton of keratinocytes. In the process, filaggrin is proteolyzed into pyrrodiline carboxylic acid and trans-urocanic acid that contribute to the composition of the water-binding so-called natural moisturizing factor (NMF) of the skin [82]. The consequence of FLG loss-of-function mutations is decreased stratum corneum hydration. The resulting impaired skin barrier is believed to, at least partly, explain an increased susceptibility to allergic sensitization, microbial colonization and infections in patients with AD. In addition to mutations in FLG, genomic profiling of mRNA in AD has detected defects in expression of several other genes encoding structural proteins of the cornified envelope [80]. The FLG mutations are not only associated with AD but also with asthma, but only in patients who suffer from AD [83-85]. This association together with the current knowledge of the impaired skin barrier and the altered immune response is considered to support the idea of a march towards atopy initiated in early life. The decrased hydration and the defective structure of the skin makes it permeable to allergens and microbes. The penetrating allergens and microbes are believed to trigger a dysfunctional immune response starting in the skin and disseminating, leading to the secondary development of allergies and asthma [80, 86].

The role of microbes

AD patients are characterized by a remarkably high cutaneous colonization rate with *S. aureus*, frequencies reaching 90% on lesional skin in some reports [87-91]. It is evident that atopic skin is a favourable habitat for *S. aureus*. In atopic skin, fibronectin is redistributed to the cornified layer and *S. aureus* possesses adhesins (fibronectin binding protein A and B) which bind fibronectin [19]. Colonization with *S. aureus* is a predisposing factor for SSTIs. In accordance, flare-ups of AD are frequently associated with *S. aureus* superinfection and require oral antibiotic treatment in addition to intensified topical anti-inflammatory therapy [92].

While the impact of *S. aureus* in flared superinfected AD is undisputed the role of this diversified pathogen in clinically non-infected AD is less clear. The density of *S. aureus* cutaneous colonization in AD has been reported to correlate with disease severity [88, 93]. The presence of exotoxin- and superantigen toxin-producing strains of *S. aureus* on the skin has been associated with exacerbation

of AD [94, 95]. Decreasing inflammation in AD with topical corticosteroids, TCI or UV-therapy generally decreases disease severity as well as cutaneous *S. aureus* colonization [96-99]. Taken together these data have led clinicians to assume that topical anti-inflammatory treatment in combination with staphylococcal reduction can improve AD management. This was supported by a 2009 placebo-controlled study from the U. S. where recurring use of dilute bleach baths was combined with intermittent intranasal application of mupirocin ointment in children with moderate to severe AD. The combination treatment reduced AD severity compared to placebo [100]. In contrast, a previous British study in children in from 1998 showed no effect on disease severity in children with clinically non-infected AD receiving oral flucloxacillin for 4 weeks with an 8-week post-treatment follow-up [101].

A recent Cochrane review on the effect of anti-staphylococcal interventions in patients with clinically non-infected AD could not support the efficacy of such interventions. The authors stated that there was a lack of high-quality randomized controlled clinical trials and that better and longer-term studies are needed to show clear evidence of clinical benefit [5]. It is also possible that there are different patterns of *S. aureus* carriage within the AD cohort and that this might affect the outcome of intervention studies.

Candida and *Malassezia* species have been implicated as aggravating factors in subsets of AD patients but the association is not clear since studies show conflicting results [38]. While not causative agents of AD, *Malassezia* species may play a role for AD severity in adult patients with head and neck dermatitis who do not respond to conventional therapy [102, 103].

In paper IV we investigate the longitudinal variation of different aspects of *S. aureus* colonization in adults with AD and relate our findings to disease severity.

Diagnosis and management

The diagnosis AD is criterion-based and is usually readily made on the basis of clinical inspection and patient history. Hanifin and Rajka and The U.K. Working Party have established criteria for the diagnosis of AD [104, 105]. These criteria were used in paper IV.

Hanifin and Rajka Diagnostic Criteria for AD

Major criteria: Must have three or more of:
1. Pruritus
2. Typical morphology and distribution
   - Flexural lichenification or linearity in adults
   - Facial and extensor involvement in infants and children

3. Chronic or chronically-relapsing dermatitis
4. Personal or family history of atopy (asthma, allergic rhinitis, atopic dermatitis)

Minor criteria: Should have three or more of:

1. Xerosis
2. Ichthyosis, palmar hyperlinearity, or keratosis pilaris
3. Immediate (type 1) skin-test reactivity
4. Raised serum IgE
5. Early age of onset
6. Tendency toward cutaneous infections (especially *S aureus* and herpes simplex) or impaired cell-mediated immunity
7. Tendency toward non-specific hand or foot dermatitis
8. Nipple eczema
9. Cheilitis
10. Recurrent conjunctivitis
11. Dennie-Morgan infraorbital fold
12. Keratoconus
13. Anterior subcapsular cataracts
14. Orbital darkening
15. Facial pallor or facial erythema
16. Pityriasis alba
17. Anterior neck folds
18. Itch when sweating
19. Intolerance to wool and lipid solvents
20. Perifollicular accentuation
21. Food intolerance
22. Course influenced by environmental or emotional factors
23. White dermographism or delayed blanch

There is no laboratory test pathognomonic for AD. Histopathology on a skin biopsy can support the diagnosis but does not give a definitive answer.

The European Task Force on Atopic Dermatitis (EFTAD) has recently published guidelines for the treatment of AD [106, 107]. Selected key points from these guidelines are summarized in the following. A majority of AD patients manage their dermatitis satisfactorily with continuous use of topical emollients combined with intermittent use of topical corticosteroids or calcineurin inhibitors (TCI).

- Topical emollients

  Topical emollients are the mainstay of maintenance therapy and rehydrate the skin. It is preferentially applied after a bath or a shower on slightly humid skin. Glycerol appears better tolerated than urea and propylene glycol, and is preferred in children. Emollients

should be used liberally and frequently. Quantities required are estimated to 150-200 g per week in young children and up to 500 g per week in adults. There is evidence to support that regular use of emollient (after an induction of remission with topical corticosteroids) has corticosteroid sparing effects in mild to moderate AD.

- Avoidance of non-specific provocation factors and aeroallergens
  Mechanic irritants (wool) and chemical irritants (repeated occupational or household exposure to water, detergents and solvents) should be avoided.
  The value of avoidance of allergens is less clear. There is some evidence to suggest that house dust mites (*Dermatophagoides pteronyssinus* and *D. farina*e) provoke symptoms of AD and that special encasings on mattresses and pillows may be beneficial.

- Food allergens
  Cow´s milk, hen´s egg, wheat, soy, tree nuts and peanuts can cause or exacerbate dermatitis during infancy [108]. There is little evidence to support effect of an elimination diet in unselected groups of patients with AD. The guidelines conclude that patients with moderate to severe AD should eliminate foods that provoked early or late reactions upon controlled provocation tests.

- Topical anti-inflammatory therapy
  Topical corticosteroids and TCI have significant effect on skin lesions of AD compared to placebo.
  Topical corticosteroids should be used liberally during acute flares. Potency and formulation is chosen based on body site and intensity of symptoms. Application of topical anti-inflammatory therapy should follow the finger-tip unit rule. 0.5 g of ointment is enough to cover two adult palm areas or 2% of an adult body surface. This amount is expressed from a tube with a 0.5 centimeter nozzle from the distal skin crease to the top of the index finger. After improvement, steroids are tapered to avoid withdrawal rebound. Proactive therapy with long-term twice weekly application may help to reduce relapses.
  TCI do not induce atrophy and can be an alternative to topical corticosteroids around the eyes, on the perioral skin and in skin folds. There is, to date, no scientific evidence of an increased risk of lymphoma, skin cancer or other malignancies due to TCI. Proactive therapy with twice weekly application of tacrolimus may help to reduce relapses.

- Antihistamines

  There is not enough evidence to support use of antihistamines in the treatment of pruritus in AD.

- Phototherapy

  UVB+UVA (280-400 nm), broadband UVB (280-320 nm), narrow-band UVB (peak 311-313 nm) and UVA1 (340-400 nm) can all improve the symptoms of AD. Effects of UV-light thought to be of importance in AD treatment is the modulation of the immune system through apoptosis of inflammatory cells, inhibition of Langerhans cells and alteration of cytokine production [109].

  Narrow-band UVB is less erythemogenic and preferred to broad-band UVB. UVA1 is preferred in severe cases.

- Systemic immunosuppressive treatment

  Oral glucocorticosteroids have an unfavourable risk/benefit ratio and long-term use is not recommended in the treatment of AD. Short-term use for acute flares in exceptionally severe cases could be considered.

  Ciclosporin may be used in chronic, severe cases of AD in adults. Azathioprine, mycophenolate mofetil or methotrexate may be used (off label) in adults AD patients if ciclosporin is ineffective or contraindicated.

ANTIBIOTICS AND ANTISEPTICS

FUSIDIC ACID AND FUSIDIC ACID-RESISTANT *S. AUREUS* (FRSA)

History of fusidic acid

Fusidic acid is a narrow spectrum anti-staphylococcal antibiotic which was introduced by the Danish company Leo Pharma in 1962 [110]. It was isolated from the fermentation broth of the fungus *Fusidium coccineum*. Fusidic acid has a steroid-like structure and is the only member of the fusidane class of antibiotics (Figure 2). After oral administration it is readily absorbed, shows high and long-lasting serum levels and penetrates bone tissue, synovial fluid and heart tissue [111].
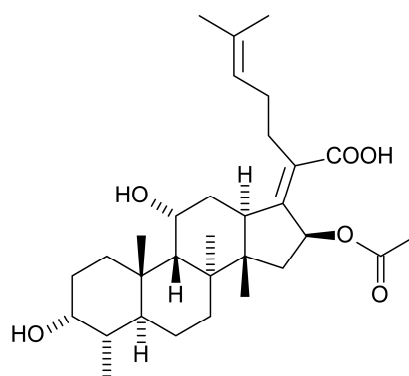
Figure 2. Structure of fusidic acid.

In Europe, fusidic acid has been available as various topical preparations for the treatment of impetigo and other superficial staphylococcal skin infections [112]. In Australia, Canada, Europe and some parts of Asia systemic fusidic acid treatment has been used for difficult-to-treat *S. aureus* infections, i. e. osteomyelitis, joint graft infections and endocarditis [113-115].

In Sweden, topical fusidic acid is available as a cream 2%, an ointment 2% and an ointment-soaked dressing 30 mg/dm^2. For use in opthamology there is an ointment 2% for application in the conjunctival sac. For systemic use, sodium fusidate is available as a 250 mg tablet and as a formulation for infusion [116].

In vitro, fusidic acid has high activity against *S. aureus*, including MRSA, and *S. epidermidis* [117]. The fact that clinical MRSA isolates can be sensitive to fusidic acid is of great clinical importance. Systemic fusidic acid is generally used in combination with other antimicrobial agents, e.g. rifampicin, since *in vitro* exposure selects for resistance in *S. aureus* [118].

Fusidic acid has never been approved in the U. S. but with the emerging epidemic of MRSA and

CA-MRSA voices from the pharmaceutical industry are being raised for the introduction of an oral preparation of fusidic acid [119].

Mechanism of action

Fusidic acid is an inhibitor of bacterial protein synthesis through binding of the bacterial GTPase elongation factor G (EF-G) [111]. EF-G interacts with the ribosome during protein synthesis to catalyze translocation of the mRNA-tRNA complex which is needed for peptide chain enlongation in the translation of mRNA to protein [120]. When fusidic acid binds EF-G it prevents the EF-G-ribosome interaction and stalls protein synthesis. This mechanism of translation inhibition is unique to fusidic acid. There is no known cross-resistance between fusidic acid and any other class of antibiotics.

The main niche for fusidic acid in Europe has been topical preparations for the treatment of superficial *S. aureus* skin infections (impetigo, secondarily infected AD). Topical antibiotics are known to exert selective pressure and promote antibiotic resistance. Even though the preparations were widely used for more than three decades there were few reports of staphylococcal resistance to fusidic acid up until the late 1990s [121].

Fusidic acid-resistant *S. aureus* (FRSA)

At the beginning of the new millennium, widespread outbreaks of bullous impetigo were noted in children in Sweden and Norway. It was discovered that these outbreaks were caused by the same fusidic acid-resistant *S. aureus* clone and the epidemiological data was published in 2002 [122, 123]. In the years that followed there was increased surveillance of FRSA in Europe. The presence of the same FRSA clone was subsequently established in the U.K., Ireland and France [124-126]. In a recently published study based on data from 2007-2008 it also appears highly prevalent in the Netherlands [127].

Fusidic acid-resistance in *S. aureus* isolates can arise through two principally different mechanisms. Mutation in *fusA* (the gene on the bacterial chromosome coding for EF-G) was the first identified cause of resistance. The altered EF-G does not bind fusidic acid as effectively and bacterial protein synthesis remains undisturbed [128]. Alternatively, *S. aureus* can harbour genes coding for proteins that protect EF-G from fusidic acid-binding. These genes (*fusB*, *fusC* and *fusD*) can be located on plasmids or the bacterial chromosome [129, 130]. It is currently not known which of the *fusA-D* determinants are the most prevalent in clinical FRSA isolates [131].

The impetigo-associated FRSA clone identified throughout Europe has been carefully characterized and named the epidemic European fusidic acid-resistant impetigo clone (EEFIC) [112, 132]. MIC for fusidic acid against the EEFIC has been shown to be approximately 4 mg/L [112].

The EEFIC carries the *fusB* determinant on its chromosome. It is further characterized by agr allelic group IV, spa type 171 and the presence of exfoliative toxin *etA* and often *etB* [112, 125]. The EEFIC is strongly associated with bullous impetigo.

Unsurprisingly, there seems to be an association between the patterns of prescription and use of fusidic acid and FRSA levels [133]. The emergence of the FRSA clone in Sweden and Norway coincided with increased prescription of topical fusidic acid products. Specifically, in 2001 a sales peak for topical fusidic acid was reached in eight Swedish counties followed by a peak in FRSA-levels in 2002. During the following two years topical fusidic acid sales and FRSA levels declined [134]. A contrasting report from the U.K. demonstrated persisting high levels of FRSA despite a drop in topical fusidic acid use. There was even an increase in FRSA levels in non-dermatology outpatients and hospital patients. The authors suggested that a reservoir of FRSA had been attained in the community and that longer follow-up was needed to witness any effects of restricted use of topical fusidic acid [135]. Recent use, prolonged use and intermittent use of topical fusidic acid have been described to influence FRSA rates in dermatology outpatients with AD [136, 137].

The emergence of the FRSA prompted the Swedish Medical Products Agency (Läkemedelsverket) to revise their guidelines on the treatment of impetigo in 2003. Avoidance of topical fusidic acid was recommended and this recommendation remained in the 2009 revision [75, 138]. Several authors outside of Sweden also advised on restrictive use of topical fusidic acid preparations [118, 139, 140].

While different aspects of FRSA and impetigo have been generously studied there are relatively few publications focusing on the prevalence of FRSA in AD. Since topical fusidic acid is used primarily in Europe most studies originate there. A dermatology inpatient department in the Netherlands reported increasing rates of FRSA in patients with AD, ranging from 9.7% in the mid-90s to 23.4% in 2001 [141]. An outpatient clinic in Cambridge reported FRSA in 50% of AD patients tested in 2002 [136]. One German study published in 2004 discovered 6% fusidic acid-resistance in *S. aureus* isolates from children with AD while another German study published in 2008 found FRSA in 25% of *S. aureus*-positive skin swabs from children and adults with AD [142, 143]. The highly variable reports are likely due to regional differences in the use of topical fusidic acid and to the fact that the investigations were conducted at different time points in the European FRSA epidemic.

ResNet, a Swedish antimicrobial resistance-surveillance database, offers monitoring of FRSA levels for each county and the whole nation [144]. The data provided by ResNet is based on antibiotic resistance profiles for 100 consecutive strains of *S. aureus* sent in by all county laboratories once yearly. Fusidic acid-resistance is consistently the most prevalent antibiotic resistance in *S. aureus* (susceptibility to erythromycin, clindamycin, gentamycin, norfloxacin, fusidic acid and cefoxitin

(screen for MRSA) is tested). FRSA levels peaked at 9.6% in 2002, declined to 3.8% in 2005 and were then stabilized at 4.0-6.0% in the years that followed. In 2011, FRSA levels were 6.0%. During the same time period (2002-2011) MRSA levels have gradually increased from virtually non-existent to 1.2% in 2011.

In paper II we have investigated the rate of FRSA in bullous impetigo, non-bullous impetigo and secondarily infected AD in patients attending a major dermatology clinic in the second-largest city in Sweden in the years 2004-2008.

ANTISEPTICS

Biocides are broad-spectrum chemical agents that inactivate microbes [145]. Biocides can be "-static" (bacteriostatic, fungistatic, sporistatic) which means they inhibit growth of microbes or "-cidal" (bactericidal, fungicidal, sporicidal) which means they kill microbes. Biocides used on living tissue are referred to as antiseptics whereas those used on surfaces and inanimate objects are referred to as disinfectants. While antibiotics inhibit specific metabolic pathways or the processing of the bacterial genome, usually at low concentrations, antiseptics have a less specific (and in most cases only partly defined) mode of action. Clinically significant antimicrobial resistance against antiseptics is much less common than resistance against antibiotics. This is probably due to the broader range of activity of antiseptics, often on multiple cellular targets. However, both innate and acquired antimicrobial resistance against antiseptics does exist. There is increasing concern that the use of biocides in health care, consumer prodcucts and in industrial settings contributes to the development and selection of antibiotic-resistant strains [146]. Acquired antimicrobial resistance against antiseptics has been described for *S. aureus* [145]. Antiseptics can also cause contact dermatitis and even anaphylactic reactions [147].

Alcohols

Alcohol formulations are commonly used for hand surface disinfecting, e. g. by health care workers. Alcohols have the ability to damage cell membranes and denature proteins of microbes. They are effective against vegetative bacteria, viruses and fungi but are not sporicidal [145].

Chlorhexidine

Chlorhexidine is a bactericidal agent. It damages and penetrates the cell wall and cell membrane, presumably by passive diffusion, causing leakage and coagulation of the cytoplasm [145].

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Cutaneous exposure to chlorhexidine can cause anaphylactic reactions [148, 149]. It has also been suggested that the low prevalence of delayed hypersensitivity to chlorhexidine is underestimated [147].

Hydrogen peroxide

Hydrogen peroxide acts as a powerful oxidant. It produces the hydroxyl radikal $\cdot$OH which oxidizes cell components such as lipids, proteins and DNA. It has been suggested that exposed sulfhydryl groups and double bindings are especially vulnerable. Hydrogen peroxide is effective against bacteria, bacterial spores, fungi and viruses [145]. Gram-positive bacteria are more susceptible than gram-negatives. If a microbe produces catalase or other peroxidases this may serve as partial protection. Catalase promotes $2H_2O_2 \rightarrow 2H_2O + O_2$, thus limiting hydroxyl radical formation. There are no known microbes that are resistant to hydrogen peroxide.

Microcid®, a hydrogen peroxide cream introduced in 1994 for the treatment of impetigo, was tested in a double-blind randomized multi-centre trial against topical fusidic acid and was found to be equally effective [150]. It was included in the 2003 Swedish Medical Product Agency guidelines for impetigo treatment as an optional complement to the recommended hygiene measures [138]. However, Microcid® did not prove to be as clinically effective as anticipated. In the current guidelines it is not included [75].

Clioquinol

Clioquinol (5-chloro-7-iodo-8-quinolinol) belongs to the chinoline colours and has been used as a drug since the 1950s. Initially, an oral formulation was prescribed for the treatment of intestinal amoebas. However, several reports of neurotoxicity in Japanese patients in the 1970s led to the withdrawal of clioquinol. Recently, clioquinol has received new attention due to its ability to dissolve copper from beta amyloid protein aggregates associated with Alzheimer´s disease. It is also known that clioquinol can induce cell death in malignant cells. It does so by inhibiting the proteasome, the "waste basket" organelle which is comprised of protelytic enzymes and degrades ubiquinated proteins destined for destruction [151]. The mechanism behind the antimicrobial effect of clioquinol is not known [152]. Clioquinol is used as a topical antiseptic in dermatology. Its origin from the group of chinoline colours is evident from the temporary yellowish discolouration of the skin which is created upon use. In Sweden, clioquinol is available as an additive (3%) to betamethasone cream or ointment. There are few reports of resistance against clioquinol. It is not a common contact allergen and has not been associated with skin malignancies [151]. Treatment periods should be kept short and use is not advised in young children [153].

Halquinol, a related substance, has previously been available in Sweden, as an additive (0.75%) to triamcinolone cream. It was discontinued for unknown reasons during the progress of this work but is featured in paper III together with clioquinol and a hydrogen peroxide formulation.

AIMS OF THE STUDIES

PAPER I

To describe the frequency and distribution of bacteria and the yeasts *Candida* and *Malassezia* in balanoposthitis.

To investigate if different microbes correspond to distinct clinical presentations of balanoposthitis.

To investigate a possible association between *Malassezia* and balanoposthitis.

To investigate a possible association between balanoposthitis and seborrhoeic dermatitis or psoriasis.

PAPER II

To investigate the bacterial spectrum in patients with impetigo, bullous impetigo and secondarily infected AD.

To investigate the prevalence of fusidic acid-resistance among *S. aureus* isolates from patients with non-bullous impetigo, bullous impetigo and secondarily infected AD.

PAPER III

To test the *in vitro* antimicrobial activity, expressed as MIC against selected microbes, of commonly used topical skin pharmaceuticals and their active substances.

PAPER IV

To describe variations in *S. aureus* colonization (body sites, density, strain identity) and relate these variations to disease severity, expressed as SCORAD, during a 5-month follow-up of adult patients with AD.

MATERIAL AND METHODS

SUBJECTS AND SAMPLES

PAPER I

The study was performed in the venereology clinic of the Department of Dermatology and Venereology at Sahlgrenska University Hospital, where we see patients with STIs and genital dermatoses. A total of 102 patients presenting with balanoposthitis between 2004 and 2006 were included in the study. Twenty-six patients with no clinical signs or medical history of balanoposthitis served as a control group.

	Balanoposthitis group $n = 102$*	Control group $n = 26$
Balanoposthitis	102	-
Bacterial STIs		
Chlamydia trachomatis urethritis	3	2
Mycoplasma genitalium urethritis	-	1
Viral STIs		
Herpes simplex type 2	1	-
Varicella zoster (right leg)	-	1
Condylomata acuminata	2	8
Molluscum contagiosum	1	2
Other		
Eczema nummularis (extragenital)	1	-
Folliculitis	1	1
Non-malignant preputial epithelial hyperplasia	1	-
Non-specific urethritis	5	2
Pruritus scrotalis	-	1
STI check-up	-	8
Zoon balanitis	1	-

Table I. Additional diagnoses in the balanoposthitis group and reasons for the visit in the control group

*The total number of diagnoses in this column exceeds the number of patients, since some patients received diagnoses in addition to balanoposthitis. STI: sexually transmitted infection.
Modified from Alsterholm et al [154] (= paper I) with permission from the publisher.

Some patients with balanoposthitis had additional diagnoses. A majority of patients in the control group attended the clinic for routine STI check-up or genital warts (Table I). It is important to note that genital warts or other dermatoses in the control group did not affect the area of the prepuce or glans penis.

It can be argued that peri-genital dermatoses such as genital warts could influence frequency and distribution of microbes on the prepuce and glans penis indirectly. To avoid this possible bias, an alternative way to recruit a control group could have been to choose age-matched patients attending the dermatology section of our clinic or to advertise for healthy volunteers. However, we could not assume that general health and lifestyle factors would be similarly distributed in such a group. In addition, we had ethical considerations regarding recruitment of a control group outside the venereology section. Inspection and swabs of the genital area are not regarded as a routine examination by most patients. It could prove difficult to find volunteers willing to participate.

Ages in the balanoposthitis group ranged from 19 to 71 years (median 28, mean 33 years). Ages in the control group ranged from 18 to 44 years (median 29, mean 29 years). Two patients in the balanoposthitis group and one patient in the control group were circumcised.

Presence or absence of predetermined clinical criteria for balanoposthitis was assessed in each patient. The criteria for the diagnosis balanoposthitis was presence of one or more of the following; erythema, papules, pustules, transudate, white membranes or fissures in the area of the prepuce and glans penis. All patients underwent clinical examination of the skin including the scalp to assess presence or absence of seborrhoeic dermatitis or psoriasis. They were also asked if they had previously experienced symptoms of seborrhoeic dermatitis or psoriasis. Swab samples from the area of the prepuce and glans penis for bacterial culture, *Malassezia* culture and semiquantitative *Candida* culture was secured from all patients.

PAPER II

A prospective study of the bacterial spectrum and prevalence of fusidic acid-resistance among *S. areus* isolates in patients with impetigo and secondarily infected AD was conducted in the time period 2004-2008. In addition a patient record review was made covering the first (2004-2005) and last (2007-2008) years of the prospective study.

At the time of the study, the Department of Dermatology ran clinics in the three hospitals serving

the greater Gothenburg area (Sahlgrenska Hospital, Östra Hospital and Mölndal Hospital). Impetigo and secondarily infected AD are considered emergency dermatology cases and are usually, but not exclusively, referred to our on-call dermatologist who works in the largest clinic located at Sahlgrenska Hospital. The authors of paper II recruited patients for the prospective study when they, on a regular basis, served as the on-call dermatologist. This arrangement explains why the number of patients included in the prospective study is low in relation to the total number of patients presenting with impetigo or secondarily infected AD at the Department of Dermatology. Also, the patients were not strictly consecutive since the reality of clinical work does not always allow time for the inclusion in a study.

The intermittent recruitment was considered a weakness of the study that could result in an involuntarily biased selection of patients. Therefore, we performed a patient record review. The review was aimed at finding all cases of impetigo and secondarily infected AD at the Department of Dermatology and collect bacterial culture data when available.

In the following, the prospective study 2004-2008 and the patient record review are described separately.

Prospective study 2004 to 2008

The study was performed at the Department of Dermatology and Venereology at Sahlgrenska University Hospital, Gothenburg, Sweden, from June 2004 to May 2008. Patients who presented to the authors at Sahlgrenska Hospital with either impetigo, bullous impetigo or secondarily infected AD were asked to participate. There was no selection based on gender or age, nor were patients selected to be seen by the authors. All patients consulting with the Department of Dermatology need a referral which is usually issued by a general practitioner at a primary health care facility. Skin lesions were examined and the diagnosis impetigo, bullous impetigo or secondarily infected AD was made based on clinical evaluation. A swab sample for bacterial culture was secured from a clinically infected skin lesion in each patient. Bacterial cultures were sent to the Department of Clinical Bacteriology, Sahlgrenska University Hospital, according to standard procedure. All *S. aureus* isolates from clincal samples are routinely tested against fusidic acid, cefoxitin (screening for MRSA), clindamycin, erythromycin and tobramycin.

Seventy-five patients, 38 presenting with impetigo (13 of whom had bullous impetigo) and 37 with secondarily infected AD, agreed to participate in the study. The age range in the impetigo group was 1–49 years, with a mean of 18 years and a median of 17 years. The age range in the bullous impetigo group was 2–30 years (mean 18 years, median 18 years). The age range in the secondarily infected AD group was 6 months to 62 years (mean 30 years, median 29 years).

Patient record review

The patient record review covered all patients attending the three clinics of the Department of Dermatology for impetigo or secondarily infected AD. All patient records at the Department of Dermatology are computerized. The review was carried out in 2009.

A search for all patients receiving the diagnostic codes for "Impetigo" (L01.0 and L01.0X), "Secondarily infected dermatoses" (L01.1), "Prurigo Besnier" (L20.0), "Otherwise specified AD" (L20.8) or "AD unspecified (L20.9)" in the time periods June 2004 to May 2005 and June 2007 to May 2008 was made. The diagnostic codes which are used come from the Swedish version of the International Statistical Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10). One or more diagnostic codes are routinely assigned to every patient visit by the dermatologist who was consulted. In the patient record review, impetigo could not be differentiated into the non-bullous and bullous types with certainty because the description of the clinical presentation was sometimes limited.

The search rendered a total of 2353 patient records (1184 from June 2004 to May 2005 and 1169 from June 2007 to May 2008). These patient records were reviewed by author MA.
A total of 283 out of the 2353 records described dermatoses other than impetigo or AD or lacked a definitive diagnosis and were excluded. All records describing ongoing impetigo or secondarily infected AD together with the securing of a bacterial culture from afflicted skin were selected for further analysis. These records also included those of the patients who were included in the first and last year of the prospective study. The results of the bacterial cultures including antibiotic resistance patterns for *S. aureus* were collected from the register at the Department of Clinical Bacteriology. An overview of the patient record review is provided (Figure 3).
In June 2004 to May 2005 there were 66 cases of impetigo and 55 cases of secondarily infected AD where a swab sample for bacterial culture was secured. The age range in the impetigo group was 9 days to 81 years (mean 26 years, median 23 years). The age range in the secondarily infected AD group was 7 months to 86 years (mean 25 years, median 21 years).
In June 2007 to May 2008 there were 53 cases of impetigo and 55 cases of secondarily infected AD where a swab sample for bacterial culture was secured. The age range in the impetigo group was 1–90 years (mean 31 years, median 21 years). The age range in the secondarily infected AD group was 1–90 years (mean 29 years, median of 24 years).

June 2004-May 2005

Impetigo, Secondarily infected dermatoses (176)

- **Impetigo** (101)
  - Bacterial culture (66)
  - No bacterial culture (35)
- Other diagnoses (75)

Prurigo Besnier, Otherwise specified AD, AD unspecified (1008)

- Other diagnoses (42)
- **Atopic dermatitis** (966)
  - Non-flaring AD (516)
  - Flare-up (450)
    - Oral antibiotics prescribed (144)
      - Bacterial culture (55)
      - No bacterial culture (395)
    - No oral antibiotics prescribed (306)

June 2007-May 2008

Impetigo, Secondarily infected dermatoses (104)

- **Impetigo** (53)
  - Bacterial culture (33)
  - No bacterial culture (20)
- Other diagnoses (51)

Prurigo Besnier, Otherwise specified AD, AD unspecified (1065)

- Other diagnoses (115)
- **Atopic dermatitis** (950)
  - Non-flaring AD (405)
  - Flare-up (545)
    - Oral antibiotics prescribed (124)
      - Bacterial culture (55)
      - No bacterial culture (490)
    - No oral antibiotics prescribed (421)

Figure 3. Patient record review

A search for diagnostic codes L01.0 + L01.0X (Impetigo), L01.1 (Secondarily infected dermatoses), L20.0 (Prurigo Besnier), L20.8 (Otherwise specified AD) and L20.9 (AD unspecified) was made and retrieved records reviewed. Number of patients at each step in brackets.

PAPER III

The MIC for topical skin pharmaceuticals and active substances from these pharmaceuticals were determined against selected microbes with agar dilution assay.

Topical skin pharmaceuticals

The following topical skin pharmaceuticals were tested. The formulations of the pharmaceuticals were all creams.

Antibiotic formulation

Fucidin® 2% (fusidic acid) (Leo Pharma, Malmö, Sweden)

Antifungal formulations

Canesten® 1% (clotrimazole) (Bayer, Solna, Sweden)

Daktar® 2% (miconazole) (Janssen-Cilag, Sollentuna, Sweden)

Lamisil® 1% (terbinafine) (Novartis, Täby, Sweden)

Antifungal formulation with corticosteroid

Daktacort® (miconazole 20 mg/g + hydrocortisone 10 mg/g) (Janssen-Cilag, Sollentuna, Sweden).

Antiseptic formulation

Microcid® 1% (hydrogen peroxide) (Bioglan Pharma, Malmö, Sweden)

Corticosteroid formulation

Betnovat® (betamethasone) (GlaxoSmithKline, Solna, Sweden)

Emovat® 0.05% (clobetasone butyrate) (GlaxoSmithKline, Solna, Sweden)

Dermovat® 0.05% (clobetasol) (GlaxoSmithKline, Solna, Sweden)

Hydrokortison CCS® 1% (hydrocortisone) (Clear Chemical Sweden, Borlänge, Sweden)

Corticosteroid formulations with antiseptic agent

Betnovat® with chinoform (betamethasone 1 mg/g + clioquinol 30 mg/g) (GlaxoSmithKline, Solna, Sweden)

Kenacutan® (triamcinolone 1 mg/g + halquinol 7.5 mg/g) (Bristol-Myers Squibb, Bromma, Sweden).

Note: Clioquinol is 5-chloro-7-iodo-8-quinolinol and halquinol is a mixture of 5,7-dichloro-, 5-

chloro- and 7-chloro-8-quinolinols.

Active substances

The following active substances were tested.

Clioquinol (5-chloro-7-iodo-8-quinolinol) (Sigma-Aldrich, Stockholm, Sweden)

Econazole nitrate salt (Sigma-Aldrich, Stockholm, Sweden)

Fusidic acid (Sigma-Aldrich, Stockholm, Sweden)

Ketoconazole nitrate salt (Sigma-Aldrich, Stockholm, Sweden)

Miconazole nitrate salt (Sigma-Aldrich, Stockholm, Sweden)

Terbinafine hydrochloride (Biomol, Immunkemi F & D, Järfälla, Sweden)

Selected microbes

The bacteria and yeast were reference strains obtained from Culture Collection University of Göteborg (CCUG); *Candida albicans* (CCUG 5594), *Escherichia coli* (CCUG 24), *Staphylococcus aureus* (CCUG 17621), *Staphylococcus epidermidis* (CCUG 39508) and *Streptococcus pyogenes* (CCUG 4207).

Comments on the selection of pharmaceuticals, substances and microbes

We wanted to explore antibacterial effects of antifungals and chose several antifungal formulations. For the antiseptics we wanted to test the species-related range of activity. In the case of the corticosteroids we wanted to investigate potential antimicrobial effects of additives and preservatives since the amount of *S. aureus* on atopic skin is reported to diminish after application of corticosteroids alone and the reason for this is not fully elucidated [96]. The topical antibiotic was chosen as a reference for anti-staphylococcal activity.

Selected active substances from the formulations were tested alone to determine if other components of the formulations influenced antimicrobial activity.

S. aureus was chosen because it is a frequent skin pathogen as well as a commensal. *S. epidermidis* was selected for being the most common coccus on human skin and a representative of the CoNS.

S. pyogenes is a common pathogen associated with SSTIs and a member of the genus *Streptococcus*. *E. coli* represented the gram-negatives and was included since it can be a relevant colonizer of inflamed skin or even a primary pathogen. *C. albicans* was added as a reference since we tested antifungal preparations.

PAPER IV

Variations in *S. aureus* colonization and disease severity in adult patients with AD were registered during a 5-month follow-up.

The study was performed at the Department of Dermatology and Venereology and the Department of Microbiology at Sahlgrenska University Hospital in 2010-2012. A search of all patient records made during the period January 1 to September 22 2010 at the Department of Dermatology was conducted. Search critera were patients 18 years or older who had received the diagnostic code "Prurigo Besnier (atopic dermatitis) L20.0" according to the Swedish version of the International Statistical Classification of Diseases and Related Health Problemn, Tenth Revision (ICD-10). 489 patient records matched these criteria and were reviewed by author MA. All patients with moderate to severe AD without ongoing systemic treatment (oral corticosteroids, other immunomodulators) or phototherapy were considered eligible for the study (*n* = 78). These patients were contacted by phone in random order and offered participation. Inclusion was stopped when 20 patients had agreed to participate.

Eleven men and ten women agreed to participate in the study. Of those, eleven men and nine women completed follow-up. Age distribution was; all patients age range 26-66 years (mean 48 years, median of 41 years); male patients age range 28-66 years (mean 41 years, median of 39 years); female patients age range 26-65 years (mean 44 years, median 42 years).

Follow-up for each patient was 5-6 months during which evaluation was made at three time-points (visit 1 at starting point, visit 2 after 2-3 months and visit 3 after 5-6 months). Follow-up took place between January 2011 and June 2011 with the exception of two patients who were followed-up from March 2011 to September 2011. The same protocol was used at each visit. A history of the patient´s treatment for AD during the last 2-3 months was taken, SCORAD assessment was performed by author MA and swab samples for qualitative and quantitative bacterial cultures were collected by authors MA and LS.

SCORAD evaluation

The severity of AD was assessed with the SCORAD index (severity <u>SCOR</u>ing of <u>A</u>topic <u>D</u>ermatitis) [155]. The SCORAD index includes estimation of the body surface area affected by AD and grading of six intensity items (erythema, oedema/papulation, oozing/crusts, excoriations, lichenification and dryness). The combination of the area estimation and intensity grading renders a score which is referred to as objective SCORAD index (range 0-83). The SCORAD index also includes two subjective items. Patients are asked to grade their average pruritus and sleep loss, respectively, during the last three days and nights, by using a visual analogue scale. This grading

renders an additional score which is added to the objective SCORAD score. The total score is referred to as the total SCORAD index (range 0-103). Objective SCORAD index as well as total SCORAD index was calculated at all visits. The intra-individual and inter-individual relations between SCORAD indices were similar for objective SCORAD index and total SCORAD index. Statistical analysis was performed with both the objective SCORAD index and the total SCORAD index with similar results (data not shown).

Due to current convention and to facilitate comparison with other studies, we chose to consistently use the objective SCORAD index in tables and figures. The intensity of AD was defined as mild (objective SCORAD index 0-15), moderate (objective SCORAD index 15-40) or severe (objective SCORAD index >40) according to the classification of Oranje et al [156].

LABORATORY METHODS

PAPER I

Culture and differentiation of bacteria and *Candida* species

One cotton swab sample for bacterial culture and one for semi-quantitative *Candida* culture were obtained from the area of the prepuce and glans penis. Bacterial cultures were sent to the Department of Clinical Bacteriology according to standard procedure. *Candida* and *Malassezia* species were cultured and differentiated at the research laboratory of the Department of Dermatology which also serves as a clinical laboratory for the detection of cutaneous yeast and dermatophyte infections.

Candida species were cultured on Sabouraud's agar at 32°C and growth visually quantified as very discrete, discrete, moderate or heavy. The isolates were then differentiated on CHROMagar™ *Candida* Medium plates (Becton Dickinson GmbH, Heidelberg, Germany). Colonies of *C. albicans* have a distinct green colour on CHROMagar medium, setting them apart from other *Candida* species [157, 158].

Culture and differention of *Malassezia* species

Samples for *Malassezia* cultures were obtained using a contact plate with Leeming-Notman agar pressed against the prepuce and glans penis. Leeming-Notman agar is the most effective culture media for isolation of *Malassezia* yeasts [159]. Semi-quantitative cultures for *Malassezia* species were made on the contact plates used for sampling followed by differentiation of the isolates. At the time of the study seven *Malassezia* species had been characterized in humans; *M. furfur*, *M. pachydermatis*, *M. sympodialis*, *M. globosa*, *M. obtusa*, *M. restricta* and *M. slooffiae*.

The contact plates were incubated at 32 °C for 7-14 days. Colonies were then counted and growth was quantified as > 5 colonies, 2–5 colonies or 1 colony. Morphologically different colonies were re-cultured on separate Leeming-Notman plates for further differentiation.

Malassezia species grow selectively on media, are sensitive to temperature and exhibit different enzymatic activities. These properties are used to distinguish species according to the combined methods of Guillot et al and Mayser et al [38, 41, 42].

Specifically, the isolates were cultured on Sabouraud dextrose agar at 32°C and modified Dixon agar at 38°C. Further differentiation was based on the catalase reaction determined by application of hydrogen peroxide, culture with Cremophor EL and β-glucosidase activity assayed by using esculin agar tubes. *M. pachydermatis* alone grows on Sabouraud´s agar. Several *Malassezia* species, but not

M. restricta, express catalase and split hydrogen peroxide into water and oxygen. *M. furfur* is the only species that can metabolize Cremophor EL (PEG-35 castor oil) and grows right up to the edge of a well containing Cremophor EL made on a Leeming-Notman plate. *M. obtusa* and *M. sympodialis*, but not *M. globosa* and *M. slooffiae*, split esculin in agartubes into esculetin and glucose turning the media black due to release of iron salts. *M. sympodialis* and *M. slooffiae* grow on Dixon agar at 38° C whereas *M. globosa* and *M. obtusa* do not. The combined growth/activity profile from the described tests determines the *Malassezia* species (Figure 4).

PAPER II

Cultures for bacteria and determination of fusidic acid-resistance in *S. aureus* isolates

A swab sample for bacterial culture was taken from a clinically infected skin lesion in each patient. Specifically, isolates of *S. aureus* were visually detected on selective plates and verified by the coagulase test. All isolates of *S. aureus* were routinely tested for antibiotic resistance against cefoxitin, fusidic acid, clindamycin, erythromycin and tobramycin by disk diffusion on Mueller-Hinton agar. The *S. aureus* isolates were characterized as FRSA or fusidic acid-sensitive according to the breakpoints defined by EUCAST [160]. Disc content of fusidic acid was 10µg. Clinical breakpoint was a zone diameter of 24 mm corresponding to a MIC breakpoint of 1 mg/L (S ≤ 1 mg/L, R > 1 mg/L).

The full genetic characterization of the FRSA clonotype later named the EEFIC was not completed when this study started [112, 132]. Consequently, the FRSA isolates were not molecularly typed to determine if they belonged to the EEFIC.

Contact plate with
Leeming-Notman agar

Sabouraud´s agar

negative → **Catalase reaction**
positive → *M. pachydermatis*

Catalase reaction

negative → *M. restricta*
positive → **Cremophor EL**

Cremophor EL

negative → **Esculin**
positive → *M. furfur*

Esculin

negative → **Dixon agar at 38° C**
positive → **Dixon agar at 38° C**

Dixon agar at 38° C (negative branch)

negative → *M. globosa*
positive → *M. slooffiae*

Dixon agar at 38° C (positive branch)

negative → *M. obtusa*
positive → *M. sympodialis*

Malassezia spp.	Sab. 32 °C	Catalase	Cremophor EL	Esculin	Dixon 38 ℃
M. pachydermatis	+	+/-	+/-	+/-	+
M. furfur	-	+	+	-	+
M. sympodialis	-	+	-	+	+
M. slooffiae	-	+	-	-	+
M. obtusa	-	+	-	+	-
M. globosa	-	+	-	-	-
M. restricta	-	-	-	-	-

Figure 4. Differentiation of Malassezia species.

All *Malassezia* isolates were cultured on Sabouraud agar (Sab.), modified Dixon agar (Dixon) and with Cremophor EL. Catalase activity was assayed by application of hydrogen peroxide (Catalase reaction). β-glucosidase activity was assayed by using esculin agar tubes (Esculin). + denotes growth or activity, - denotes abscence of growth or activity. The growth/activity profile determined the *Malassezia* species.

PAPER III

Determination of MIC with agar dilution assay

The MIC is the lowest concentration of a substance that will inhibit visible growth of a microbe after overnight incubation (this period can be extended for some slow-growing microorganisms). MIC is considered the golden standard for antimicrobial susceptibility testing and can be determined by agar or broth dilution assays [161, 162].

It is possible to dissolve creams in agar and we chose this unconventional approach to determine the MIC of entire formulations against selected microbes. The colour and turbidity of the agar is affected when high concentrations of cream are dissolved but bacterial and yeast colonies are still easily recognizable on the plates.

When using the agar dilution assay the substance that is being tested is dissolved in agar on plates in a range of concentrations (doubling dilutions steps up and down from 1 mg/L as appropriate). An inoculum of the microbe is applied on the plates and allowed to absorb. After overnight incubation, the plate with the lowest concentration of the substance that inhibits microbial growth is noted and represents the MIC of the substance against the microbe (Figure 5).
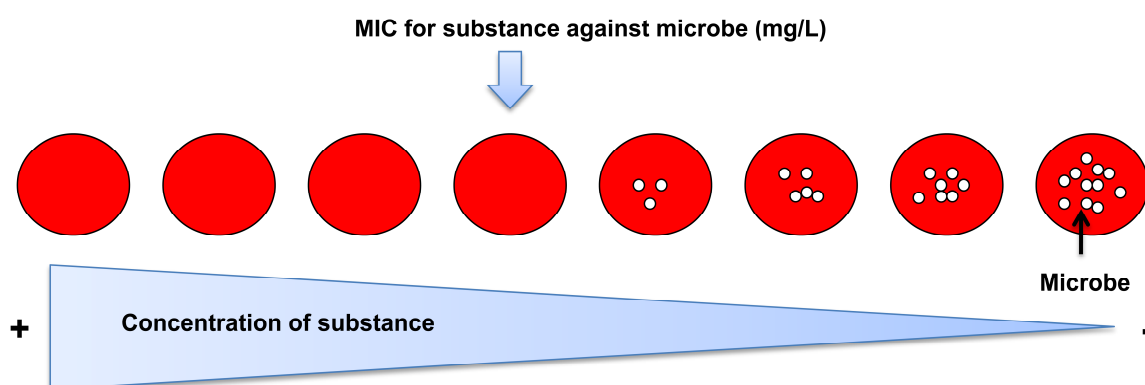
Figure 5. Basic concept of MIC determination by agar dilution assay.

The agar dilution assay was used to determine MIC for topical skin pharmaceuticals (below referred to as formulations) and active substances from these pharmaceuticals against *S. aureus*, *S. epidermidis*, *S. pyogenes*, *E. coli* and *C. albicans*.

Dilution series for each formulation/substance were prepared in blood agar medium (Columbia agar (Oxoid, Basingstoke, U.K.) + 5% defibrinated horse blood) for *S. pyogenes* and in Diagnostic

Sensitivity Test (DST) agar (Oxoid) for *S. aureus*, *S. epidermidis*, *C. albicans* and *E. coli*. All dilution series were made with freshly prepared and autoclaved agar. The agar was allowed to cool to a temperature of 48–50°C in an incubator before a formulation or substance was added. Formulations were weighed and dissolved directly in agar for the concentrations 20% (10 g cream in a final volume of 50 ml agar, weight/volume (w/v)), 10% (w/v), 5% (w/v) and 1% (w/v). For the lower concentrations a stock solution of 1% (0.5 g cream in a final volume of 50 ml agar) was prepared and further diluted with agar to obtain concentrations 0.5–0.0001% (w/v). A pre-warmed oven with a constant temperature of 50°C kept the stock solution at an appropriate temperature during experimental procedures. Final concentration ranges were; Betnovat® 1–20% (w/v), Betnovat® with chinoform 0.005–20% (w/v), Emovat® 1–20% (w/v), Dermovat® 1–20% (w/v), Canesten® 1% 0.001–20% (w/v), Daktar® 2% 0.001–5% (w/v), Daktacort® 0.001–20% (w/v), Fucidin® 2% 0.0001%–20% (w/v), Hydrokortison CCS® 1% 1–20% (w/v), Kenacutan® 0.005–20% (w/v), Lamisil® 1% 0.001–20% (w/v) and Microcid® 1% 0.001–20% (w/v). Substances were dissolved in dimethylformamide (DMF) to prepare a stock solution of 10 mg/ml and this solution was further diluted and mixed with agar. When testing *S. pyogenes*, the stock solution was instead prepared with ethanol 99.5% (Kemetyl AB, Haninge, Sweden) because DMF was no longer recommended for laboratory use. Final concentration ranges were: clioquinol 0.125–100 μg/ml, econazole nitrate salt 1.0–50 μg/ml, fusidic acid 0.03125–25 μg/ml, ketoconazole 0.125–100 μg/ml, miconazole nitrate salt 0.125–100 μg/ml and terbinafine hydrochloride 0.5–50 μg/ml. The MICs against all microbes for DMF (range tested 0.625–10% (volume/volume = v/v)) or ethanol (range tested 0.625–10% (v/v)) were also determined to ensure that antimicrobial effect could not be attributed to the solvent for the active substances.

Standard Petri dishes (92 × 16 mm (diameter × height)) were used and the agar volume in each plate was 25 ml. After the agar was poured, plates were allowed to cool and were then used immediately. There were duplicate plates for all dilution steps.

Bacterial colonies from blood agar plates were used for preparation of the inoculums. *C. albicans* was grown on Sabauroud's agar medium. Colonies were suspended in PBS. The appropriate colony-forming units (CFU)/ml for a microbe was the concentration producing sufficient growth on pure agar plates when inoculated with a 20 μl spot and incubated at 37 °C for 24 hours. According to this suspensions were adjusted to approximately 2×10^6 CFU/ml for *C. albicans*, 2×10^4 CFU/ml for *E. coli*, 2×10^5 CFU/ml for *S. aureus*, 8×10^5 CFU/ml for *S. epidermidis* and 6×10^5 CFU/ml for *S. pyogenes* using a turbidimeter (DEN-1 McFarland Densitometer, Biosan).

The surface of the agar plates were inoculated with a 20 μl spot using a replicator and incubated at 37°C for 24 h. MIC was the lowest concentration of the formulation/substance inhibiting growth determined by visual inspection. MIC for the active substance as part of a cream was calculated as

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the concentration of active substance in the cream multiplied by the MIC for the entire cream.

Limitations of the *in vitro* model

When reviewing the results of paper III it is important to consider that *in vitro* results cannot always be extrapolated to the *in vivo* situation. The *in vitro* and *in vivo* milieu can differ greatly with regards to factors such as pH, salt concentrations and temperature. A potential weakness with the agar dilution assay is the heating of the formulation which occurs when it is dissolved in the agar. When agar plates are made the formulation or substance is dissolved in liquid agar which keeps a temperature of 48-50°C as described. The agar has to keep this temperature during dissolving because it sets quickly when cooled below 45°C. The agar, with the dissolved formulation, is immediately poured into Petri dishes at room temperature where it quickly cools and sets. As a consequence, all formulations tested have briefly reached a temperature which is above body temperature and higher than that recommended for storage. It is possible that this heating affects properties of the formulations.

Considering these limitations, the MIC value for a formulation needs to be viewed in relation to MIC values obtained for other formulations with the same assay and, most importantly, in relation to clinical experience.

Quantitative culture for *S. aureus*

Quantitative culture from the skin surface allows for determination of the density of a particular microbe expressed as CFU/cm^2.

Quantitative cultures for *S. aureus* were obtained from representative sites of lesional and uninvolved skin at all patients' visits during follow-up. Sampling and culture was performed at the Department of Dermatology, Sahlgrenska University Hospital. The method was an adaptation of methods previously described [163, 164].

A stainless steel ring with an inner area of 5.5 cm^2 was placed on the skin and held in place with moderate pressure by two fingers (Figure 6). One milliliter of dispersed detergent (PBS, pH 7.9, with 0.1% Triton X-100) was applied onto the skin circumscribed by the ring. Diluted Triton X-100 helps suspend bacteria to single cells without damaging their cell membranes. The skin was then rubbed with a glass rod for exactly one minute. The microbe-containing suspension was then aspirated and aliquoted. Serial ten-fold dilutions (undiluted to 1:100 000) were immediately prepared. 100 µl of each dilution was plated on individual blood agar plates (Columbia agar (Oxoid, Basingstoke UK) + 5% defibrinated horse blood). Plating serial dilutions of the microbe suspension ensured that there would be plates with an appropriate number of colonies for manual counting. Plates were incubated for 24-48 hours in 37°C.

All morphologically different colonies found on the plates after incubation were counted separately and tested with Slidex® Staph Plus (Biomérieux, Lyon, France), a rapid *S. aureus*-specific agglutination test. All Slidex® Staph Plus-positive isolates were verified with the tube coagulase test. The density of *S. aureus* on the skin expressed as CFU/cm^2 was calculated.

$$\frac{\text{Number of } S.\ aureus \text{ CFU on plate} \times 10 \times \text{dilution factor}}{5.5 \text{ cm}^2} = \text{Number of } S.\ aureus \text{ CFU/cm}^2$$

All *S. aureus* isolates were cultured on new blood agar plates for purity. Isolates were then kept in freezing medium at -80°C.
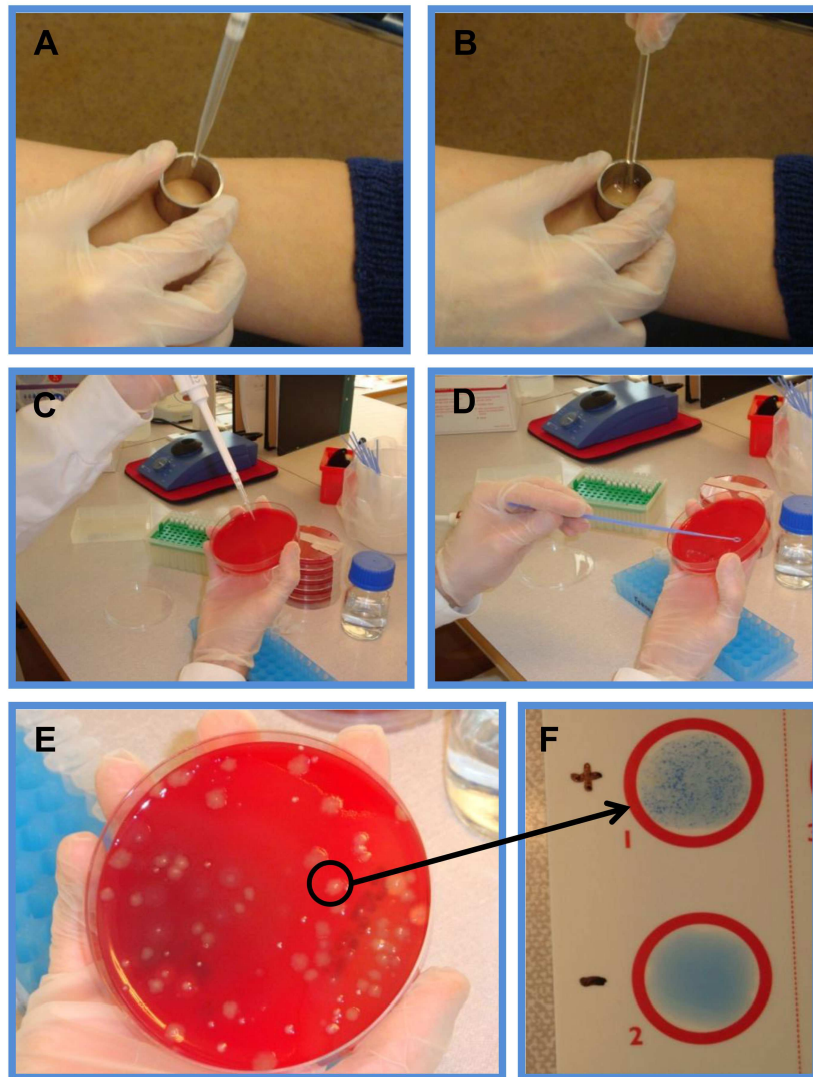
Figure 6. Quantitative culture for S. aureus.

A. 1 mL Triton X 0.1% in PBS applied onto skin. B. Skin is rubbed for one minute with a glass rod. C+D. The aspirate is plated on blood agar plates. E. Multiple colonies after incubation. F. Slidex® Staph Plus shows agglutination for *S. aureus* colony. Photo: Mikael Alsterholm and Lina Hagvall

Considerations regarding sampling site

For several reasons, the preferred sampling site for quantitative *S. aureus* culture was the arm. In adult patients with AD both lesional skin and uninvolved skin is often present on the arms. The arm is also a body part where the stain-less steel ring used for sampling is easily applied and held in place. Since *S. aureus* colonization rates vary depending on body site it was important to keep sampling within the same body area, whenever possible, in order to avoid over- or underestimation of differences between lesional and uninvolved skin. Other body sites were sampled when there were no representative areas of lesional or uninvolved skin on the arms; preferably the trunk or

upper leg. The meticulous sampling technique for quantitative culture proved highly sensitive. In fact, we took conventional swabs for qualitative culture on skin immediately adjacent to the sampling sites for the quantitative cultures. The *S. aureus* detection level was lower for swab samples than for the quantitative cultures (data not shown).

Qualitative culture for *S. aureus*

Swab samples for *S. aureus* cultures were secured from the anterior nares, tonsils and perineum at each visit. Swab samples were also taken from the body sites where quantitative cultures were obtained for comparison of the detection level of the two techniques as described above. Cultures were made at the Department of Microbiology, the Sahlgrenska Academy at the University of Gothenburg.

S. aureus isolates were verified with Slidex® Staph Plus and the tube coagulase test, re-cultured for purity on blood agar plates and kept in freezing medium at -80°C.

Pulsed-field gel electrophoresis (PFGE)

All *S. aureus* isolates found on lesional skin, in the anterior nares and perineum (*n* = 94) and twenty-five *S. aureus* isolates found on uninvolved skin were typed for strain identity with PFGE. Low counts of *S. aureus* on uninvolved skin in the absence of *S. aureus* on lesional skin were not typed.

We chose to type for strain identity with PFGE because the primary aim was to establish isolate interrelationships and because PFGE remains the gold standard in clinical bacteriology [165].

S. aureus isolates were grown on blood agar plates over night. Ten colonies were suspended in 500 μl TEN-buffer (Trizma® HCL (Sigma T3253), EDTA (Titriplex II Merck 8417), sodium desoxycholate (Merck 6504)), the suspension centrifuged (13 000 G, 20 seconds) and the supernatant discarded. The bacteria were re-suspended in 125 μl EC-buffer (Trizma® HCl (Sigma T3253), sodium chloride (Fischer Scientific), EDTA (Titriplex II Merck 8417, polyoxyethylene 20 cetyl ether (Sigma P5884), sodium deoxycholate (Merck 6504), N-laurylsarcosyl (Sigma L5125)). The suspensions were placed on a heating block set to 56°C for 15 minutes and 12 μl Lysosome A (0.5 u/μl, Sigma Aldrich) was added. Plugs were created by adding 125 μl 2% low-melting-point agarose to each suspension (Sigma Aldrich) and pipetting 125 μl of the resulting mix into plug molds. Plugs were kept at 5°C for 15 minutes and then incubated at 37°C for one hour in tubes containing 1 ml EC-buffer. Subsequently, plugs were sliced in half and placed in tubes containing 250 μl TE-buffer (Trizma® HCl (Sigma T3253), EDTA (Titriplex II Merck 8417)) and 25 μl protein kinase K (5 mg/ml) (Roche Applied Science). Plugs were then incubated with fresh TE-buffer at 5°C for ten minutes and this washing step was repeated six times.

The bacterial DNA was digested with 20 units of smaI enzyme in 200 µl of the corresponding restriction enzyme buffer and separated by electrophoresis with the Gene Path system (Bio-Rad Laboratories, Sweden). Total runtime was 23 hours. The first block switch time was 5-15 seconds for 10 hours and the second block switch time was 15-60 seconds for 13 hours. The voltage for the run was 6 V/cm or 200 V. The included angle was 120°. The ramp factor was linear. After completed runtime, gels were stained in ethidiumbromide (1µg/ml) followed by destaining for one hour or more in 500 ml distilled water.

The digital comparison of profiles was made with the BioNumerics software (Version 6.6; Applied Maths, St-Martens-Latem, Belgium). Cluster analysis of Dice similarity indices based on the unweighted- pair group method using average linkages (UPGMA) was used to generate a dendrogram describing the relationships among PFGE profiles. Isolates were considered to belong to the same PFGE group if their Dice similarity index was $\geq 80\%$.

Carriage patterns of *S. aureus* strains on <u>lesional skin</u> were analyzed.

<u>Persistent carriage</u> was defined as *S. aureus*-positive with same strain at three visits or same strain at two visits and negative at remaining visit.

<u>Intermittent carriage</u> was defined as *S. aureus*-positive with low counts at one visit, negative at remaining visits.

<u>Non-carriage</u> was defined as *S. aureus*-negative at all three visits.

STATISTICS

All data were analyzed using R version 2.14.2 or previous versions (The R Foundation for Statistical Computing, Vienna, Austria) [166].

Paper I
The frequencies of microbes in the balanoposthitis group and in the control group were compared with Fisher's exact test. The same statistical instrument was used to compare prevalence of seborrhoeic dermatitis between groups. The significance level was $p < 0.05$.

Paper II
The statistical test used to compare frequencies of FRSA between groups was Fisher's exact test. The significance level was $p < 0.05$.

Paper III

No statistical calculations were used in the interpretation and presentation of the data.

Paper IV

Comparison of *S. aureus* density on lesional and uninvolved skin was made with Wilcoxon sign-rank test. Mean objective SCORAD index for persistent *S. aureus* carriers and non- or intermittent carriers was compared with Wilcoxon rank-sum test. Correlation between the density of *S. aureus* on lesional skin and objective SCORAD index was analysed with linear regression. The number of *S. aureus*-positive body sites was related to objective SCORAD index with generalized least square regression. The significance level was $p < 0.05$.

ETHICAL APPROVAL

Paper I, II and IV

The design of the studies presented in papers I, II and IV were all individually reviewed and approved by the Ethics Committee at the University of Gothenburg. Participants received oral and written information about the studies before their written consent was obtained.

Paper III

For paper III no ethical approval was required since the study did not involve patients, patient data or animals.

SUMMARY OF RESULTS AND DISCUSSION

PAPER I

A total of 102 patients with clinical signs of balanoposthitis and 26 control patients were examined. Investigation revealed that one patient from the balanoposthtitis group suffered from Zoon balanitis and another from HSV type 2. The data of these two patients are not included in the following since Zoon balanitis and viral aetiology were outside the scope of the study.

General microbial colonization

Overall, we found that microbial colonization was more frequent in the area of the prepuce and glans penis in patients with balanoposthitis than in the control group (59% and 35%, respectively, $p < 0.05$). *S. aureus* was found in 19 % of patients in the balanoposthitis group and in none of the control patients, $p < 0.05$. Results are summarized in Table II.

	Balanoposthitis group $n = 100$	Control group $n = 26$
S. aureus	19 (19%) *	-
Group B streptococci	9 (9%)	1 (3.8%)
Group C streptococci	3 (3%)	-
C. albicans	18 (18%)	2 (7.7%)
Malassezia species	23 (23%)	6 (23%)

Table II. Frequency of bacteria and yeasts in balanoposthitis and in the control group.
$*p < 0.05$
Modified from Alsterholm et al [154] (= paper I) with permission from the publisher.

C. albicans

We found no increase in *C. albicans* (or group B streptococci) in patients with balanoposthitis compared with controls (Table II, Table III). No other *Candida* species than *C. albicans* were detected.

	Balanoposthitis group *n* = 100	Control group *n* = 26
C. albicans		
Heavy growth	10 (10%)	-
Moderate growth	8 (8%)	2 (7.7%)

Table III. Quantification of growth of C. albicans in patients with balanoposthitis and in the control group.

Modified from Alsterholm et al [154] (= paper I) with permission from the publisher.

This result is in contrast with previous investigators, who have reported *C. albicans,* followed by group B streptococci, to be the most common microbe in balanoposthitis. Only two previous studies have included a control group for comparison and they report the prevalence of *C. albicans* in balanoposthtitis to 30-31% [51, 52]. Age range and rate of circumcision were similar in our study and the studies of others and cannot explain the difference.

In our study the growth of *C. albicans* was visually quantified. We found very discrete or discrete growth of *C. albicans* in 13 additional patients in the balanoposthitis group and in 2 additional patients in the control group (not displayed in Table III). We felt that the clinical importance of very discrete or discrete growth of *C. albicans* was uncertain, especially since colonization of the sulcus coronarius is frequent in asymptomatic men [68, 69]. Therefore we chose to present statistics based only on patients with moderate and heavy growth of *C. albicans* in paper II. Even if we include the patients with very discrete and discrete of *C. albicans* in our statistical analysis we do not reach a significant difference in the frequency of *C. albicans* between the balanoposthitis group and the control group (31% vs 15%, $p = 0.14$). Previous investigators do not describe or consider quantification of *C. albicans* and this could possibly lead to an overestimation of clinical significance.

Malassezia species

There was no difference in the frequency of *Malassezia* species between groups (Table II). Five different *Malassezia* species were found (Table IV).

	Balanoposthitis group n = 100			Control group n = 26		
	> 5 col.	2-5 col.	1 col.	> 5 col.	2-5 col.	1 col.
M. globosa	-	5	3	-	-	1
M. obtusa	1	4	2	-	-	1
M. sympodialis	-	-	3	-	-	2
M. obtusa + M. globosa	1	1	-	-	-	-
M. obtusa + M. sympodialis	-	1	-	-	-	-
M. obtusa + M. restricta	1	-	-	-	-	-
M. sympodialis + M. restricta	-	1	-	1	-	-
M. globosa + M. slooffiae	-	-	-	1	-	-

Table IV. Frequency, distribution and quantification of Malassezia species in the balanoposthitis and control groups.

For each *Malassezia* species quantification and number of patients are shown. When more than one *Malassezia* species was detected the combined number of colonies is presented.
Modified from Alsterholm et al [154] (= paper I) with permission from the publisher.

Observations of *Malassezia* species were too few to establish if the distribution of species differed between groups. The most commonly found species appeared to be *M. globosa* and *M. obtusa*. For the differentiation of *M. obtusa* and *M. sympodialis* growth at 38°C in a Dixon medium is used (Figure 4, page 51). During the study, this step proved to be unexpectedly sensitive to the general state of the medium (date of preparation, viscosity). Therefore, we cannot exclude that some of the isolates have been incorrectly identified as *M. obtusa* while they in fact were *M. sympodialis*. Assuming that we, due to these methodological issues, have underestimated the number of *M. sympodialis* isolates in favour of *M. obtusa*, we find that *M. globosa* and *M. sympodialis* might have been two of the most common isolates among our patients. This would be similar to the results of Mayser et al who described the frequency and spectrum of *Malassezia* species in the area of the prepuce and glans penis in healthy men, a majority of which were uncircumsized. The most common isolates in that study were *M. sympodialis* and *M. globosa* [49].

The lack of overrepresentation of *Malassezia* species in our study does not necessarily rule out a role for this yeast in the aetiology of balanoposthitis. The association between specific *Malassezia* species, the amount of those species on the skin and diseases like pityriasis versicolor and seborrhoeic dermatitis has proven to be complicated and incompletely understood [40]. For instance, patients with seborrhoeic dermatitis have been shown to harbour increased, decreased or unchanged levels of yeasts on lesional skin compared with uninvolved skin [47, 167]. It has been proposed that in those skin diseases where the association with *Malassezia* is clearly established, e. g. seborrhoeic dermatitis, the host immunological response to the yeast is of importance [168]. In seborrhoeic dermatitis, treatment to reduce *Malassezia* yields a parallel improvement of symptoms [169]. Suggested ways for *Malassezia* to induce symptoms are through precipitating antibodies against the yeast, production of toxin, lipase activity and activation of complement [170, 171].

S. aureus

In the balanoposthitis group 19% of patients exhibited *S. aureus* in the area of the prepuce and glans penis while no *S. aureus* were found in the control group ($p < 0.05$) (Table II).

An overrepresentation of *S. aureus* has not been described in any previous study on the microbial flora of balanoposthitis. In fact, *S. aureus* has rarely been detected at all. This is somewhat surprising since *S. aureus* is a very common skin pathogen as well as a commensal. In the general population, 22% of adults are colonized in the perineum, a number rising to 60% for those who are nasal carriers of *S. aureus* [12].

There are several possible explanations for the difference in *S. aureus* prevalence in our and other comparable studies [51-54].

- The definition of balanoposthitis in our study could differ from the definitions used by others and this could in turn influence the microbiological data. There are no universally acknowledged criteria for the diagnosis balanoposthitis, nor is there a scoring system to grade severity. Most patients attending our clinic present with mild to moderate balanoposthitis. We suggest that mild to moderate balanoposthitis in many cases is caused primarily by irritants such as excessive hygiene, frequent sexual intercourse or other dehydrating or abrasive trauma to the prepuce and glans penis. This association has been described by others [60]. Dehydrated or otherwise irritated skin could be vulnerable to colonization with microbes that can maintain or increase inflammation. A probable candidate for colonization would be *S. aureus*, explaining why we find a high percentage of this staphylococcus in our study.

- Individual patient factors, i.e. diabetes, obesity, personal hygiene customs and sexual habits were not described by us or others. Differences in such factors might explain why we find and increased rate of *S. aureus* (and no increase in *C. albicans*) in contrast with other studies. Another factor to consider in this context is climate. Our studie was performed in a relatively cold climate. The microbial panorama of balanoposthitis might be different in hot and humid climates.

- Another possible explanation for the increased isolation rate of *S. aureus* could be that the frequency and distribution of microbes in any population are not constant. Community patterns of antibiotic consumption, the use and composition of hygiene products as well as sexual habits change and can in turn alter the composition of the microbiome. The comparable studies of microbes in balanoposthitis were published in the years 1983-1995 and might represent a microbial milieu that has now evolved [51-54].

- The increased rate of *S. aureus* could also be due to a hypothetical higher rate of patients with atopy in our study compared to others. An association between balanoposthitis and AD has been suggested [60]. In retrospect, we regret not taking a patients' history regarding AD and other manifestations of atopy. Patients with AD are frequently colonized with *S. aureus* and the skin lesions of balanoposthitis and AD are similar. Balanoposthitis could in some cases be a manifestation of AD and *S. aureus* colonization would be expected.

Normal bacterial skin flora

41% of patients in the balanoposthitis group and 65% of patients in the control group exhibited no *S. aureus* or group B/C streptococci and no growth of *Candida* or *Malassezia* species. Cultures from these patients showed normal bacterial skin flora which was defined as one or several of alpha streptococci, CoNS, *Corynebacterium* species, enterococci, *E. coli*, gram-negative mixed flora or gram-positive mixed flora.

The association between clinical presentation and different microbes

Clinical findings were similar among patients in the balanoposthitis group, irrespective of microbe. Erythema of the prepuce and/or glans penis was the most common finding (83%) followed by papules (48%), transudation (30%) and white membranes (27%).

In this material there was no correlation between clinical findings and specific microbes. The clinical presentation of balanoposthitis did not predict the presence or absence of microbes or the type of microbe present in the preputial sac or on the glans penis. *Candida* infection of the skin is sometimes associated with pustules. It has been shown that the presence of pustules in balanoposthitis is highly suggestive of *Candida* [51]. This was not replicated in our study. In fact,

pustules were rare, present in only five patients, two of whom presented with *C. albicans*.

The presence of a particular microbe was not correlated with the number of clinical criteria noted in an individual patient, meaning it was not apparent that any of the microbes were associated with a more severe clinical picture (data not shown).

The association between balanoposthitis, seborrhoeic dermatitis and psoriasis

No association between balanoposthitis and seborrhoiec dermatitis or psoriasis could be demonstrated.

Patients with balanoposthitis reported a similar frequency of seborrhoeic dermatitis compared with controls, 29/100 patients (29%) and 9/26 patients (35%), respectively. Clinical findings of seborrhoeic dermatitis at time of visit were present in 13/29 (45%) of those patients with balanoposthitis who reported ever having seborrhoeic dermatitis and in 3/9 (33%) of those control patients who reported ever having seborrhoeic dermatitis.

Patients with balanoposthitis were subdivided based on the results of the cultures. The frequency of seborrhoeic dermatitis was not significantly higher in any of the subgroups (44% of patients with *Malassezia* vs 5.4% of patients with *C. albicans* vs 26% of patients with *S. aureus*, NS). The observed *Malassezia* isolates were too few to establish whether one or several species were associated with balanoposthitis and or seborrhoeic dermatitis.

There was no patient with active psoriasis in the study but one patient in the balanoposthits reported previous psoriatic skin lesions.

Conclusion

For the first time, the spectrum of bacteria, *Candida* and *Malassezia* species in patients with balanoposthitis and a control group is described. There was a significantly higher frequency of microbes in the area of the prepuce and glans penis in the balanoposthitis group compared to the control group. *S. aureus* was found in 19% of balanoposthitis patients but not at all in the control group. An increase in *C. albicans* in balanoposthitis, previously described by others, was not confirmed. Clinical findings did not predict presence of specific microbes. There was no association between balanoposthtitis and seborrhoeic dermatitis or psoriasis.

Our findings and the results of other investigators suggest that patients with mild to moderate balanoposthitis are vulnerable to colonization with microbes in the area of the prepuce and the glans penis. We propose that the condition reflects an increased susceptibility to irritants causing dermatitis and subsequent colonization with microbes. These microbes could increase and maintain pre-existing inflammation. The irritants could be excessive hygiene habits or repeated minor

abrasive trauma during sexual intercourse.

The primary therapeutic target in mild to moderate cases of balanoposthtitis should be to decrease inflammation with a mild topical corticosteroid (group I or II). The addition of antimicrobial agents to the topical preparation can reduce microbial load and thereby probably increase the anti-inflammatory effect. It is interesting that some azoles, for instance miconazole, in addition to their effect on yeasts are active against *S. aureus* and *S. epidermidis in vitro* (shown and discussed in paper III). In our clinic, tapered topical hydrocortisone-miconazole or triamcinolone-econazole application (twice daily for one week, once daily for one week and once every other day for one week) is generally effective. In severe cases of balanoposthitis systemic antibiotics are sometimes needed to resolve symptoms.

PAPER II

Bacterial spectrum in prospective study 2004-2008

Thirty-eight patients with impetigo and 37 patients with secondarily infected AD were included in the study.

S. aureus was by far the most common bacteria found in cultures from skin lesions in impetigo and AD (76-93%) (Table V, Table VI).

	Impetigo *n* = 25	Bullous impetigo *n* = 13
S. aureus	17 (68%)	11 (85%)
Methicillin-resistant *S. aureus* (MRSA)	1 (4.0%)	-
S. aureus + Group A streptococci	1 (4.0%)	1 (7.6%)
Coagulase negative staphylococci	2 (8.0%)	-
Bacillus cereus	1 (4.0%)	-
Normal skin flora	1 (4.0%)	-
Negative culture	2 (8.0%)	1 (7.6%

Table V. Prospective study 2004-2008. Frequency and distribution of bacteria in impetigo and bullous impetigo.

For each bacteria/group of bacteria the number of patients is presented.
Modified from Alsterholm et al [172] (= paper II) with permission from the publisher.

	Secondarily infected AD *n* = 37
S. aureus	28 (76%)
Methicillin-resistant *S. aureus* (MRSA)	1 (2.7%)
S. aureus + Group A streptococci	3 (8.1%)
S. aureus + Group C streptococci	1 (2.7%)
Coagulase negative staphylococci	2 (5.4%)
Negative culture	2 (5.4%)

Table VI. Prospective study 2004-2008. Frequency and distribution of bacteria in secondarily infected AD.

For each bacteria/group of bacteria the number of patients is presented.
Modified from Alsterholm et al [172] (= paper II) with permission from the publisher.

FRSA in prospective study 2004-2008

FRSA were a frequent finding in impetigo, particularly bullous impetigo, but less common in AD. Specifically, 75% of *S. aureus* isolates were fusidic-acid resistant in bullous impetigo, 32% in impetigo and 6.1% in secondarily infected AD. There was a statistically significant difference between groups (Figure 7).
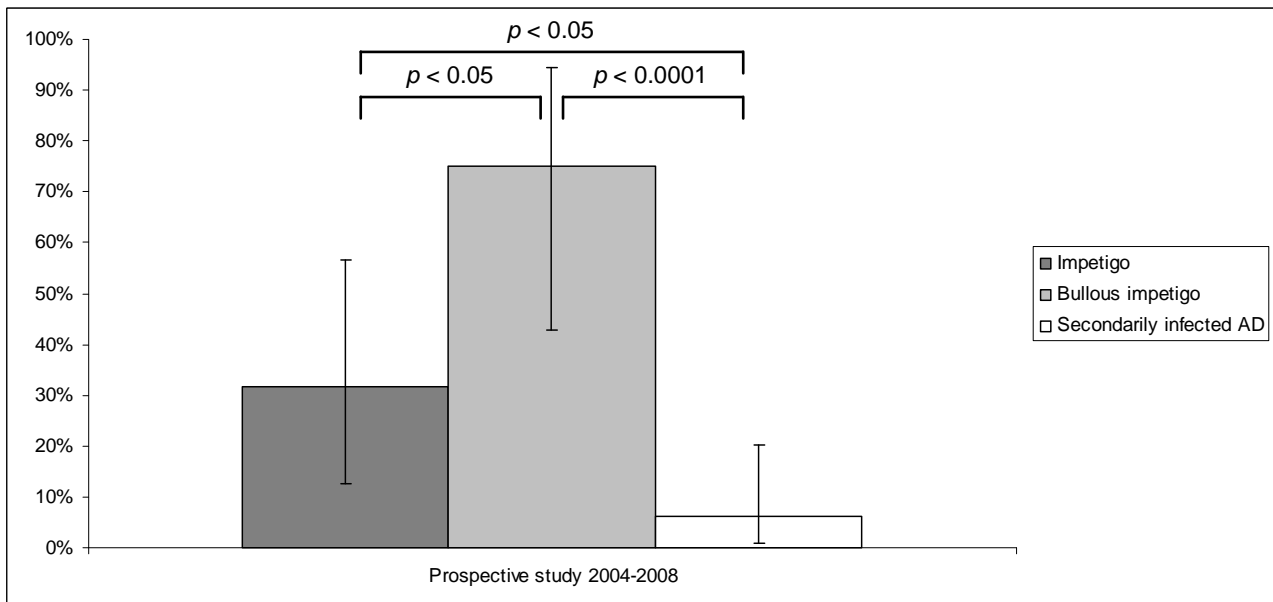
Figure 7. Prospective study 2004-2008. Frequency (%) of fusidic acid-resistance in S. aureus-isolates from patients with impetigo, bullous impetigo or secondarily infected AD.

Frequency of FRSA was compared between groups with Fisher's exact test. Values are presented with a 95% confidence interval. Significance level was $p < 0.05$.
From Alsterholm et al [172] (= paper II) with permission from the publisher.

Bacterial spectrum in the patient record review

S. aureus was by far the most common bacteria found in cultures from skin lesions in impetigo (76-88%) and AD (78-84%) (Table VII, Table VIII). Bacterial spectrum and frequency of *S. aureus* isolates were similar in the prospective study and the patient record review.

	Impetigo	
	June 2004-May 2005 *n* = 66	June 2007-May 2008 *n* = 33
S. aureus	45 (68%)	21 (64%)
Methicillin-resistant *S. aureus* (MRSA)	-	1 (3.0%)
S. aureus + Group A streptococci	2 (3.0%)	
S. aureus + Group B streptococci	5 (7.6%)	2 (6.1%)
S. aureus + Group G streptococci	-	1 (3.0%)
S. aureus + other bacteria	6 (9.1%)	-
Coagulase negative staphylococci	2 (3.0%)	2 (6.1%)
Group A streptococci	1 (1.5%)	1 (3.0%)
Bacillus cereus	1 (1.5%)	-
Other bacteria	-	2 (6.1%)
Negative culture	4 (6.1%)	3 (9.1%)

	Secondarily infected AD	
	June 2004-May 2005 *n* = 55	June 2007-May 2008 *n* = 55
S. aureus	30 (55%)	22 (40%)
Methicillin-resistant *S. aureus* (MRSA)	1 (1.8%)	1 (1.8%)
S. aureus + Group A streptococci	3 (5.5%)	6 (11%)
S. aureus + Group B streptococci	2 (3.6%)	8 (15%)
S. aureus + Group C streptococci	2 (3.6%)	1 (1.8%)
S. aureus + Group G streptococci	2 (3.6%)	5 (9.1%)
S. aureus + Group A & G streptococci	-	1 (1.8%)
S. aureus + other bacteria	3 (5.5%)	2 (3.6%)
Group G streptococci	-	1 (1.8%)
Coagulase negative staphylococci	5 (9.1%)	2 (3.6%)
Other bacteria	3 (5.5%)	3 (5.5%)
Negative culture	4 (7.3%)	3 (5.5%)

Table VII and VIII. Patient record review. Frequency and distribution of bacteria in impetigo (VII) and secondarily infected AD (VIII).

For each bacteria/group of bacteria the number of patients is presented.
Modified from Alsterholm et al [172] (= paper II) with permission from the publisher.

FRSA in the patient record review

In the time period June 2004 to May 2005 33% of *S. aureus* isolates found in impetigo were fusidic acid-resistant whereas the corresponding number for *S. aureus* isolates found in secondarily infected AD was 12%. In the time period June 2007 to May 2008 24% of *S. aureus* isolates found in impetigo were fusidic acid-resistant whereas the corresponding number for *S. aureus* isolates found in secondarily infected AD was 2.2% (Figure 8). These results were similar to those of the prospective study.
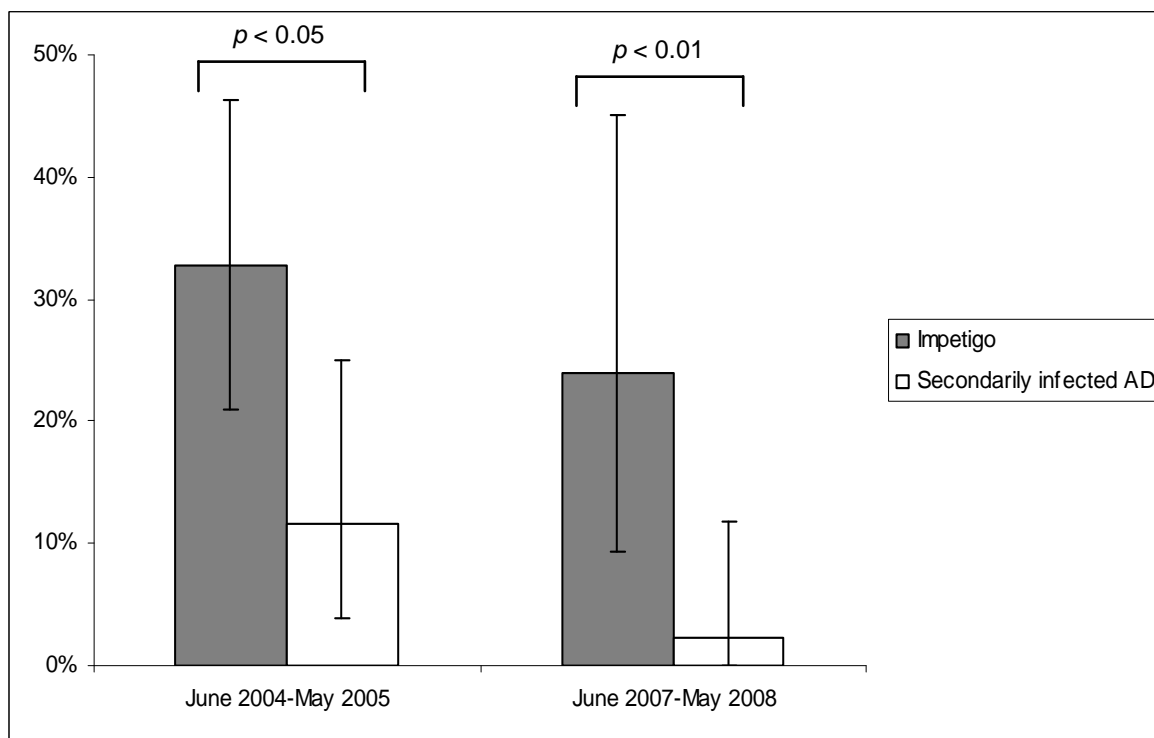
Figure 8. Patient record review. Frequency (%) of fusidic acid-resistance in S. aureus-isolates from patients with impetigo or secondarily infected AD.

Frequency of FRSA was compared between groups with Fisher's exact test. Values are presented with a 95% confidence interval. Significance level was $p < 0.05$.
From Alsterholm et al [172] (= paper II) with permission from the publication.

It is important to point out that the patients in this study represent a selected group of impetigo and AD patients in the sense that they have been referred to an outpatient dermatology clinic. Mild cases of impetigo and secondarily infected AD are often treated by general practitioners and other health care professionals. This means that the patients in our study do not necessarily represent the spectrum of impetigo and secondarily infected AD in the general population. For instance, bullous or extensive lesions could be a clinical presentation of impetigo suggesting differential diagnoses and could make a general practitioner more inclined to refer the patient to a dermatologist. While

we note that a similar study in a primary health care setting might have rendered another result, it appears unlikely that the frequencies of FRSA in our study are an underestimation of those in the general population.

In our clinical setting, the prospective study did not seem to reflect a biased selection of patients. The patient record review gave a similar result regarding age-distribution, bacterial spectrum and frequency of fusidic acid-resistance in *S. aureus* isolates.

S. aureus was, unsurprisingly, the most common finding in non-bullous impetigo, bullous impetigo and secondarily infected AD, constituting over 75% of isolates. Fusidic acid-resistant isolates of *S. aureus* were a frequent finding in bullous impetigo. This is consistent with the established association between FRSA, production of exfoliative toxin and bullous impetigo lesions [112]. However, FRSA were also cultured from lesions of clinically non-bullous impetigo and a small fraction of infected AD lesions. This demonstrates that FRSA infection is not always associated with blistering skin disease.

The patient record review indicated a decline in the proportion of FRSA isolates from 2004 to 2008 (from 33% to 24% for impetigo and from 12% to 2.2% for secondarily infected AD, NS). A statistically significant change in the same direction is documented in the well defined population of the Norwegian island community Austevoll where impetigo and FRSA incidence is continuously monitored. A gradual decrease in impetigo-derived *S. aureus* isolates resistant to fusidic acid was recorded from 2001 to 2009 [74].

National data on the proportion of FRSA among SSTI-derived *S. aureus* isolates from Norway and Sweden also show a decline. The Norwegian Surveillance System for Antimicrobial Resistance demonstrated that the proportion of FRSA from SSTIs was steadily falling from 25% in 2004 to 10% in 2008 [173]. Data from the Swedish surveillance system ResNet showed the same trend but on a lower scale [144]. Swedish FRSA levels peaked at 9.6% in 2002, declined to 3.8% in 2005 and stabilized at 4.0-6.0% in the years that followed.

Although FRSA levels seem to decline it is noteworthy that resistance against fusidic acid remains the most prevalent antibiotic resistance in the panel of antibiotics tested on *S. aureus* isolates reported to ResNet (page 35) [144].

In addition to investigating fusidic acid-resistance in *S. aureus* isolates derived from lesions of impetigo this study focused on the less investigated prevalence of FRSA in secondarily infected AD. The atopic skin is highly susceptible to colonization with *S. aureus*. We speculated that patients

with AD could be at risk of becoming carriers of fusidic acid-resistant clones of *S. aureus,* in turn serving as community reservoirs and increase further spread. With the high prevalence of both impetigo and AD in young children, FRSA could rapidly become widespread through daycare centers and schools.

In contrast to this proposed scenario, the results show a low frequency of fusidic acid-resistance among *S. aureus* isolates from patients with secondarily infected AD. Encouragingly, the frequency remained low during 2004-2008 and there was even indication of a decline. This was in contrast with the trend indicated by scattered European reports from the mid-90s and onward where FRSA were found in up to 50% of patients [136, 141-143]. One reason for the low rates of FRSA in AD in our study is probably the restrictive use of topical fusidic acid implemented in Sweden in 2003 [75, 138]. There could also have been differences between Sweden and other European countries in the pattern of prescription and use of fusidic acid prior to 2003. Increased use of topical fusidic acid in the community promotes the emergence of resistant strains. Conversely, limitations in use have been followed by declining FRSA levels [134-137].

Table IX illustrates topical fusidic acid sales and FRSA levels in Sweden in the years following our study. The comparatively low and stable frequency of fusidic acid- resistance among *S. aureus* isolates from SSTIs has been paralleled with diminishing national sales of fusidic acid cream and ointment.

	Sales of fusidic acid cream and ointment (number of 15 g packs*)	FRSA (% of total *S. aureus* isolates)
2008	58539	5.3
2009	53076	4.7
2010	50030	6.2
2011	44833	6.0
2012	40700**	4.8***

Table IX. Swedish sales of fusidic acid cream and ointment and FRSA reported to ResNet 2008-2012.

Sales data available on request from Apotekens Service: statistic@apotekensservice.se
* Fusidic acid cream and ointment are available in 15 g and 30 g packs, numbers have been recalculated to 15 g packs.
** Estimated value for entire year, report available for January to August (27248).
*** Report currently available from 12 out of 29 reporting laboratories.

It is difficult to determine to what extent a decline in total FRSA levels depends on restrictive use of topical fusidic acid and what is attributable to the normal dynamics of an epidemic. In the patient record review we found 101 cases of impetigo in the first year but only 53 cases in the last year. In similarity, there is data from Austevoll showing a decrease in the incidence of impetigo between 2001 and 2009. Given that impetigo remains the SSTI primarily associated with FRSA, fewer impetigo cases would also mean a decrease in the total number of FRSA isolates. While the incidence of impetigo and, as a consequence, total FRSA levels in Austevoll have faded, the percentage of FRSA isolates has remained high among impetigo cases. In 2008, 44% of *S. aureus* isolates from impetigo lesions were fusidic acid-resistant and 89% of those isolates belonged to the EEFIC [74].

One lesson we learned during the patient record review was that in many cases no bacterial cultures were secured from patients with impetigo or secondarily infected AD. The reason for this is probably that dermatologists are aware that in a vast majority of cases these conditions are caused by *S. aureus*. There is however another increasingly important reason for collecting swabs for bacterial cultures: monitoring of trends in antibiotic resistance. Sweden has excellent surveillance of antibiotic resistance on a national and regional level, comparatively low frequencies of multi-resistant bacteria in both outpatient and inpatient care and the authorities work actively to reduce non-evidence based prescription of antibiotics. To compliment this, clinicians need to be vigilant of emerging antibiotic resistance in bacterial strains from specified patient groups.

It appears widely accepted that long term treatment with topical antibiotics should be avoided as it can select for resistant strains [174]. For instance, topical mupirocin for SSTIs has promoted resistance in *S. aureus* and CA-MRSA [175, 176]. In contrast, short duration topical antibiotic therapy remains controversial [152]. Fusidic acid teaches us that widespread use of brief courses can promote resistance on a community level.

Conclusion

Management of impetigo and other SSTIs is possible without the use of topical fusidic acid formulations. Fusidic acid resistance remains a common form of antibiotic resistance among *S. aureus* isolates in Sweden. There is strong evidence that the community pattern of topical fusidic acid use influences the proportion of FRSA isolates cultured from patients with impetigo and other SSTIs. Fusidic acid is an antibiotic that, due to its unique mechanism of action, does not exhibit cross-resistance with β-lactam antibiotics. Fusidic acid can therefore be effective against MRSA and may become an increasingly important systemic antibiotic in the years to come [118]. Rapidly increasing antibiotic resistance is a global health threat and in view of that it seems wise not to waste fusidic acid. Indiscriminate use could potentially make this antibiotic worthless.

PAPER III

The *in vitro* antimicrobial properties of commonly used topical skin pharmaceuticals and their active substances, expressed as MIC-values against selected microbes were investigated with agar dilution assay. Table X provides an overview of the results to the help the reader follow the discussion. Tables XI-XV show the results in more detail.

Antibiotic formulation and substance

The MICs for Fucidin® 2% cream (fusidic acid) and fusidic acid are presented in Table XI. The strongest activity was, as predicted, seen against *S. aureus* and *S. epidermidis* (MIC 0.1 µg/ml for fusidic acid as part of the cream or alone and 5 µg/ml for the entire cream), but there was also an effect on *S. pyogenes* (MIC 10 µg/ml for fusidic acid as part of the cream, 6.25 µg/ml for fusidic acid alone and 500 µg /ml for the entire cream). Fusidic acid did not inhibit growth of *C. albicans* or *E. coli*.

Fusidic acid is primarily an anti-staphylococcal drug, but activity against streptococci has been described *in vitro*. In an evaluation of 242 strains of streptococci isolated from SSTIs, MIC levels ranged from 8 mg/l to 16 mg/l for fusidic acid [177]. In our study, MIC for fusidic acid as part of cream against *S. pyogenes* is equivalent to 10 mg/l and MIC for fusidic acid tested as active substance alone is equivalent to 6.3 mg/l. It is possible that fusidic acid can have a clinically relevant activity against streptococci when used as a topical preparation. In comparison, skin concentrations after twice daily *oral* administration of 500 mg fusidic acid are reported to be 39-79 mg/l which could explain a described clinical effect [178]. However, development of resistance against fusidic acid in *S. aureus*, argues against the use of topical fusidic acid formulations as discussed in paper II.

Antifungal formulations and substances

Clotrimazole as part of Canesten® 1% and miconazole nitrate, econazole nitrate and terbinafine hydrochloride were effective against *C. albicans* (MICs 3.125–25 µg/ml) (Table XII). Miconazole in Daktar® 2% and Daktacort® had a high MIC against *C. albicans* (1000 µg/ml for miconzole as part of the cream, 50,000 µg/ml for the entire cream) compared with miconazole nitrate alone (MIC 3.1 µg/ml). The same pattern was noted for terbinafine in Lamisil® 1% (MIC 2000 µg/ml for terbinafine as part of the cream, 200,000 µg/ml for the entire cream) and terbinafine hydrochloride alone (MIC 25 µg/ml).

	C. albicans	*E. coli*	*S. aureus*	*S. epidermidis*	*S. pyogenes*
Fucidin®	-	-	+++	+++	++
Fusidic acid	-	-	+++	+++	++
Canesten®	+	-	+++	+++	-
Daktar®	-	-	+++	+++	-
Daktacort®	-	-	+++	+++	-
Miconazole nitrate	++	ND	+++	+++	++
Lamisil®	-	-	-	-	++
Terbinafine HCl	++	ND	-	-	-
Econazole nitrate	+++	ND	+++	+++	++
Ketoconazole	++	ND	++	++	ND
Microcid®	-	++	+++	+++	++
Hydrokortison CCS®	-	-	-	-	-
Emovat®	-	-	-	-	-
Betnovat®	-	-	-	-	-
Dermovat®	-	-	-	-	-
Betnovat® with chinoform	++	++	++	++	+
Clioquinol	++	++	++	++	ND
Kenacutan®	++	++	++	++	NA

Table X. Overview of in vitro antimicrobial activity of topical skin pharmaceuticals.

Arbitrarily choosen ranges of activity: +++ MIC < 2 µg/ml (for active substance as part of cream or active substance alone), ++ MIC 2-50 µg/ml, + MIC > 50 µg/ml, - MIC > 1000 µg/ml. ND = not determinable. NA = not analyzed.

Clotrimazole, miconazole and econazole nitrate showed an effect against staphylococci (MIC 1.0 µg/ml for clotrimazole and miconazole as part of creams, MIC 1.6 µg/ml for miconazole nitrate alone and MIC 1.0 µg/ml for econazole nitrate alone).

Activity against *S. pyogenes* was variable. Lamisil[®] 1% cream had the strongest effect, with a MIC of 10 µg/ml for terbinafine as part of the cream while terbinafine hydrochloride alone did not have any effect.

There was no effect on *E. coli* for any of the antifungal formulations or substances.

No effect against *E. coli* was detected for the formulations Canesten[®], Daktar[®] or Daktacort[®]. The possible effect of the azoles against *E. coli* was not determined because at 50 µg/ml the concentration of the solvent DMF (used to dissolve the substances before added to the agar) is 5%. MIC for DMF against *E. coli* is 5%.

One aim of this investigation was to test whether base components of a formulation had additive or synergistic effects with the active substance of that formulation. Our data does not support the presence of such effects. MIC values for the active substance as part of a formulation and for the same substance tested separately were roughly the same (Table X). There were two exceptions to this, miconazole and terbinafine, respectively, which will be discussed in the following.

- The lack of acitivity of miconazole in Daktar[®] and Daktacort[®] (MIC 1000 µg/ml) against *C. albicans* while miconanzole nitrate alone appeared effective (MIC 3.1 µg/ml) was an unexpected finding. A possible explanation for this discrepancy could be interactions between cream and agar components inhibiting the effect of miconazole. It is also noted that glucocorticoids have been reported to inhibit the effect of some antimicrobials *in vitro* [179]. High concentrations of glucocorticoids have been shown to protect yeasts, but not staphylococci, *in vitro* against the action of econazole nitrate [180]. A glucocorticoid-azole interaction may explain the high MIC for miconazole in Daktacort[®], which is a combination of miconazole and hydrocortisone, but not the MIC of Daktar[®], which does not contain any glucocorticoid.

- Terbinafine as part of Lamisil[®] exhibited MIC 2000 µg/ml against *C. albicans*, while terbinafine hydrochloride alone had a MIC of 25 µg/ml. A possible explanation for this, as proposed for Daktar[®] and Daktacort[®], could be conditions in the *in vitro* situation, such as interactions between cream and agar components.

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Our results show an effect of azoles against staphylococci, both as part of formulations and as single substances. The members of the azole class of drugs are known to possess antibacterial properties against some gram-positive bacteria [181, 182]. Clotrimazole and econazole have been shown to be effective against *Mycobacterium smegmatis* and *Streptomyces* strains but not *E. coli in vitro* [183]. These bacteria contain P450 mono-oxygenases (P450s), some of which are homologues to 14α-sterol demethylase, the target enzyme of azoles. It can be speculated that the *in vitro* effect of azoles against *S. aureus* and *S. epidermidis* demonstrated in our study are mediated through 14α-sterol demethylase homologues in staphylococci.

	C. albicans		E. coli		S. aureus		S. epidermidis		S. pyogenes	
	ASPC/AS	EC	ASPC/AS	EC	ASPC/AS	EC	ASPC/AS	EC	ASPC/AS	EC
Fucidin® 2% (fusidic acid)	No effect	No effect	No effect	No effect	0.1	5.0	0.1	5.0	10	500
Fusidic acid	No effect	-	No effect	-	0.13	-	0.13	-	6.3	-

Table XI. Minimal inhibitory concentration (MIC) for antibiotic cream and substance. MIC expressed as µg/ml of active substance as part of cream (ASPC)[a], entire cream (EC) and active substance alone (AS).

[a] MIC of active substance as part of cream (ASPC) is calculated as the concentration of active substance in the cream multiplied by MIC for the entire cream. Modified from Alsterholm et al [184] (= paper III) with permission from the publisher.

	C. albicans		E. coli		S. aureus		S. epidermidis		S. pyogenes	
	ASPC/AS	EC	ASPC/AS	EC	ASPC/AS	EC	ASPC/AS	EC	ASPC/AS	EC
Canesten® 1% (clotrimazole)	10	1000	No effect	No effect	1.0	100	1.0	100	500	50000
Daktar® 2% (miconazole)	1000	50000	No effect	No effect	1.0	50	1.0	50	1000	50000
Daktacort® (miconazole)	1000	50000	4000	200000	1.0	50	1.0	50	1000	50000
Miconazole nitrate	3.1	-	-[b]	-	1.6	-	1.6	-	25	-
Lamisil® 1% (terbinafine)	2000	200000	No effect	No effect	No effect	No effect	2000	200000	10	1000
Terbinafine HCl	25	-	-[b]	-	No effect	-	No effect	-	No effect	-
Econazole nitrate	3.1	-	-[b]	-	1.0	-	1.0	-	25	-
Ketoconazole	3.1	-	-[b]	-	25	-	13	-	-[c]	-

Table XII. Minimal inhibitory concentration (MICs) for antifungal creams and substances. MIC expressed as µg/ml of active substance as part of cream (ASPC)[a], entire cream (EC) and active substance alone (AS).

[a] MIC of active substance as part of cream (ASPC) is calculated as the concentration of active substance in the cream multiplied by MIC for the entire cream. [b] MIC 50 µg/ml. At 50 µg/ml of miconazole nitrate, terbinafine HCL, econazole nitrate and ketoconazole there is 5% DMF in agar. MIC for DMF against *E.coli* is 5%. [c] MIC 50 µg/ml. At 50 µg/ml of ketoconazole there is 5% ethanol in agar. MIC for ethanol against *S. pyogenes* is 5%. Modified from Alsterholm et al [184] (= paper III) with permission from the publisher.

Corticosteroid formulations

The corticosteroid creams showed high MICs against all microbes. In some cases there was still growth of microbes on agar plates with 20% (w/v) of cream (Table XIII). This was not unexpected. Still it was important to explore any potential antimicrobial effects of preservatives or other additives in these formulations.

Corticosteroid formulations with antiseptic agent

Corticosteroid creams with antiseptic agents showed an effect on all microbes tested (Table XIV). Betnovat® with chinoform (betamethasone with clioquinol) had similar activity against all microbes with the exception of *S. pyogenes* (MIC 15 µg/ml against *C. albicans*, *E. coli*, *S. aureus* and *S. epidermidis* and MIC 150 µg/ml against *S. pyogenes*, for clioquinol as part of the cream). Clioquinol tested alone had comparable effects. At 50 µg/ml of clioquinol no growth of *S. pyogenes* was seen. However, at 50 µg/ml of clioquinol there was 5% ethanol in the agar and MIC for ethanol against *S. pyogenes* was 5%, thus the true effect of clioquinol against *S. pyogenes* was not determined.

Kenacutan® (triamcinolone with halquinol) was similarly effective against all microbes (MIC 7.5 µg/ml for halquinol as part of the cream). MIC of Kenacutan® against *S. pyogenes* was not determined because this cream was discontinued during the study.

Antiseptic formulation

The antiseptic cream Microcid® 1% (hydrogen peroxide) had effect primarily on *S. aureus* (MIC 1.0 µg/ml for hydrogen peroxide as part of the cream and 100 µg/ml for the entire cream) and *S. epidermidis* (MIC 0.5 µg/ml for hydrogen peroxide as part of the cream and 50 µg/ml for the entire cream) with weaker activity against *E. coli, S. pyogenes* and *C. albicans* (Table XV).

The corticosteroids with antiseptic agents and the hydrogen peroxide cream Microcid® were the formulations with the widest species-related range of activity (Table X). In constant effort to limit the use of antibiotics, non-resistance-promoting antiseptic formulations with activity against bacteria and yeasts can be very useful in the treatment of superficial skin infections and in secondarily infected skin diseases. Betnovat® with chinoform and Kenacutan® had MICs of a magnitude which are probably reached *in vivo*. It is unfortunate that Kenacutan® is no longer available.

Our results showed *in vitro* activity of Microcid® against the staphylococci but less so against *S. pyogenes* and *E. coli* and the effect was even weaker against *C. albicans*. The MIC for hydrogen peroxide as part of Microcid® cream was 500 µg/ml against *C. albicans*. This is equivalent to 0.05 g

cream/ml and it is conceivable that a concentration of that magnitude can be reached *in vivo* by applying a relatively modest amount of cream. This line of thought demonstrates the difficulty of interpreting and extrapolating *in vitro* data to the *in vivo* situation. Specifically, it is hard to transform a MIC-value obtained with the agar dilution assay into suggestions on the amount of cream needed to be applied in order to reach a similar concentration on the skin. The *in vitro* results of our study indicate that hydrogen peroxide preparations can be useful. The drawbacks of hydrogen peroxide are short time of action because of a high degradation rate and local irritation. Formulations need to stabilize as well as release hydrogen peroxide in order to be effective *in vivo* [150].

	C. albicans		E. coli		S. aureus		S. epidermidis		S. pyogenes	
	ASPC	EC	ASPC	EC	ASPC	EC	ASPC	EC	ASPC	EC
Betnovat® (betamethasone)	200	200000	No effect	No effect	No effect	No effect	No effect	No effect	100	100000
Emovat® 0.05% (clobetasone butyrate)	100	200000	100	200000	100	200000	50	100000	100	200000
Dermovat® 0.05% (clobetasol)	50	100000	100	200000	100	200000	100	200000	50	100000
Hydrokortison CCS® 1% (hydrocortisone)	2000	200000	No effect	No effect	No effect	No effect	2000	200000	500	50000

Table XIII. Minimal inhibitory concentrations (MICs) for corticosteroid creams. MIC expressed as µg/ml of active substance as part of cream (ASPC)[a]

and for the entire cream (EC).

[a] MIC of active substance as part of cream (ASPC) is calculated as the concentration of active substance in the cream multiplied by MIC for the entire cream.

Modified from Alsterholm et al [184] (= paper III) with permission from the publisher.

	C. albicans		E. coli		S. aureus		S. epidermidis		S. pyogenes	
	ASPC/AS	EC	ASPC/AS	EC	ASPC/AS	EC	ASPC/AS	EC	ASPC/AS	EC
Betnovat® with chinoform (clioquinol)	15	500	15	500	15	500	15	500	150	5000
Clioquinol	6.3	-	13	-	6.3	-	6.3	-	-[b]	-
Kenacutan® (halquinol)	7.5	1000	7.5	1000	7.5	1000	7.5	1000	-[c]	-[c]

Table XIV. Minimal inhibitory concentrations (MICs) for corticosteroid creams with antiseptic agent. MIC expressed as µg/ml of active substance as

part of cream (ASPC)[a] and for the entire cream (EC).

[a] MIC of active substance as part of cream (ASPC) is calculated as the concentration of active substance in the cream multiplied by MIC for the entire cream. [b] MIC 50 µg/ml. At 50 µg/ml of clioquinol there is 5% ethanol in agar. MIC for ethanol against *S. pyogenes* is 5%. [c] Not investigated because Kenacutan® was taken off the market during this work.

Modified from Alsterholm et al [184] (= paper III) with permission from the publisher.

	C. albicans		E. coli		S. aureus		S. epidermidis		S. pyogenes	
	ASPC	EC	ASPC	EC	ASPC	EC	ASPC	EC	ASPC	EC
Microcid® 1% (hydrogen peroxide)	500	50000	50	5000	1.0	100	0.50	50	50	500

Table XV. Minimal inhibitory concentration (MIC) for antiseptic cream. MIC expressed as µg/ml of active substance as part of cream (ASPC)[a] and for the entire cream (EC).

[a] MIC of active substance as part of cream (ASPC) is calculated as the concentration of active substance in the cream multiplied by MIC for the entire cream.

Modified from Alsterholm et al [184] (= paper III) with permission from the publisher.

Benefits and hazards with antiseptics

The results of the study highlight the potential usefulness of azoles and antiseptic preparations. While skin bacteria, especially *S. aureus*, have developed resistance to various antibiotics, the topical azoles and allylamines used against dermatophytes and yeast infections have been prescribed extensively with less development of clinically relevant resistance among fungi [185, 186]. Therefore it was interesting to explore any antibacterial effects of antifungals. We find that the azoles are effective against *S. aureus* and *S. epidermidis in vitro*. If this activity is present *in vivo* as well it could be beneficial in the treatment of mixed superficial skin infections.

To be able to replace or complement antibiotics with topical antiseptics is an appealing concept. In contrast with the targeted action of antibiotics, antiseptics are not as pharmacologically precise and act on multiple cellular targets making it harder for microbes to evade their damaging effects. It can be argued that increased proper use of antiseptics for localized superficial skin infections could decrease the use of antibiotics, thus minimizing selective pressure on bacterial strains.

There is however concern that the use of biocides in health care, households and in industrial settings can promote resistance to antiseptics and contributes to the selection of antibiotic-resistant strains [146]. For instance, increased antiseptic MICs, due to the presence of the *qacAB* family of genes encoding multi-drug efflux pumps, have been described for MRSA strains [187]. These efflux pumps can confer resistance to chlorhexidine and other antiseptics. The *qacAB* family of genes is present predominantly on multi-resistance plasmids, and has been found in methicillin-sensitive *S. aureus* and CoNS as well. The significance of this with regards to clinical effect (e.g. hand disinfectants) and the ecological consequences (cross-resistance with antibiotics and selection for resistant strains) remains to be discovered.

Another important consideration is the risk of allergic reactions to antiseptics. For instance, chlorhexidine can provoke life-threatening anaphylactic reactions, even after application on unbroken skin [147-149].

Conclusion

Microbes are believed to trigger, exacerbate and/or sustain inflammatory skin disease, such as AD (discussed in paper IV), balanoposthitis and seborrhoeic dermatitis (discussed in paper I). Treatment of these and other skin diseases can require intermittent use of antibiotics and antifungals in addition to anti-inflammatory agents. We are facing a global rapid dissemination of multi-resistant bacteria. Therefore, dermatologists and all other health care professionals need to carefully consider every prescription of antibiotics and make us of antiseptic alternatives including the antimicrobial potential of already existing topical skin pharmaceuticals.

This study highlights the broad *in vitro* antimicrobial effect of the currently available clioquinol and

hydrogen peroxide formulations and of the discontinued halquinol formulation. The azole class of antifungal formulations have an anti-staphylococcal effect.

The development of new antiseptic and antibiotic topical preparations is an important task for the future. Responsible use of existing and future formulations is essential for the successful management of skin infections.

PAPER IV

The variations in *S. aureus* colonization (measured with qualitative and quantitative culture and PFGE) and disease severity (measured with objective SCORAD index) were described over a 5-month period in twenty adult patients with moderate to severe AD.

At total of 21 patients (eleven men and ten women) participated in the study. Nineteen patients attended all three visits. Patient number 10 attended the first visit but declined further participation. Patient number 12 was unable to attend the second visit due to a traumatic elbow injury but attended the first and third visit.

The objective SCORAD index showed that 19 patients (90%) exhibited moderate AD at one or more visits, for some fluctuating to mild (6/19) or severe (8/19). One patient had consistently mild AD and one patient had consistently severe AD during follow-up (Table I*).

S. aureus colonization rate on lesional and uninvolved skin

The proportion of patients colonized with *S. aureus* at least once during follow-up was 71% for lesional skin and 90% for uninvolved skin (Table II).

S. aureus density on lesional and uninvolved skin

The mean *S. aureus* density for the patients was higher on lesional skin than on uninvolved skin at all visits ($p = 0.04$, $p = 0.02$ and $p = 0.02$ for visits 1, 2 and 3, respectively) (Table II, Figure 1). Higher density of *S. aureus* on lesional skin corresponded to more severe disease, measured as objective SCORAD index (visit 1, $p = 0.0003$, $R^2 = 0.49$; visit 2, $p = 0.0003$, $R^2 = 0.52$; visit 3, $p = 0.004$, $R^2 = 0.35$) (Figure 2).

Number of body sites colonized with *S. aureus*

Five different body sites were sampled for *S. aureus* at each visit; lesional skin, uninvolved skin, the anterior nares, tonsils and perineum. The number of body sites colonized with *S. aureus* was positively correlated with the objective SCORAD index ($p = 0.007$, $R^2 = 0.46$) (Figure 3). Statistical analysis was made with generalized least square regression for all observations from all visits and with linear regression for all observations from each visit separately (data not shown) with similar result.

*Paper IV is currently in manuscript. For tables and figures the reader is referred to the manuscript.

Variations in carriage of *S. aureus* strain

PFGE was performed on all *S. aureus* isolates from lesional skin, the anterior nares and perineum (*n* = 94) and a majority of isolates from uninvolved skin (*n* = 25). Low counts of *S. aureus* on uninvolved skin in the absence of *S. aureus* on lesional skin were not typed.

There were few intra-individual changes in carriage of strain during follow-up. Eleven patients (11/20, 55%) were persistent carriers of a specific strain on lesional skin. Persistent carriage was defined as same *S. aureus* strain at three visits *or* same strain at two visits and *S. aureus*-negative at remaining visit. Seven patients (7/20, 35%) were non-carriers of *S. aureus* on lesional skin (5/7) or intermittent carriers (2/7). Non-carriage was defined as *S. aureus*-negative on lesional skin at all three visits. Intermittent carriage was defined as *S. aureus*-positive on lesional skin with low counts at one visit, negative at remaining visits (Table IV). The mean objective SCORAD index was significantly higher among persistent carriers than among non- and intermittent carriers (38.0 vs 17.2, *p* = 0.002) (Figure 4).

In order to understand the clinical influence of *S. aureus* in AD it is important to investigate the temporal variations in colonization in relation to disease severity. Flare-ups of AD are often associated with *S. aureus* super-infection and one treatment strategy is the use of topical antimicrobial agents and oral antibiotics [92]. Despite the strong link between flare-ups of AD and *S. aureus* super-infection, the importance of *S. aureus* colonization in AD pathogenesis and the impact of colonization in patients with clinically non-infected AD remain controversial.

AD is not just a disease affecting children and teenagers but often persists in later life why research should focus on adults as well [188]. The temporal shift in *S. aureus* colonization in relation to the severity of AD has, to our knowledge, rarely been described in adults.

The patients participating in this study were adults with moderate to severe AD which they felt was satisfactorily managed with emollients and topical corticosteroids. They were not treated with topical or oral antimicrobials, phototherapy or oral immunomodulators during follow-up. Phototherapy was excluded because UV-light affects the microbial flora on atopic skin, including *S. aureus*, and decreases disease severity [97-99]. We chose to conduct the study during the first six months of the year (January to mid-June 2011) when the climate in Sweden offers little outdoor living and UV-exposure. The UV-exposure that can occur in late spring and early summer did not affect mean objective SCORAD index or mean number of *S. aureus* isolates (no statistically significant difference between visit 1, 2 and 3, data not shown).

Patients treated with immunomodulating drugs (e.g. cyclosporine or oral corticosteroids) were not

included since immunomodulation might influence the composition of the skin microbiome, potentially complicating the interpretation of data.

The data in this study represents the normal variations in disease severity and life circumstances for adult patients with moderate to severe AD. There were only two flare-ups of AD during follow-up. Those flare-ups required anti-staphylococcal antibiotic treatment (patient number 17 and patient number 21). In addition, there was a one antibiotic treatment for a post-traumatic soft tissue infection and one short term prophylactic treatment after minor skin surgery (patient number 12 and patient number18). Two of the patients were colonized with a new *S. aureus* strain on lesional skin after antibiotic treatment (patient number 18 and patient number 21).

The patients in this study were colonized with *S. aureus* on lesional skin, uninvolved skin, in the anterior nares, tonsils and perineum to a much higher extent than the general population. In the general population, *S. aureus* carriage rates on different body sites are highly variable and influenced by nasal carriage status. In nasal non-carriers the rate of colonization on different body sites averages 15% with the hands, forearm and the perineum being the most common site of carriage. In persistent nasal carriers the rate of colonization averages 40% with colonization of the hands in 90%, the forearm in 45% and the perineum in 60% of cases [12]. The colonization rates on different body sites among the patients in this study were similar to those of persistent nasal carriers in the general population even though 24% (5/21) of patients were non-carriers in the anterior nares during follow-up (Table III, Table IV).

It is well known that nasal colonization is a risk factor for staphylococcal skin infection [12]. We propose that nasal colonization is a risk factor for colonization/recolonization of atopic skin as well and this has been indicated by others [189]. It is also possible that atopic skin can serve as a significant reservoir for *S. aureus*, equally or more important than the anterior nares, with colonization of the skin influencing the colonization of the anterior nares rather than the reverse. An early study of colonization and infection with *S. aureus* in AD found nasal carriage in 84% of patients, comparable to 53-67% in this study [190]. Interestingly, five patients in our study (5/21, 24%) carried one *S. aureus* strain in the anterior nares and another strain on lesional skin at one or several visits, demonstrating that nasal carriage does not always dictate which strain is carried on the skin.

Persistent carriers of *S. aureus* on lesional skin had significantly higher mean objective SCORAD index than non-/intermittent carriers. This data support previous findings that *S. aureus* carriage on

lesional skin is associated with more severe AD [93, 191-193]. In addition, linear regression analysis showed that patients with more severe AD had higher density of *S. aureus* on lesional skin and carried *S. aureus* on multiple body sites.

This study shows that the patients with the most severe AD were persistent carriers of the same strain on lesional skin during follow-up. They were also carriers of the same strain at multiple other body sites. It has been proposed that colonization with a new *S. aureus* strain could provoke an exacerbation of AD by triggering the immune system [191]. Atopic skin obviously provides a beneficial environment for *S. aureus* and the concept of frequent recolonization with a new strain is not inconceivable. The results of this study (although follow-up was short) do not support colonization with a new *S. aureus* strain as a prominent trigger of disease activity. In fact, even though there were large intra-individual fluctuations in SCORAD indices, a change of *S. aureus* strain was a rare event for any of the participants. The results indicate that variations in AD intensity, at least in the absence of acute flare-ups and antibiotic treatment, are associated with a change in *S. aureus* density on lesional skin but not necessarily with a change of strain.

Is *S. aureus* a primary trigger of dermatitis or is colonization a secondary phenomenon due to the fact that the altered skin barrier in AD provides a beneficial environment for *S. aureus*? The question remains controversial. Wherever truth lies, the abundance of virulence factors associated with *S. aureus* supports a role for this fascinating microbe as a driving force in the skin inflammatory response. Reducing the amount of *S. aureus* on the skin could very well be beneficial for AD severity although this is still not conclusively demonstrated in a large randomized clinical trial. It is important to consider that anti-staphylococcal treatment might be effective for patients who are persistent *S. aureus* carriers but less so for intermittent carriers. We believe that persistent carriers need to be identified in the inclusion process of future clinical trials. There is a possibility that AD patients who are persistent carriers of one *S. aureus* strain in high numbers and on multiple sites could benefit from anti-staphylococcal treatment in the absence of clinical infection. Changes in the skin microbiome before, during and after disease flares have recently been described in children with AD using 16S ribosomal RNA bacterial gene sequencing [194]. The authors show that microbial diversity on lesional skin was dramatically reduced during flare-ups but changed towards higher diversity post-treatment. Flare-ups were characterized by a build-up to high numbers of one species, typically staphylococci, while an increasing proportion of *Streptococcus*, *Propionibacterium, Corynebacterium* and other species were observed following therapy. We suggest that intermittent treatment with topical antimicrobials could prevent the domination of *S. aureus* and promote microbial diversity on the skin. This could potentially reduce overall disease

severity and number of flare-ups for those AD patients who are persistent *S. aureus* carriers on lesional skin. Preferably, antiseptics should be used since it is important not to further increase the use of topical or oral antibiotics. As with all cutaneous application the risk for allergic reactions (contact dermatitis and anaphylaxis) has to be considered when designing new treatment strategies. If intermittent use of antiseptics proves effective it could be an antibiotic-sparing treatment strategy. This area of research warrants further exploration.

Conclusion

We describe the temporal shift in *S. aureus* density and strain distribution along with changes in disease severity in adult patients with AD. Colonization with *S. aureus* on lesional skin is associated with more severe disease. Even in the absence of acute flare-ups the objective SCORAD index varies greatly. In our study, these variations occur without a shift in strain but with concomitant changes in *S. aureus* density on lesional skin. The data implies that intermittent anti-staphylococcal treatment could be beneficial for persistent *S. aureus* carriers by reducing bacterial density, breaking the reign of a single *S. aureus* strain and thereby promote a more diverse microbiome. Clinical trials investigating the effects of anti-staphylococcal treatment in AD patients persistently colonized with *S. aureus* are needed.

CONCLUSION OF PAPER I-IV

Patients with balanoposthtitis have a significantly higher frequency of microbes in the area of the prepuce and glans penis compared to healthy controls. *S. aureus* was found in 19% of balanoposthitis patients but not at all in the control group. An increase in *C. albicans* in balanoposthtitis, previously described by others, was not confirmed. Clinical findings did not predict presence of specific microbes. There was no association between balanoposthtitis and seborrhoeic dermatitis or psoriasis. The primary therapeutic target in mild to moderate cases of balanoposthtitis without overt signs of infection should be to decrease inflammation with a mild topical corticosteroid (group I or II). The addition of antimicrobial agents to the topical preparation can reduce microbial load and further help to decrease inflammation.

FRSA was a common cause of impetigo, especially bullous impetigo, in the period studied. It was a relatively rare finding in secondarily infected AD. Rates of FRSA isolation from impetigo and secondarily infected AD did not increase in the period studied. There is strong evidence that the community pattern of topical fusidic acid use influences the proportion of FRSA isolates cultured from patients. Management of impetigo and other SSTIs is possible without topical fusidic acid formulations and use should be avoided.

Topical skin pharmaceuticals with clioquinol, halquinol and hydrogen peroxide have a broad antimicrobial effect *in vitro*. The azole class of topical antifungal formulations have an anti-staphylococcal effect *in vitro*. These effects, when present *in vivo*, should be used when appropriate to avoid unnecessary antibiotic treatment of limited superficial skin infections. In view of increasing antibiotic resistance the development of new antiseptic and antibiotic topical preparations is an important task for the future. Responsible use of existing and future formulations is essential for the successful management of skin infections.

In adults with AD, variations in disease severity were positively correlated with *S. aureus* density on lesional skin, number of body sites colonized and persistent carriage of the same *S. aureus* strain. The data suggest that patients with this pattern of *S.aureus* colonization might benefit from anti-staphyloccocal interventions even in the absence of signs of infection.

FUTURE PERSPECTIVES

Our current practice is to treat mild to moderate balanoposthitis with a topical hydrocortisone-miconazole combination. It would be interesting to test this treatment against hydrocortisone alone and miconazole alone in a double blind study. Results could determine if anti-inflammatory interventions, anti-microbial interventions or a combinantion of both are the most effective.

There is a continuous discussion regarding the transmission of *Candida* between partners in heterosexual relationships. Evidence to support male to female sexual transmission of *Candida* as a clinically significant cause of recurrent vulvovaginal candididas is lacking [195-197]. At the same time, the 2008 U.K. National Guidelines on the Management of Balanoposthtitis state that there is a high rate of candidal infection in sexual partners of patients with *Candida* balanoposthitis and that these partners should be offered screening [71]. The fact that transmission of *Candida* species from men to women seem to be of limited clinical importance for recurrent vulvovaginal candidiasis does not necessarily mean that vulvovaginal candidiasis will not increase the risk for balanoposthitis. We have found no studies describing the prevalence of *Candida* balanoposhtitis in partners of women with recurring vulvovaginal candidiasis. A prospective study of men in monogamous relationships with women suffering from recurrent vulvovaginal candidiasis could help shed light on possible effects for these men.

To complement the yearly FRSA-surveillance from ResNet it would be interesting to perform a new culture-based study at the Department of Dermatology at Sahlgrenska University Hospital. A suggested design would be to prospectively collect cultures from all patients with impetigo and AD (this time including clinically non-infected AD as well) during three months.

The impact of *S. aureus* colonization on AD deserves further investigation. We plan to analyze for virulence factors with polymerase chain reaction in the isolates which have already been collected and typed for strain specificity.

An exciting prospect would be a late follow-up of the patients described in paper IV. What shifts occur in the characteristics of *S. aureus* colonization and AD severity over longer periods of time? Ultimately, a randomized controlled trial investigating the effect of an anti-staphylococcal intervention on AD severity in patients with significant and persistent *S. aureus* carriage could prove rewarding.

KOLONISATION OCH INFEKTION MED STAFYLOKOCKER OCH ANDRA MIKROORGANISMERS VID HUDSJUKDOM

Introduktion

Mikroorganismer såsom bakterier och svampar koloniserar ständigt vår hud. Kolonisation innebär att mikroorganismer lever på huden utan att orsaka skada. De utgör dessutom ett skydd genom att hindra sjukdomsframkallande bakterier och svampar från att få fäste och ge upphov till en infektion. Koloniserande mikroorganismer tros kunna underhålla och försämra hudsjukdomar som uppstått av annat skäl.

Bakterien *Staphylococcus aureus* (*S. aureus*) kan kolonisera huden men även ge upphov till hudinfektion. Studierna som ingår i den här avhandlingen har undersökt förekomst och betydelse av *S. aureus* och andra mikroorganismer vid hudsjukdom.

Artikel I

Den första studien har undersökt förekomsten av mikroorganismer på ollon och förhud vid balanit, en eksemliknande reaktion på penis som drabbar de flesta män. Överdrivet tvättande och skav vid samlag är vanliga orsaker. Tidigare studier har visat att infektion med svampen *Candida albicans* (*C. albicans*) kan vara en orsak. Studien som presenteras i avhandlingen visar att mikroorganismer är vanligare på ollon och förhud hos patienter med balanit än hos en frisk kontrollgrupp. Framförallt förekom *S. aureus* ofta (19%) vid balanit men inte alls hos friska. Svamp var inte vanligare vid balanit i denna studie.

Slutsats: Patienter med balanit är koloniserade med mikroorganismer, framförallt *S. aureus*, oftare än friska och det kan underhålla och förstärka eksemreaktionen på ollon och förhud. Behandlingen bör inriktas på att minska eksemreaktionen och mängden mikroorganismer med en mild kortisonkräm med bakterie- och svampdödande tillsats.

Artikel II

Den andra studien har undersökt vilka bakterier som växer på huden vid hudinfektionen impetigo (svinkoppor) och vid infekterat eksem. *S. aureus* var den vanligaste bakterien och hittades i 76-93% av fallen. De *S. aureus* som hittades var i varierande utsträckning motståndskraftiga mot antibiotikakräm innehållande fusidin som tidigare använts mycket vid behandling av impetigo. 75% av *S. aureus* var motståndskraftiga mot fusidin vid blåsbildande impetigo, 32 % vid icke-blåsbildande impetigo och 6.1% vid infekterat eksem.

Slutsats: *S. aureus* motståndskraftiga mot fusidin är en vanlig orsak till impetigo men är relativt ovanliga vid infekterat eksem. Användning av fusidinkräm kan ge ökning av motståndskraftiga *S. aureus* och bör undvikas.

Artikel III

Den tredje studien har undersökt den bakterie- och svampdödande effekten hos vanligt förekommande hudläkemedel (krämer). Krämerna har testats genom att gjutas in i odlingsplattor där olika mikroorganismer som kan finnas på huden fått växa. Krämer som innehöll substanserna clioquinol, halquinol och väteperoxid var effektiva mot många olika mikroorganismer. Azoler, som är svampläkemedel, hade dessutom effekt mot bakterier av typen stafylokocker, t ex *S. aureus*.

Slutsats: Den bakterie- och svampdödande effekten hos nu tillgängliga läkemedel bör utnyttjas. Nya läkemedel i krämform som inte främjar utveckling av motståndskraft hos mikroorganismer behövs för att i framtiden behandla hudinfektioner.

Artikel IV

Den fjärde studien har undersökt hur kolonisation med *S. aureus* på huden och andra ställen på kroppen varierar över tid hos vuxna patienter med eksem. Samtidigt har svårighetsgraden av eksemet mätts. De patienter som hade svårt eksem bar oftare *S. aureus* på den eksemdrabbade huden och hade tätare växt av *S. aureus* än patienter med lindrigare eksem. De bar också oftare *S. aureus* på frisk hud, i näsan och svalget.

Slutsats: Det är viktigt att fortsätta studera samspelet mellan *S. aureus* kolonisation och svårighetsgrad av eksem för att avgöra det eventuella värdet av att behandla ner mängden *S. aureus* hos patienter med eksem.

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