Department of Dermatology, Allergology and Venereology
Helsinki University Hospital and
University of Helsinki

THE SKIN MICROBIOME

INVESTIGATIONS ON SKIN MALIGNANCIES AND PRETERM NEWBORN SKIN

Alexander Salava

ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Medicine of the University of Helsinki for public examination at the Skin and Allergy Hospital Auditorium, Meilahdentie 2, Helsinki, on the 6th of April 2018 at 12 o'clock noon.

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Supervised by:
Professor Antti Lauerma, M.D., Ph.D.
Department of Dermatology and Allergology
University Hospital Helsinki, Finland

Reviewed by:
Docent Sirkku Peltonen, M.D., Ph.D.
Department of Dermatology
University of Turku, Finland
and
Docent Jenni Hultman, Ph.D.
Department of Microbiology
Faculty of Agriculture and Forestry
University of Helsinki, Finland

Opponent:
Professor Tilo Biedermann, M.D., Ph.D.
Department of Dermatology and Allergology
Technical University of Munich, Germany

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Geheimnisvoll am lichten Tag
Läßt sich Natur des Schleiers nicht berauben,
Und was sie deinem Geist nicht offenbaren mag,
Das zwingst du ihr nicht ab mit Hebeln und mit Schrauben.

Johann Wolfgang von Goethe:
Faust - Der Tragödie erster Teil. 1808, Verse 672-675.

To Margarita, Julia and Victoria

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LIST OF ORIGINAL PUBLICATIONS

This doctoral thesis is based on the following publications that are referred to in the text by their Roman numerals.

I. Skin microbiome in melanomas and melanocytic nevi
Salava A, Aho V, Pereira P, Koskinen K, Paulin L, Auvinen P, Lauerma A. *European Journal of Dermatology* 2016; 26(1): 49-55.

II. Skin microbiome in small- and large-plaque parapsoriasis
Salava A, Pereira P, Aho V, Väkevä L, Paulin L, Auvinen P, Ranki A, Lauerma A. *Acta Dermato-Venereologica* 2017; 9;97(6): 685-691.

III. Loss of cutaneous microbial diversity during first three weeks of life in very low birthweight infants.
Salava A, Aho V, Lybeck E, Pereira P, Paulin L, Nupponen I, Ranki A, Auvinen P, Andersson S, Lauerma A. *Experimental Dermatology* 2017; 26(10): 861-867.

ABBREVIATIONS

CRP	C-reactive protein
CTCL	Cutaneous T-cell lymphoma
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
HF	High fidelity
IL	Interleukin
LPP	Large plaque parapsoriasis
NICU	Neonatal intensive care unit
NMDS	Non-metric multidimensional scaling
NS	Neonatal sepsis
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
RDP	Ribosomal Database Project
RNA	Ribonucleic Acid
SALT	Skin associated lymphoid tissue
SEA	Staphylococcal enterotoxin A
SOP	Standard Operating Procedures
SPP	Small plaque parapsoriasis
STAT	Signal transducer and activator of transcription
UVA	Ultraviolet A
VLBW	Very low birth weight

ABSTRACT

Background:
Skin disorders have been associated with specific microbiome changes and raised an interest in developing new diagnostic methods and treatments.

Objectives:
To investigate the microbiome in skin cancer (melanoma, study **I**) and inflammatory skin disorders (parapsoriasis, study **II**), to explore microbiome swab sampling as a non-invasive diagnostic method, and to investigate the skin microbiome in very low birth weight infants in intensive care and possible association of skin staphylococci and neonatal sepsis (study **III**).

Methods:
Microbiome samples were taken of 15 cutaneous melanomas and 17 benign melanocytic nevi (study **I**), of parapsoriasis lesional skin and contralateral healthy skin in 13 patients (study **II**) and in 12 very low birth weight infants during treatment in intensive care (study **III**).

Sequencing was carried out on 454 GS-FLX Titanium (study **I**) and Illumina MiSeq (studies **II**-**III**) platforms and the data was analysed by bioinformatics. In studies **I**-**II** alpha diversity indices were calculated with bioinformatic tools. In study **II** the staphylococcus sequences were investigated by oligotyping.

Results:
In study **I** there were no significant differences in the microbiome of melanomas, melanocytic nevi and controls. A notable interpersonal variation was observed. No significant differences in bacterial diversity could be detected.

In study **II** the microbiome showed no significant differences between parapsoriasis and the same patient's healthy looking skin. No differences could be demonstrated between small and large plaque parapsoriasis. Based on oligotyping *Staphylococcus aureus* was not identified in parapsoriasis skin.

In study **III** a high cutaneous microbial diversity was observed in most infants at birth. There was a decrease in diversity during the first three weeks of life in both septic and non-septic infants. In 50 % of the infants the microbial diversity recovered. There was no association between microbiome changes and sepsis.

Conclusions:

Study **I** was about the microbiome in melanomas and melanocytic nevi. There was a substantial variation between body sites and individual patients. The results suggest that microbiome swab sampling may not be helpful in diagnostics of melanoma or melanocytic nevi. Limitations were a small and heterogeneous patient group and possible bias in sampling and analysis.

Study **II** was about the skin microbiome in parapsoriasis. No significant differences could be demonstrated between parapsoriasis and healthy skin. Recently it has been shown that Staphylococcal enterotoxin A from the affected skin of cutaneous lymphoma patients induced in vitro IL-17 production in primary malignant T-cells of Sezary syndrome patients when cocultured with autologous nonmalignant T-cells. Parapsoriasis is known to precede mycosis fungoides, the most common form of cutaneous T-cell lymphoma. The role of staphylococci has, to our knowledge, not been investigated in parapsoriasis. We could not identify *S. aureus* in parapsoriasis lesions. This suggests that *S. aureus* and staphylococcal enterotoxins do not to play a role in parapsoriasis. Specific differences between cutaneous lymphoma and parapsoriasis or metabolomics may explain the observations. Limitations were a small patient group and notable interpersonal variations.

Study **III** was about the skin microbiome in very low birth weight infants. A high microbial diversity was present during the first days of life regardless of the way of delivery, prematurity causes or perinatal infections. The diversity decreased during the first weeks of life possibly due to intensive care treatment and antibiotics.

Neonatal sepsis showed no time-based link with the decrease of diversity and shifting into a staphylococci dominated microbiome. It is likely that other factors play a role, e.g. skin injury by medical devices and concomitant infections. Limitations were a small patient group and irregularly carried out sampling during intensive care.

TIIVISTELMÄ (ABSTRACT IN FINNISH)

Tausta:
Ihosairauksissa todetut mikrobiomin muutokset ovat herättäneet kiinnostusta uusien diagnoosimenetelmien ja hoitomuotojen löytämiseksi.

Tavoitteet:
Väitöskirjan tavoitteena oli selvittää ihon mikrobiomi ihosyövässä (melanooma, tutkimus **I**) ja tulehduksellisissa ihosairauksissa (parapsoriasis, tutkimus **II**) sekä tutkia näissä ei-invasiivisen mikrobiomi pyyhkäisynäytteen käyttökelpoisuutta. Lisäksi tavoitteena oli kartoittaa ihon mikrobiomi erittäin alhaisen syntymäpainon keskosissa tehohoidossa ja sen mahdollista yhteyttä ihon stafylokokkeihin ja sepsikseen (tutkimus **III**).

Menetelmät:
Mikrobiominäytteitä otettiin 15 melanoomasta ja 17 hyvänlaatuisesta melanosyyttiluomesta (tutkimus **I**), 13 potilaan parapsoriasis ihomuutoksista sekä terveeltä iholta (tutkimus **II**) ja 12 erittäin alhaisen syntymäpainon keskosesta tehohoidon aikana (tutkimus **III**).

Sekvensointi toteutettiin 454 GS-FLX Titanium (tutkimus **I**) ja Illumina MiSeq (tutkimukset **II-III**) alustoilla ja tietoja analysoitiin bioinformatiikan avulla. Tutkimuksissa **I-II** mikrobiomin diversiteetin indeksit laskettiin bioinformatiikan apuvälineillä. Tutkimuksessa **II** stafylokokkien sekvenssit tutkittiin tarkemmin käyttäen oligotyypitystä.

Tulokset:
Tutkimuksessa **I** ihon mikrobiomi oli samankaltainen melanoomissa ja hyvänlaatuisissa melanosyyttiluomissa. Näytteissä todettiin merkittävää yksilöllistä vaihtelua. Merkittäviä eroja bakteeriston diversiteetissä ei tullut esille melanoomien ja hyvänlaatuisten luomien välillä.

Tutkimuksessa **II** ei todettu merkittäviä eroja mikrobiomissa parapsoriasis ihottuman ja terveen näköisen ihon välillä. Myöskään pientäpläisen ja suurtäpläisen parapsoriasiksen välillä ei todettu merkittäviä eroja. *Staphylococcus aureus* bakteeria ei havaittu oligotyypityksellä parapsoriasis ihottumassa.

Tutkimuksessa **III** havaittiin useimmilla keskosilla syntyessä korkea ihon mikrobien diversiteetti. Kolmen ensimmäisen elinviikon aikana diversiteetti kaventui sekä septisillä että ei-septisillä keskosilla. 50 %:lla keskosista nähtiin diversiteetin elpyminen, mutta tämä ei ollut ajallisessa yhteydessä sepsikseen.

Johtopäätökset:

Tutkimus **I** käsitteli ihon mikrobiomia melanoomassa ja melanosyyttiluomissa. Mikrobiomi vaihteli merkittävästi tutkitun ihoalueen ja yksittäisten potilaiden välillä. Löydöksemme viittaavat siihen, että mikrobiomin tutkimisesta ei ole hyötyä melanooman tai melanosyyttiluomien diagnostiikassa. Tutkimuksen rajoituksia olivat pieni ja heterogeeninen potilasryhmä sekä näytteenottoon ja analyysiin liittyvät metodiset ongelmat.

Tutkimus **II** käsitteli ihon mikrobiomia parapsoriasiksessa. Mikrobiomin ei todettu eroavan merkittävästi terveestä ihosta. Vastikään on *in vitro* tutkimuksissa osoitettu, että stafylokokkien enterotoksiini A voi indusoida ihon lymfoomaa sairastavilla IL-17-tuotantoa Sezaryn syndrooman potilaiden primaarisissa pahanlaatuisissa t-soluissa, kun näitä viljeltiin autologisilla, ei-maligneilla t-soluilla. Parapsoriaasiksen tiedetään edeltävän Mycosis fungoidesta, joka yleisin ihon t-solulymfooma.

Stafylokokkien roolia ei ole tietääksemme tutkittu aikaisemmin parapsoriasiksessa. Tutkimuksessa emme havainneet *S. aureus* bakteeria parapsoriasis ihottumassa. Tämä viittaa siihen, että *S. aureuksella* tai sen enterotoksiineilla ei ole merkitystä parapsoriasiksessa. Iholymfoomien ja parapsoriasiksen selvät erot patogeneesissa ja metabolomiikka voivat selittää nämä tulokset. Tutkimuksen rajoituksia olivat pieni potilasryhmä ja ihon mikrobiomin merkittävät yksilölliset vaihtelut.

Tutkimus **III** käsitteli erittäin alhaisten syntymäpainon keskosten ihon mikrobiomia. Pystyimme osoittamaan mikrobiomin korkean diversiteetin ensimmäisinä elinpäivinä riippumatta syntymistavasta, keskosuuden syystä tai infektioista. Diversiteetti kaventui ensimmäisen elinviikon aikana johtuen mahdollisesti tehohoidossa annetuista antibiooteista.

Vastasyntyneiden sepsis ei ollut yhteydessä diversiteetin kaventumiseen eikä siihen, että mikrobiomia hallitsi tämän jälkeen stafylokokkikolonisaatio. Sepsiksen taustalla on luultavasti muita tekijöitä kuten ihoa lävistävät lääkinnälliset laitteet tai samanaikaiset infektiot. Tutkimuksen rajoitukset olivat pieni potilasryhmä ja ajallisesti epätasainen näytteenotto tehohoidossa.

1 INTRODUCTION

1.1 THE SKIN MICROBIOME

Human skin is a multifaceted ecosystem *(Dréno et al. 2017, Wilson et al. 2005)*. The term microbiome is used to describe the collective genomes of the microorganisms that are resident on human skin (i.e. all of the microbes' genes). Seen as an ecological community it is called microbiota. This complex ecosystem houses wide-ranging habitats on its nearly two square meter surface, reaching from humid environments in the axillary folds to dry areas of the shins and the back *(Kong et al. 2017)*. Seen from the microbiome perspective, areas on human skin are usually divided into three environments, which all have their unique ecosystem and characteristic bacterial community (Figure 1, page 14, modified after *Fyhrqvist et al. 2016*): dry environment (e.g. on the back and the shins), moist environment (e.g. in the axillary and inguinal folds) and sebaceous environment (e.g. on the face and the scalp) *(Prescott et al. 2017)*. The composition of the microbiome in each location is believed to be primarily defined by physical properties, but modulated by extrinsic and intrinsic factors *(Grice et al. 2009)*.

As a boundary to the outside environment, the skin is colonized by many micro-organisms, i.e. bacteria, fungi, mites and viruses *(Grice et al. 2011)*. Most of these are now considered harmless and are even able to provide protection. For compensation the commensal bacterial community receives a body site or a biological niche to colonize in a symbiosis-based relationship *(Zeeuwen et al. 2013, Grice et al. 2009)*. The cohabitation of microbes and host is multifaceted and should not be regarded as unilateral or stationary *(Brandwein et al. 2016)*. Microbes have been reported to modify the host's innate and adaptive immune system and play an important and dynamic role in the skin's immune defense and barrier function *(Campbell et al. 2017, Belkaid et al. 2016, 2014)*. Permanent interactions with the colonizing microbes teach cutaneous T-cells to recognize appropriate antigens and form immune responses correctly and they can be regarded as a part of the immunologic barrier of the skin *(Naik et al. 2012)*.

New molecular research methods have changed our view of the cutaneous microbial community *(Kong et al. 2017)*. PCR-based methods have enabled us to understand the complex architecture and the dynamics of the human skin's microbiome. Due to specific microbiome changes observed recently in inflammatory skin disorders, an interest has risen in developing new diagnostic methods and treatments *(Eck et al. 2017)*.

2 REVIEW OF THE LITERATURE

2.1 FACTORS THAT INFLUENCE THE MICROBIOME

The microbiome of a specific body site is mainly defined by its physical characteristics: skin thickness, the number of skin folds and the amount of hair follicles, sweat and sebaceous glands *(Szabó et al. 2017, Costello et al. 2009, Dréno et al. 2016)*. Individual features, e.g. age, gender and the immune system, also influence the microbial community *(Baviera et al. 2014)*. Based on these endogenous and exogenous factors the resident bacterial community shows a wide variability in different regions and body sites of human skin (Figure 1, page 14) *(Grice et al. 2009, Oh et al. 2014)*. Skin microbiome differences have also been explained by physiological and anatomical gender factors, such as sweat and sebum quality and amount, as well as hormonal differences. In studies conducted on healthy patient cohorts it has been shown that profession, environmental factors, e.g. use of antibiotics and skin care products modify the skin microbiome *(Fierer et al. 2008, Ursell et al. 2012)*. Additionally, studies based on residence and cultural aspects have identified that a warm and humid climate is associated with increased microbial diversity of the skin *(Costello et al. 2009)*. Once formed, the skin microbiome shows a remarkable stability in the same individual and can remain unchanged even despite various external factors, such as irritation, humidity and UV-radiation *(Patra et al. 2016)*. Recent research indicates that the skin microbiome has an important role in maintaining a functional skin barrier *(Baldwin et al. 2017)*.

Skin colonization takes place instantly after birth *(Costello et al. 2009, Costello et al. 2013)*. A new and interesting research field is the development and subsequent formation of the cutaneous microbiome during early life and its possible associations with skin disorders *(Capone et al. 2011, Zeeuwen et al. 2013)*. Here, many exogenous and endogenous factors influence the formation of the skin microbiome *(Costello et al. 2013)*. Intravenous antibiotics are known to have a negative impact on the bacterial diversity, whereas links of feeding habits and skin-to-skin contact or mode of delivery have showed inconsistent results in microbiome studies *(Cooijmans et al. 2017)*. It has been reported that for example gestational age has an effect on the cutaneous structure and function and is thus associated with the development of the skin microbiome in preterm infants *(Pammi et al. 2017)*.

In intensive care of preterm infants exogenous factors, such as medical devices (e.g. central venous line) and colonization from hospital employees or members of the family, have been reported to impact the skin microbiome *(Groer et al. 2015)*. The microbial selection caused by antibiotic treatment, is a frequent problem in neonatal intensive care and seems to play a role also in the skin microbiome *(Hartz et al. 2015)*. Especially the colonization of the

neonatal skin with *Staphylococcus epidermidis* and other coagulase negative commensal bacteria seem to play a role here *(Bradford et al. 2011)*. There have been reports of a special susceptibility to coagulase negative commensal bacterial colonization due to skin and immunological immaturity *(Costello et al. 2013)*. New molecular methods have enriched our understanding of the evolution and maturation of the healthy skin microbiome *(Underwood et al. 2017)*. It is possible that in the nearer future the modulation of pathogen colonization, e.g. staphylococci, in preterm and low birth weight infants can be done *(Scharschmidt et al. 2017)*.

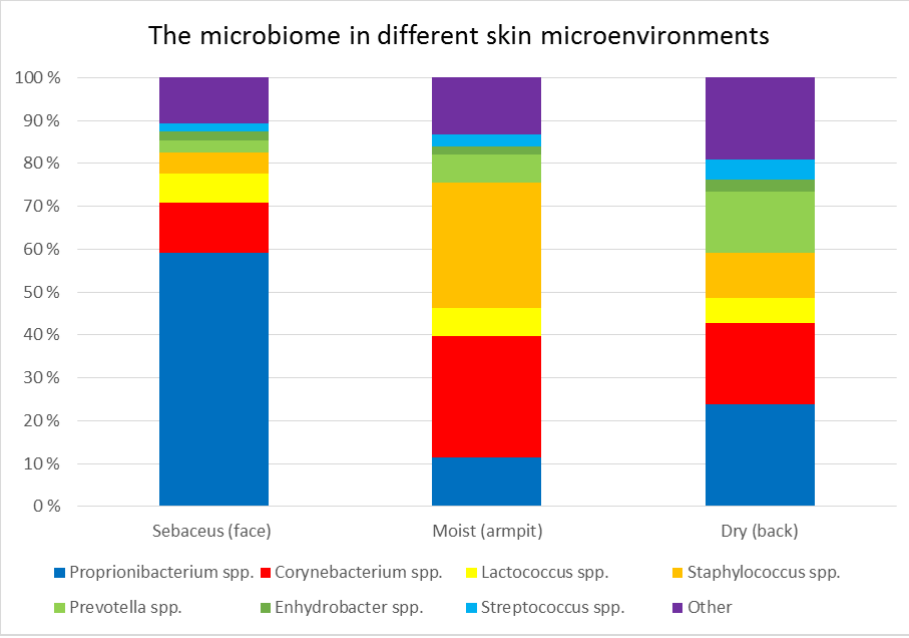

Figure 1 The cutaneous microbiome in different body sites and environments, mean relative abundances of the most common genera (figure modified from data presented in *Fyhrqvist et al. 2016)*

2.2 INTERACTIONS OF THE MICROBIOME AND IMMUNE SYSTEM

Besides the physical properties the skin is also as an immunological barrier *(Belkaid et al. 2016)*. Maintenance and changes of the cutaneous microbiome are thus influenced by immunological factors *(Campbell et al. 2017, Chen et al. 2013)*. In particular, the innate immune system has been shown to play an important role in the epidermal function *(Pasparakis et al. 2014)*. There have been observations of a constant and dynamic interaction with commensal and

pathogenic microbes *(Skabytska et al. 2015, Dziarski et al. 2012, Dréno et al. 2016)*.

The innate immune system should consequently be viewed as a key element of the immunologic cutaneous barrier *(Baldwin et al. 2017, Boguniewicz et al. 2011, Gallo et al. 2011)*. Malfunctions have been shown to lead to insufficient responses to pathogens or persistent inflammatory states *(Kuo et al. 2013, Lai et al. 2009)*. In recent studies it was demonstrated that also cells of the adaptive immune system (e.g. T-lymphocytes in the epidermis) are involved in supporting the function of the skin barrier *(Belkaid et al. 2016, Brestoff et al. 2013)*. T-lymphocytes seem to be important in keeping up a tolerance to the commensal flora and in initiating immune responses against pathogens *(Belkaid et al. 2014, Scharschmidt et al. 2017)*.

2.3 NEW MOLECULAR RESEARCH METHODS

Novel molecular research methods have had a strong impact on the understanding of the microbiome *(Hugerth et al. 2017, Schaffer et al. 2017, Kong et al. 2017)*. Recent genomic studies on 16S ribosomal RNA (rRNA) gene sequencing *(Kong et al. 2017, Dekio et al. 2007)* have shown that the colonization of the skin is characterized by a great diversity and variability *(Kong et al. 2011)*.

These new molecular methods offer the possibility to investigate microbial communities without culture mediums and have thus broad application areas *(Dréno et al. 2016)*. It is also relatively easy and noninvasive to obtain the cutaneous specimens *(Prescott et al. 2017)*. Negative aspects of the PCR-based research methods are the absence of bacterial resistance testing possibilities and that the methods detect also dead micro-organisms *(Brandwein et al. 2016)*. With PCR-based microbiome research methods the current microbiome can be determined in a given point of time, but the dynamic content of bacterial genomes, including virulence factors and resistance mechanisms remain largely obscure *(Findley et al. 2014)*. Due to their high sensitivity a frequent problem is furthermore contamination during sampling and laboratory analysis *(Salter et al. 2014)*. A possible source of contamination are the person taking samples and the environment where the samples are obtained. It has also been shown that DNA extraction kits can be an important source of contamination. Other potential sources are the reagents used in the work-flow and the laboratory personnel handling the samples *(Kong et al. 2017, Salter et al. 2014)*.

The standard sequencing process in investigating cutaneous specimens consist of swabs, scrapings or skin biopsies *(Maguire et al. 2017, Kong et al. 2011)*. The results of most of the published studies on skin microbiome are based on skin swabbing techniques *(Egert et al. 2017)*. But it is acknowledged that the sampling technique may have an impact on the observed microbiome

data *(Meisel et al. 2016, Grice 2015)*. Recently, in addition to the surface microbiome research, the investigation of deeper skin structures (i.e. the compartment-specific microbiome) has gained interest *(Kong et al. 2017)*. Based on skin biopsy material some newer studies have also investigated the cutaneous microbiome in deeper cutaneous structures, such as the hair follicles and dermis *(Ring et al. 2017)*.

When investigating the cutaneous bacterial community the bacterial DNA is isolated and the 16S rRNA gene is amplified using PCR. It is recognized that some regions of the prokaryotic 16S rRNA gene, primarily the regions V1-V3, are the most suitable for investigating the skin microbiome *(Egert et al. 2017)*. It has been noted, that especially the 16S hypervariable regions V1-V3 are best at discriminating bacterial taxa and identifying the bacteria at the genus level *(Egert et al. 2017)*. Finally the amplified genes are sequenced on commercial platforms, e.g. MiSeq sequencer *(Kozich et al. 2013)*. The immense microbiome data is analyzed using bioinformatic computer programs and tools such as mothur *(Schloss et al. 2009)*. The final result of this approach is obtained by clustering and constructed based on the amount of sequence similarity (> 97 %) and named operational taxonomic unit (OTU) *(Meng et al. 2017)*. Based on this information it is possible to identify and classify most cutaneous bacteria to genus level, some even down to species level *(Kong et al. 2017)*.

On the basis of rRNA gene polymorphisms the bacteria normally colonizing human skin can be divided into four phyla: Actinobacteria, Firmicutes, Proteobacteria and Bacteroidetes *(Schommer et al. 2013)*. The proportions of these phyla seem to vary in different regions *(Oh et al. 2014)*, but once the microbiome is formed and stabilized, it remains remarkably constant *(Kong et al. 2011, Oh et al. 2016)*. Skin microbiome studies based on PCR-sequencing can provide us information about the occurrence of bacteria, the abundance and diversity of the bacterial community and whether specific bacteria are associated with a specific disease state or phenotype of skin disorder. They provide us information of the bacterial community at single time points, however they cannot prove a causal relationships between the skin microbiome and investigated disease *(Grice et al. 2009)*.

2.4 ASSOCIATION OF THE MICROBIOME AND INFLAMMATORY SKIN DISORDERS

Inflammatory skin disorders, such as atopic dermatitis, have been shown to cause specific microbiome changes *(Salava et al. 2014, Kong et al. 2012)*. It is still uncertain if these changes are caused by the microbiome itself or if they represent an epiphenomenon. Some studies have demonstrated a possible association to skin barrier malfunctions and immunologic dysfunctions *(Belkaid et al. 2016, Seite et al. 2015, Zeeuwen et al. 2013)*.

Most extensive microbiome investigations have been conducted in patients with atopic dermatitis *(Biedermann et al. 2015, Marrs et al. 2016, Fyhrqvist et al. 2016)*. In some studies the effect of commensal and pathogenic microbes has been suggested as an aggravating factor *(Kong et al. 2012)*. Clinical improvement has also been demonstrated with treatments affecting the cutaneous microbiome (e.g. antimicrobial treatments) *(Bjerre et al. 2017, Kong et al. 2012)*. During disease flares of atopic dermatitis a decrease in cutaneous microbial diversity has been observed *(Harkins et al. 2017)*. A recovery to high bacterial diversity was shown after effective topical treatments (Figures 2 and 3, page 18, modified after *Salava et al. 2014) (Seite et al. 2015)*.

In a recent study *(Harkins et al. 2017)* on pediatric atopic dermatitis nasal and skin colonization by clonal *Staphylococcus aureus* populations (containing a single sequence type) was observed in both atopic dermatitis and controls. In the same study the later phylogenetic analyses showed that the disease flares that occurred over months, were associated with the clonal expansion of the *S. aureus* population *(Harkins et al. 2017)*. It has also been shown that *S. aureus* colonization is increased in atopic dermatitis patients with filaggrin mutations (and other mutations of genes important for the epidermal barrier) *(Clausen et al. 2017)*. Based on the results of this newer study the authors suggest that host-microbe interactions and differences in the *S. aureus* clones play an important role in the skin colonization of atopic dermatitis. There have also been reports of skin microbiome changes in psoriatic disease *(Yan et al. 2017)*. In a recent study the authors detected an increased relative abundance of streptococci in the skin of psoriasis patients *(Yan et al. 2017)*.

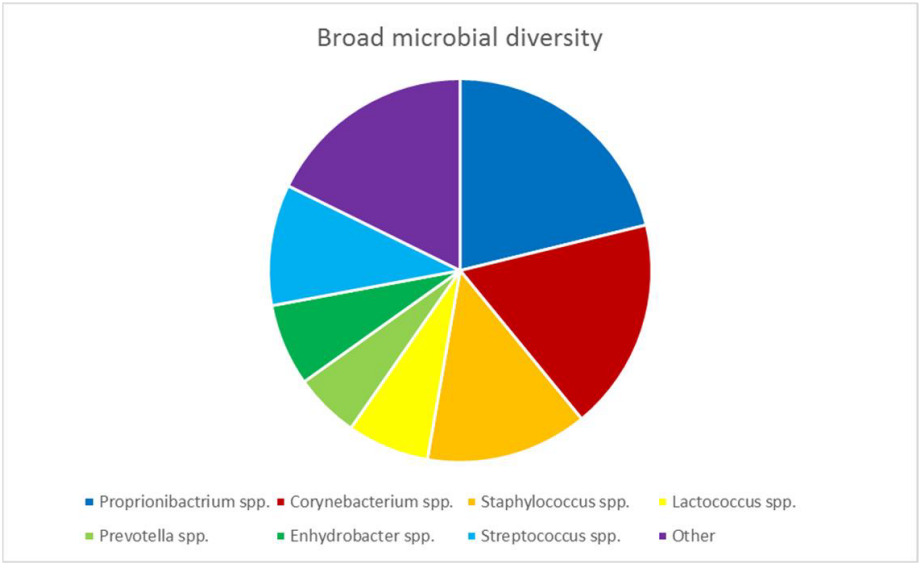

Figure 2 Relative abundances of the most common genera of the microbiome, patient with atopic dermatitis, stable disease state, moist environment (armpit), (figure modified from data presented in *Salava et al. 2014*)

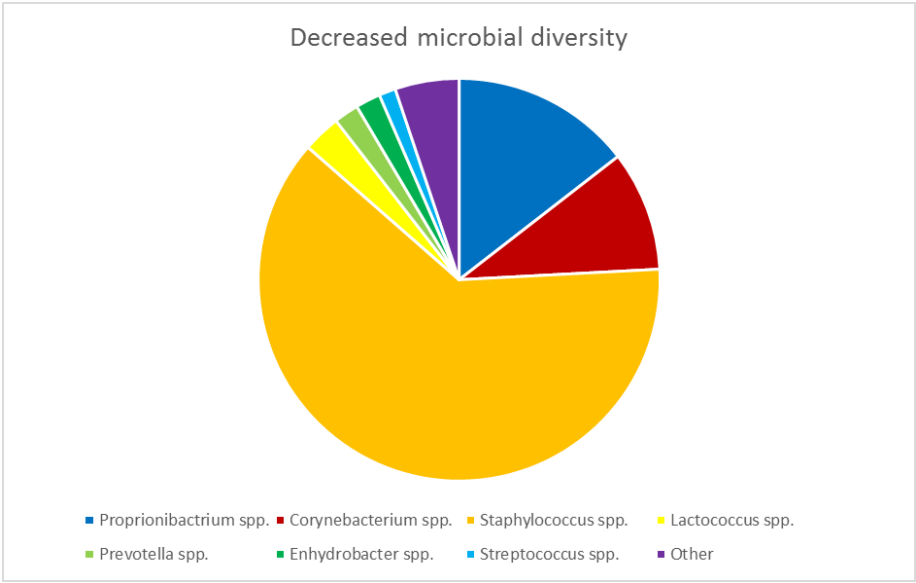

Figure 3 Relative abundances of the most common genera of the microbiome, patient with atopic dermatitis, during disease flare, moist environment (armpit), (figure modified from data presented in *Salava et al. 2014*)

The role of *S. aureus* in inflammatory skin disorders has been under widespread investigation in the preceding years *(Iwase et al. 2010, Lai et al. 2009, Otto et al. 1999)*. There has been some evidence that during disease flares of atopic dermatitis the microbiome changes into more susceptible towards *S. aureus* colonization *(Kong et al. 2012, Oh et al. 2013)*. There have also been reports that the commensal bacterial community inhibits the colonization of pathogens, especially *S. aureus (Coates et al. 2014, Kong et al. 2012)*.

Interactions between the microbiome and the immune system seem to play a role in many skin disorders *(Miodovnki et al. 2017, Zeeuwen et al. 2013)*. However, we have still only a limited understanding about their impact on the clinical picture and disease pathogenesis *(Cerf-Bensussan et al. 2012, Atarashi et al. 2011)*. Many questions have remained unclear about the cutaneous microbiome, the host-microbiome relationship and its significance *(Lee et al. 2017)*.

2.5 CUTANEOUS MELANOMA

Cutaneous melanoma is a malignant tumor arising from skin melanocytes *(Tsao et al. 2017, Reginster et al. 2012)*. Its incidence and the need for therapeutic resources are expected to rise in the near future *(Apalla et al. 2017, Tuong et al. 2012)*. Despite profound research the exact cause of melanoma has remained uncertain, but great progress has been made in recent years in terms of understanding the evolution and molecular pathogenesis of the tumor *(Rajkumar et al. 2016)*.

It has been shown that genetic and environmental causes play important roles in the initiation and progression of this malignant tumor *(Hawkes et al. 2016)*. Recognized environmental risk factors are UV-exposure and cumulative amount of sunburns *(Kanavy et al. 2011)*. Mutations in proteins that regulate the cell-cycle are known to have an important role in the development of the malignancy *(Tsao et al. 2017, Nikolau et al. 2012)*.

Interactions between melanoma cells and host immune system have been investigated intensely *(Tsao et al. 2017, Tietze et al. 2011)*. There have been reports of immune mediated regression of melanoma tissue *(Byrne et al. 2017)*. The individual immune response and immunologic characteristics are recognized to be important factors determining the initiation and progression of the disease *(Mignogna et al. 2017)*. Some *in vivo* studies have reported of endogenous anti-melanoma immunity *(Byrne et al. 2017, Pandolfi et al. 2011)*.

Novel therapies that target the immune system, cell-cycle regulation and pathways have been developed and are used in disseminated disease *(Amann et al. 2017, Chapman et al. 2011)*. However in localized disease surgical excision still remains the first line therapy *(Garbe et al. 2016)*. Recognized important factors in patient management are an early detection and surgical

removal of melanoma, which lead to a high cure rate and relatively good prognosis *(Garbe et al. 2016, Bichakijan et al. 2011)*. In cases with metastatic disease response rates are still unfavorable and all current therapy regimens are not curative *(Garbe et al. 2016)*.

2.6 MELANOCYTIC NEVI

Melanocytic nevi are very common and are based on localized proliferations of melanocytes *(Huang et al. 2017)*. Histopathologically they can also have dysplastic features and then consequently are called dysplastic melanocytic nevi *(Piccolo et al. 2016, Barnhill et al. 2010, Friedman et al. 2009)*. Regardless of the profound research in melanocytic nevi, our understanding about the etiology and pathogenesis have remained incomplete *(Barnhill et al. 2010)*. There are still distinct controversies about dysplastic nevi and their place as an entity in the range of melanocytic skin lesions *(Rosendahl et al. 2015)*.

Genetics, sexual hormones and exposure to UV-radiation have been recognized as the most relevant etiologic factors in the development of melanocytic nevi *(Huang et al. 2017, Lens et al. 2008)*. Similar mutations found in melanoma tissue (proteins that regulate the cell-cycle, e.g. BRAF V600E), have been detected in eruptive melanocytic nevi and dysplastic melanocytic nevi *(Turner et al. 2005, Sekulic et al. 2010, Hawkes et al. 2016)*.

There have been observations that immunological factors, such as immunosuppression, may play a role in both the development and growth of melanocytic nevi *(Kim et al. 2016, Zattra et al. 2009)*. In patients with immunosuppression, e.g. patients receiving chemotherapy, a greater incidence of melanocytic nevi and dysplastic nevi has been described *(Kim et al. 2016, Chen et al. 2014)*. There have also been reports of eruptive melanocytic nevi in association of immunosuppressive therapy in children *(Vena et al. 2017, Reutter et al. 2007)*.

2.7 THE MICROBIOME AND CANCER

The possible association between the microbiome and malignancies is a recent hypothesis and has to our knowledge not been formerly investigated in skin cancer *(Yu et al. 2015)*. The microbiome is present predominantly in epidermal and mucosal locations: skin, mucosal surfaces, lung and the intestine. It is now known that the microbiome interacts profoundly with its host and especially with the epithelial surface *(Chen et al. 2017)*. A dysbiosis of the microbiome is believed to play a role in some human cancers, but strong evidence for this is still missing *(Chen et al. 2017)*. Most microbiome studies

have been conducted in gastrointestinal and oral cancer *(Mima et al. 2017, Schmidt et al. 2014)*, where results have been inconsistent and conflicting and the role of the gastrointestinal microbiome in the pathogenesis of intestinal malignancies has remained unclear *(Singh et al. 2017, Yu et al. 2015)*.

It is known that specific bacterial pathogens such as *Helicobacter pylori* may initiate gastrointestinal carcinogenesis *(Noto et al. 2017)*. On the other hand, in a recent study *(Ferreira et al. 2017)* the microbiome in gastric carcinoma was found to have a reduced microbial diversity and decreased abundance of *Helicobacter*. The authors also found an enrichment of intestinal commensal bacteria and calculated a dysbiosis index, which is believed to discriminate between chronic inflammation (gastritis) and malignant proliferation (gastric carcinoma). Reports of commensal bacteria-induced chronic inflammation and progression of malignancies of other organ systems have also been published *(Oke et al. 2017, Arthur et al. 2012, Maslowski et al. 2009, Mima et al. 2017)*. In another recent study on lung cancer the authors found a significant decrease in the bacterial diversity of the lower airway (bronchial brushing sample) on the side of lung cancer, when compared to the same patients' healthy contralateral bronchi *(Liu et al. 2017)*. The authors used the contralateral lower airway microbiome as control and discussed that investigating the microbiome could possibly be used as a lung cancer screening method in the future. Other authors have discussed microbiome studies as a possible target for prevention and treatment of chronic inflammatory airway diseases, such as asthma *(Chung 2017)*.

In inflammatory bowel diseases bacterial components such as lipopolysaccharides have been demonstrated to maintain gastrointestinal inflammation and the disease progression *(Baillie et al. 2017)*. In a recent study on colitis ulcerosa associated colon cancer the intestinal microbiome was reported to be significantly different from sporadic colon cancer and healthy individuals *(Richard et al. 2017)*. The authors also did a comparison of the tumour site and normal mucosa near the tumour site, but the microbiome did not differ significantly and showed a similar pattern. In gastrointestinal cancer the global mucosa-associated bacterial microbiome has been characterized as decreased in microbial biodiversity *(Kang et al. 2017)*. It has been postulated that a chronic inflammatory state initiates carcinogenesis by Toll-like receptor signalling *(Bhatt et al. 2017, Mazmanian et al. 2008)*, however it is still mainly unclear how changes in the microbiome and the host inflammatory response lead to malignancy, e.g. colorectal cancer *(Louis et al. 2014)*.

Recently there have been reports of a possible role of the vaginal microbiome in gynaecologic malignancies. The results have been controversial *(Champer et al. 2017)*. Additionally, studies about the role of the microbiome in malignancies of other organ systems, e.g. lung cancer, have been now initiated *(Cameron et al. 2017, Rajagopala et al. 2017)*. The role of the cutaneous and lactic gland microbiome in the pathogenesis of breast cancer has been discussed *(Mani 2017)*. Microbiome studies have raised an interest in finding potential biomarkers (primarily characteristics in the microbiome

data) for cancer diseases, e.g. lung cancer. In a recent microbiome study on lung cancer the authors concluded that spontaneous sputum appears to have potential for bacterial microbiome-based biomarkers, which may be utilized also clinically *(Cameron et al. 2017)*.

Microbiome studies have evolved as interesting novel perspectives in cancer research. However, many questions about the role of the microbiome in initiation and progression of malignancies still remain unclear. Altogether, these novel microbiome studies have contributed to our understanding of how the normally symbiotic and commensal bacterial community can effect processes like chronic inflammation and cancer development and progression. Additionally, we have gained more insights into the role of the microbiome in modulating the environmental effects on the host and carcinogenesis. The interest has been particularly in exogenous factors (e.g. diet, antibiotics) which directly affect the commensal bacterial communities *(Dmitrieva et al. 2017)*. In case of the skin microbiome the most important factors appear to be the exposition to UV-radiation, but this has not been studied systematically *(Patra et al. 2016, Chen et al. 2017)*. There is a complex relationship between, on one side the commensal and pathogenic microbes and the other side, host immunity and metabolism *(Fulbright et al. 2017)*. In cancer research microbiome studies have revealed unexpected and exciting results, which have to be confirmed in larger patient groups. It will be interesting to see, if the investigation of the microbiome will influence future therapies and possible prevention of malignant diseases.

2.8 THE MICROBIOME AND LYMPHOPROLIFERATIVE DISEASE

Parapsoriasis belongs to the spectrum of cutaneous lymphoproliferative disorders *(Cerroni 2017)*. On one side there is a picture resembling chronic dermatitis and on the other a picture similar to cutaneous T-cell lymphoma *(Burg et al. 2002)*. At present it is still unknown what initiates parapsoriasis and what are the factors that lead to progression of the disease. The development and disease course cannot be efficiently predicted *(Lewin et al. 2012)*. To our knowledge microbiome studies have not been conducted before in lymphoproliferative diseases or in parapsoriasis.

Traditional classifications classify parapsoriasis into small plaque (SPP) and large plaque parapsoriasis (LPP). Both subtypes may follow a chronic course for many years. Molecular studies have shown that parapsoriasis is a monoclonal cutaneous T-cell disorder *(Sibbald et al. 2016)*. T-cell receptor gene rearrangement and sensitive polymerase chain reaction (PCR)-based tests have demonstrated, that the pathogenic T-cells in parapsoriasis belong to the skin-associated lymphoid tissue (SALT) *(Leloup et al. 2014)*.

T-cell infiltration in parapsoriasis can be divided into two main patterns:

1. Superficial eczema-like type of inflammation (with a sparse infiltration of mature lymphocytes around the superficial capillaries and only some lymphocytes invading into the epidermis)
2. Atypic lymphocyte infiltration-type (with a band-like lymphocytic infiltration with some lymphocytes infiltrating diffusely into the epidermis) *(Baderca et al. 2014, Leloup et al. 2014, Väkevä et al. 2005)*.

The typical histologic picture of parapsoriasis in comparison with normal skin is demonstrated in figure 4, page 23 and figure 5, page 24. There is current consensus that both clinical subtypes can progress into cutaneous T-cell lymphoma, mainly mycosis fungoides *(Berg et al. 2017, Lindahl et al. 2014)*. The risk for malignant transformation is known to be higher in large plaque parapsoriasis *(Cerroni 2017)*.

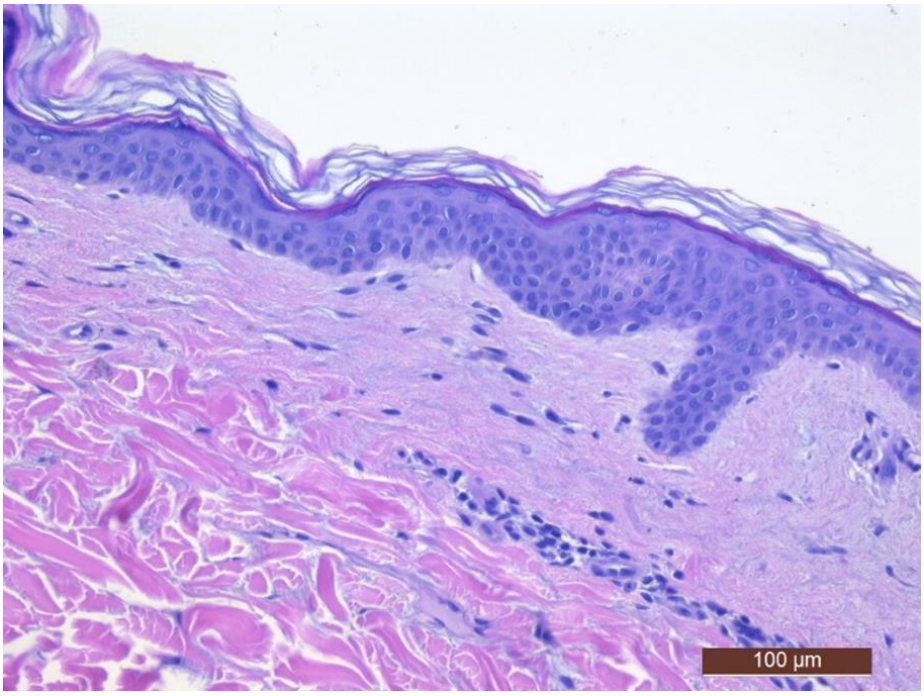

Figure 4 Histopathology of normal skin, HE-staining, 20-fold magnification, there is no notable T-cell infiltration in the dermis, figure by Katriina Lappalainen M.D., Ph.D., with permission

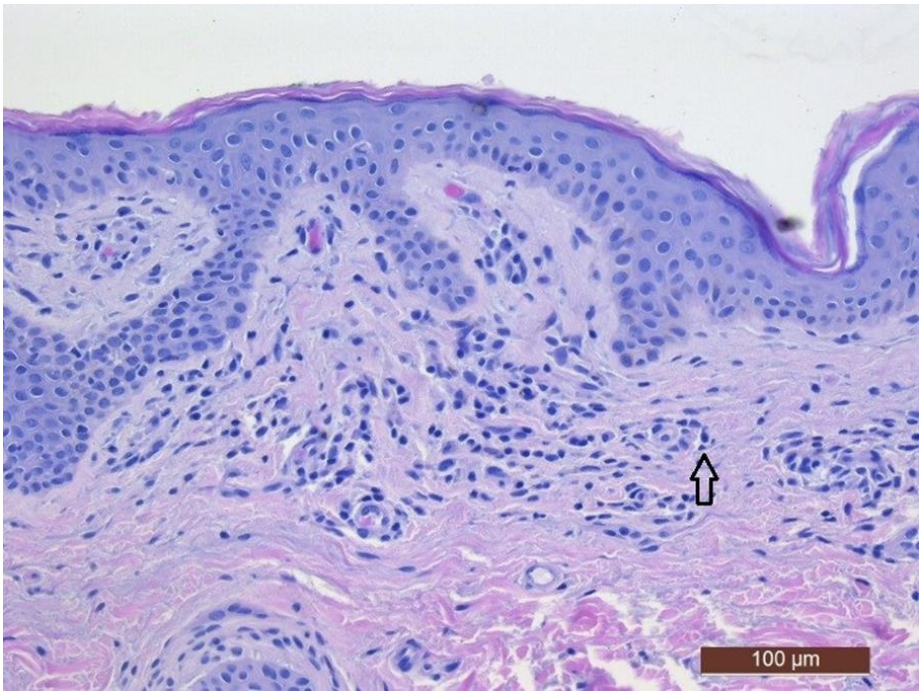

Figure 5 Histopathology of parapsoriasis, HE-staining, 20-fold magnification, T-cell infiltration takes mainly part in the dermis, the epidermis contains only single atypical T-lymphocytes (arrow), figure by Katriina Lappalainen M.D., Ph.D., with permission

2.9 PROGRESSION INTO CUTANEOUS T-CELL LYMPHOMA

It has been observed that approximately 30 % of the cases of large plaque parapsoriasis progress into cutaneous T-cell lymphoma, mostly mycosis fungoides *(Talpur et al. 2017, Väkevä et al. 2005)*.

Based on the clinical picture or on histopathology it can be impossible to differentiate LPP from early cutaneous T-cell lymphoma (for example mycosis fungoides) *(Kikuchi et al. 2003, Eklund et al. 2016)*. No marker has been found to recognize the cases that are susceptible to lymphoma transformation *(Leloup et al. 2013)*. Prognostic factors that would indicate a possible progression into cutaneous T-cell lymphoma are currently not available. There are also no tests existing to differentiate between benign and malignant cutaneous T-cell infiltration and if the disease is going to develop into malignancy *(Sibbald et al. 2016)*.

In large plaque parapsoriasis the quantity of malignant monoclonal cells in the skin has been reported 1 % to 10 %, while in mycosis fungoides it can extent to 50 % *(Howard et al. 2000)*. Although some cases progress to lymphoma, parapsoriasis in the most cases has a benign course and stays indolent for many years. There have been cases with complete resolution. Various prognostic parameters such as histopathology, immunohistochemistry, PCR and T-cell receptor gene rearrangement studies have been investigated. The main goal has been to characterize the atypical lymphocytes, and thus to predict the disease course *(Scarisbrick 2017, Costa et al. 2003, Väkevä et al. 2005)*.

Parapsoriasis patients require a dermatological follow up and often repeated skin biopsies to exclude cutaneous T-cell lymphoma. Small or large plaque parapsoriasis can be treated symptomatically with topical corticosteroids or phototherapy, e.g. psoralen plus UVA-therapy *(Duarte et al. 2013)*. Systemic therapies can be considered in severe cases and when there are signs of progression into cutaneous T-cell lymphoma *(Trautinger et al. 2017)*.

2.10 CUTANEOUS T-CELL LYMPHOMA AND STAPHYLOCOCCAL ENTEROTOXINS

Several recent studies have demonstrated an intense interaction between the cutaneous microbiome and the host's immune system *(Belkaid et al. 2016, Kikuchi et al. 2003, Cerf-Bensussan et al. 2012)*. In addition to the skin's physical functions, the innate immune system and the commensal bacteria have shown to play a role in the cutaneous immunological barrier *(Wanke et al. 2011, Burg et al. 2002)*.

There have been reports that staphylococcal enterotoxins (e.g. enterotoxin A, SEA) from affected skin of cutaneous T-cell lymphoma induces in vitro IL-17 production in primary malignant T cells of lymphoma patients *(Litvinov et al. 2016)*. Here, malignant T-cells produced interleukins when they were cultured with autologous nonmalignant T-cells. This observation was not made in monocultures of malignant T-lymphocytes *(Willerslev-Olsen et al. 2016)*.

2.11 THE SKIN MICROBIOME IN PRETERM INFANTS

It has been shown that the cutaneous microbiome in preterm neonates and infants undergoes significant changes in early life *(Costello et al. 2013, Capone et al. 2011)*. Observations of microbiome studies suggest that before the skin microbiome is established during infancy and consequently turns into a

relatively stable state, it is initially characterized by substantial dynamics and variations *(Pammi et al. 2017)*. The main reasons for this development have remained obscure. A study with healthy neonates found that neonates were colonized with cutaneous bacterial communities that were undifferentiated across many body sites, regardless of the birth delivery mode *(Dominguez-Bello et al. 2010)*. This was observed in direct contrast to the complex and differentiated bacterial communities of their mothers. Although the skin microbiome of healthy neonates is characterized quite well, we have only a limited amount of knowledge of it in preterm and very low birth weight infants (birth weight < 1500 g) *(Costello et al. 2013, Pammi et al. 2017)*. To our knowledge microbiome studies in very low birth weight infants in intensive care have not been carried out before.

The variety of microorganisms that colonize the new-born skin differs depending on many intrinsic and extrinsic factors *(Underwood et al. 2017)*. The skin microbiome has been shown to change when physiological functions, e.g. the barrier function of the skin, are disturbed *(Costello et al. 2013)*. *S. epidermidis* is a typical bacterium of the commensal bacterial community of human skin. Apart from its commensal role, recent studies *(Byrd et al. 2017, Soerg et al. 2017)* have suggested that it could also be an important factor to the general health of neonates *(Pammi et al. 2017)*. *S. epidermidis* has a role in the inhibition of virulent pathogens and interactions with the immune system *(Bradford et al. 2011, Becker et al. 2014)*. In a recent study it was observed that skin colonization by commensal bacteria (including *S. epidermidis*) and local chemokine production together recruit regulatory T-cells into neonatal skin *(Scharschmidt et al. 2017)*. The authors suggested that because regulatory T-cells are important for immune tolerance, early life interactions with commensal microbes seem to play an important role in establishing this tolerance. The results of another study on the microbiome of preterm neonates showed that the characteristics of the neonatal skin microbiome did not differ between different body sites for in term and preterm infants during the neonatal period *(Pammi et al. 2017)*. It was however shown, that the cutaneous microbial diversity was positively associated with the gestational age during early life. As observed in a former study *(Costello et al. 2013)*, the authors concluded that in preterm neonates intravenous antibiotics are likely to have a negative impact on the skin's bacterial diversity. Contrary to the results of a previous study on healthy neonates *(Dominguez-Bello et al. 2010)*, the authors did not see differences with respect to feeding (e.g. breast feeding or milk substitutes) or mode of delivery.

The skin colonization with commensal bacteria, such as *S. epidermidis* and other coagulase negative bacteria, takes place shortly after birth *(Rogers et al. 2016, Costello et al. 2013)*. During intensive care treatment, many factors seem to have an effect on the cutaneous bacterial community *(Pammi et al. 2017)*. There have been reports that invasive procedures and devices, colonization from health care workers or family members and the microbial selection caused by antibiotic treatment, alter the microbiome *(Chu et al. 2017, Power*

Coombs et al. 2013). Our understanding of the skin microbiome and its dynamics in early life of neonates and especially that of preterm and very low birth weight neonates is still incomplete. Above all the impact of skin microbiome changes on health and development of infants and the possible association with skin disorders remains to be elucidated.

2.12 NEONATAL SEPSIS AND STAPHYLOCOCCI

Neonatal sepsis is a frequent clinical problem in intensive care of premature and low to very low birth weight neonates *(Shane et al. 2017, Bekhof et al. 2013)*. Sepsis diagnosis can be challenging because the presenting symptoms are frequently unspecific *(Larson et al. 2005, Tsai et al. 2014)*. Microbiome studies investigating the possible link between neonatal sepsis and the gastrointestinal microbiome have been carried out, but results have been controversial *(Berrington et al. 2014)*. In a twin study it was shown that preterm twins share a similar gut microbiome, which is modulated by the complex environment of the intensive care unit *(Stewart et al. 2013)*. The authors concluded that it was likely a result of genetic and immunologic factors as well as exposure to the same maternal microbiome during birth, skin-to-skin contact and breast milk. No association to neonatal sepsis was detected *(Stewart et al. 2013)* and the authors suggested that skin colonization and invasive medical devices would more likely to be linked to neonatal sepsis. Until now, microbiome studies that investigate the possible association between the cutaneous microbiome of preterm neonates and neonatal sepsis have to our knowledge not been carried out. It seems however, that environmental factors including prematurity of the skin barrier, invasive medical procedures and antibiotic exposure play a role in colonization of the neonates' skin by staphylococci *(Chu et al. 2017)*. Other factors that have been discussed are the structure of the microbial community and host genetics *(Mutic et al. 2017, Pammi et al. 2017)*. The premature skin of neonates is known to be susceptible to colonization by coagulase negative commensal bacteria *(Groer et al. 2015)*. The main reasons for this are believed to be immaturity in the skin barrier function and the immune system *(Underwood et al. 2017, Capone et al. 2011)*.

It has been demonstrated that coagulase-negative staphylococci, in particular *S. epidermidis*, are the main pathogens in neonatal sepsis *(Dong et al. 2014)*. Over 80 % of the sepsis cases in preterm low birth weight neonates are caused by *S. epidermidis (Garite et al. 2017, Rupp et al. 2014)*. Intensive treatment and the use of invasive medical treatments, e.g. with a central venous line, are believed to increase the risk for neonatal sepsis *(Garite et al. 2017)*. Different factors of intensive care seem also to have an additive role in influencing the skin microbiome: invasive medical devices, colonization from hospital employees and members of the family, as well as microbial selection

by antibiotics *(Alcock et al. 2017)*. Subsequently, in most preterm neonates the skin is initially colonized with *S. epidermidis* and other coagulase negative commensal bacteria *(Dong et al. 2014, Li et al. 2013)*.

2.13 THE SKIN MICROBIOME AND NEONATAL SEPSIS

Previous microbiome studies have shown, that based on the immature cutaneous barrier and undeveloped immune system neonates have a predisposition to cutaneous bacterial diversity changes (mainly decreased diversity) and to skin colonization by coagulase negative commensal bacteria *(Nakamizo et al. 2015, Bokulich et al. 2016)*.

The microbiome of preterm neonates seems to undergo profound changes in early life *(Pammi et al. 2017)*. It seems to be dominated by coagulase-negative staphylococci, mainly *S. epidermidis (Capone et al. 2011)*. Because these bacteria are also the accepted pathogens of neonatal sepsis, there has been an interest in investigating the skin microbiome in preterm neonates *(Li et al. 2013)*. In general, intensive care is known to have a profound effect on the cutaneous microbiome of preterm neonates, but our knowledge about its relationship to neonatal sepsis remains unclear *(Groer et al. 2015, Costello et al. 2013)*. Environmental factors of the intensive care unit, such as caregiving equipment (especially invasive medical device), colonization from medical personnel and antibiotic use seem to play a major role for the skin microbiome, but the causality to neonatal sepsis is still controversial *(Hartz et al. 2015)*.

Altogether, there have been only a limited amount of studies investigating the possible association between the observed skin microbiome changes and neonatal septic infections, particularly neonatal sepsis. Currently, our knowledge about the skin microbiome in preterm infants is still incomplete and there is only a small quantity of skin microbiome data in this patient group. In a recent review article it has been discussed *(Rodriguez et al. 2017)* that various factors in the neonatal intensive care ward influence the skin microbiome and that this information could also be used to establish clinical guidelines in neonatal intensive care. The authors point out that the prevalence of nosocomial infections can be decreased by concrete skin targeted measures: infection control (e.g. venous catheters), encouraging of skin-to-skin care and breast feeding and whenever possible decreasing the use of antibiotics *(Rodriguez et al. 2017, Hartz et al. 2015, Groer et al. 2015)*.

3 OBJECTIVES OF THE PRESENT STUDY

1.
In study **I** the aim was to characterize the microbiome in cutaneous melanoma and in benign melanocytic nevi. The study was targeted to investigate whether there are any differences in diversity or specific bacterial taxa between the same patients' healthy skin, melanomas and melanocytic nevi.

2.
In study **II** the aim was to characterize the skin microbiome in small and large plaque parapsoriasis. Parapsoriasis is known to precede mycosis fungoides, the most common form of cutaneous lymphoma. Study **II** was targeted to investigate whether the chronic parapsoriasis lesions would have a different skin surface microbiome compared to the individual's healthy (nonlesional) skin sites. The goal was to explore if there is an association of any specific bacterial community (especially *S. aureus*) with the chronic T-cell proliferation of parapsoriasis. In addition, we wanted to investigate if microbiome swab sampling could be helpful as a diagnostic method in parapsoriasis.

3.
Based on previous studies and suggested links between neonatal sepsis, staphylococci and microbiome changes, our aim in study **III** was to characterize the cutaneous microbiome of very low birth weight infants during the first postnatal weeks in neonatal intensive care. The goal was also to explore the possible association of microbiome changes and neonatal sepsis. Because of limitations in the clinical setting (intensive care, sepsis treatment) it was only possible to conduct study **III** as a case study.

4 METHODS

4.1 PATIENT STUDIES

The ethics committee of the Helsinki-Uusimaa Hospital District, Helsinki, Finland, approved the study protocols (approval number studies **I**-**II**: 12/13/03/01/2012, study **III**: 103/13/03/03/2012).

4.1.1 STUDY I

The clinical part was carried out between February 2012 and January 2014 at the Department of Dermatology and Allergology, Helsinki University Hospital, Finland.

Microbiome swab samples of melanomas (Figure 6, page 32) and melanocytic nevi from 52 different patients were examined. Samples from 20 patients had to be excluded from the microbiome analysis because of inappropriate lesion type (e.g. dysplastic nevus, blue nevus) or unsatisfactory amount of DNA.

4.1.2 STUDY II

The clinical part was carried out between January and September 2014 at the Department of Dermatology and Allergology, Helsinki University Hospital, Finland.

Altogether 13 patients with histologically confirmed parapsoriasis (6 with small plaque parapsoriasis and 7 with large plaque parapsoriasis) were investigated.

4.1.3 STUDY III

The clinical part was carried out between March and October 2013 in the neonatal intensive care unit (NICU), Children's Hospital, Helsinki University Hospital, Finland.

Microbiome samples from 12 randomly selected preterm very low birth weight (VLBW) infants were taken on several days during intensive care. The patients were given mother-infant skin-to-skin care and prophylactic primary antibiotics after birth. Neonates with sepsis were given antibiotic treatment according to their symptoms and sepsis treatment guidelines.

4.2 SAMPLE COLLECTION

4.2.1 STUDIES I AND II

Skin microbiome samples were obtained with a sterile swab that was immersed into a buffered solution. The borders of the investigated skin lesions were not overlapped and the skin was not handled or disinfected before sampling (Figure 7, page 32) *(Dréno et al. 2016)*.

Control specimens were taken using the same method from the patients' healthy skin on the contralateral body sites (Figure 8, page 33) *(Ursell et al. 2012)*. The microbiome samples were deposited directly in liquid nitrogen and conserved. Melanomas and nevi were subsequently removed surgically and parapsoriasis lesions were biopsied for histopathology.

4.2.2 STUDY III

The samples were taken in the intensive care ward by three educated nurses from the infants' abdomen and the incubators.

The same sampling protocol as in studies **I** and **II** was used. All samples were obtained from the same body sites at the same time of the day. The samples of the first day were taken after relocation of the infants from the delivery room and after the administration of primary antibiotics.

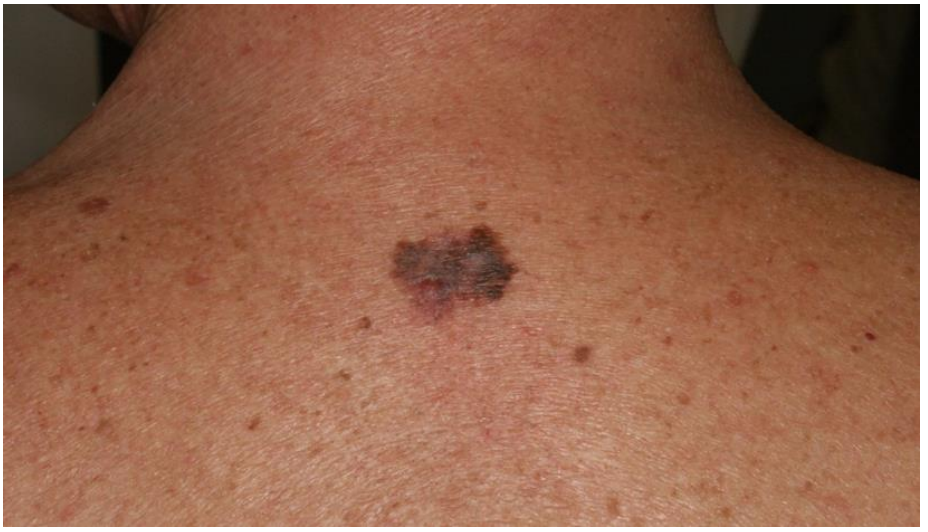

Figure 6 Superficially spreading melanoma, Breslow 0,85 mm, Clark IV, on the upper back of a patient, non-invasive microbiome swab samples were taken from the overlying skin (patient from study **I**), figure with permission of the patient

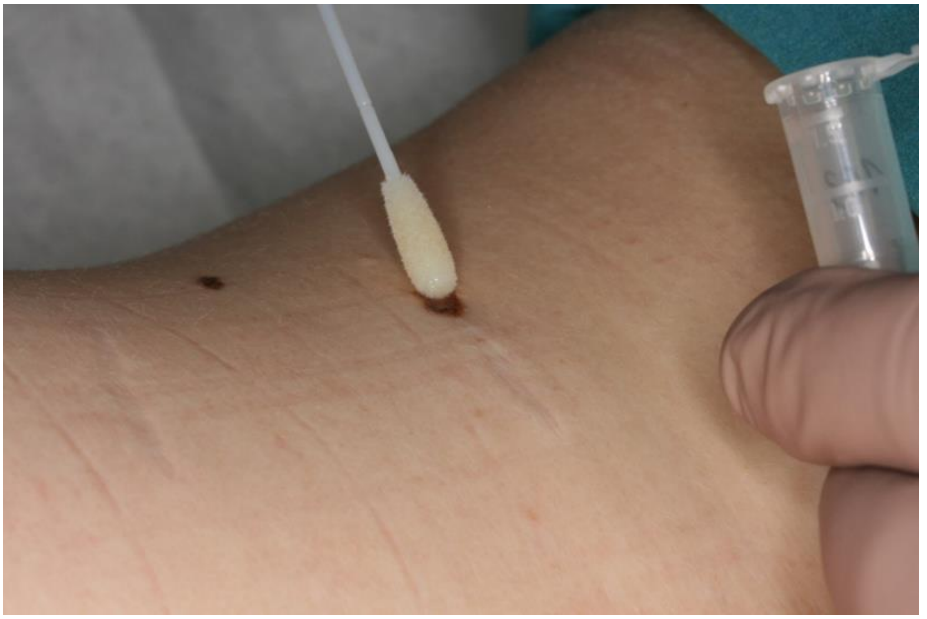

Figure 7 Swab sampling from a melanocytic nevus (patient from study **I**), figure with permission of the patient

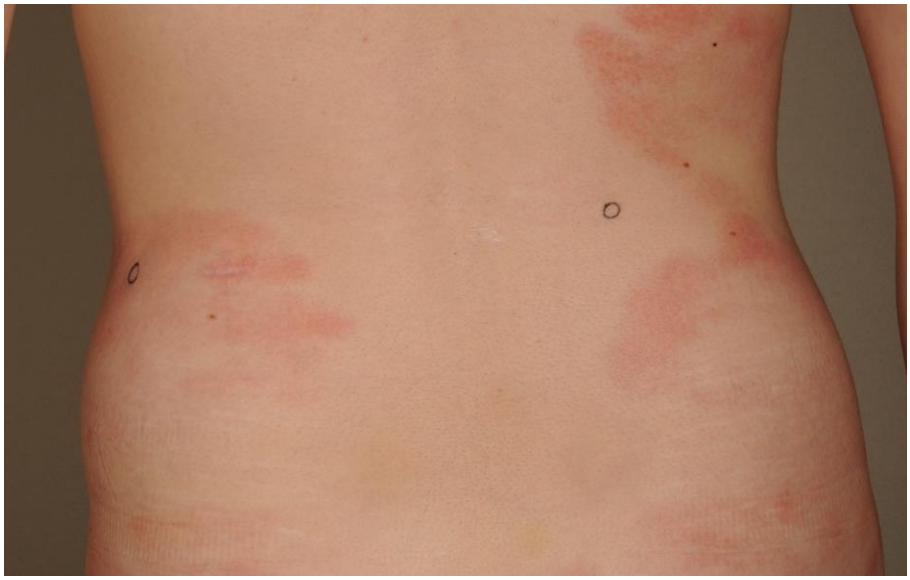

Figure 8 Microbiome swab specimens were collected from lesional parapsoriasis skin (left flank) and contralateral healthy skin (right flank), both marked with black circles (patient from study **II**), figure with permission of the patient

4.3 DNA EXTRACTION, PCR AMPLIFICATION AND SEQUENCING

All molecular studies were conducted at the Institute of Biotechnology, University of Helsinki, Finland. Laboratory analysis followed the general rules and regulations of the University of Helsinki.

The bacterial DNA was extracted from the conserved swab sticks by using the commercially available FastDNATM Spin Kit for Soil (MP Biomedicals) based on the manufacturer's instructions. In all studies the PCR amplification was performed with the ARKTIK Thermal Cycler (Finnzymes Diagnostics, Thermo Scientific) in two stages *(Grice et al. 2009)*.

The PCR protocol was carried in the following steps: In the first step 25 µl of technical replicates of each sample were analyzed. The template DNA quantity ranged from 23 to 58 ng. Primers of the first step targeted the variable V1-V3 regions of the bacterial 16S rRNA gene. The primer sequences were the following: pA (AGAGTTTGATCMTGGCTCAG) *(Lane et al. 1991)* and pD' (GTATTACCGCGGCTGCTG) *(Edwards et al. 1989)* and partial Illumina TruSeq adapter sequences (ATCTACACTCTTTCCCTACACGACGCTCTTCCGA TCT and GTGACTGGAGTTCAGACGTGTGCTCT TCCGATCT,
correspondingly) added to the 5' ends of the primers.

The subsequent PCR program was carried out in the following way: Firstly, DNA denaturation at a temperature of 98 °C, followed by 15 cycles at 98 °C for 10 s, 65 °C for 30 s, and 72 °C for 10 s, and finally an extension for 5 min at 72 °C. During every PCR run a PCR blank without any template DNA was included in the analysis. Before moving to the second step, a purification of the PCR products was carried out with Exonuclease I (Thermo Scientific) and Thermosensitive Alkaline Phosphatase (FastAP; Thermo Scientific). A sample (5 µl) of the first PCR step was used also for the second PCR step. During the second step the PCR run was carried out with full-length TruSeq P5 and Index-containing P7 adapters, and a PCR program identical to the first, except with 18 cycles. Finally, the PCR products were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter). In study **I** sequencing was carried out using the 454 GS FLX Titanium chemistry (Roche Diagnostics) and in studies **II** and **III** using the Illumina MiSeq platform in two separate sequencing runs. Additionally, two kit controls (extraction made without sample material) and three PCR blanks (PCR run without template DNA) were sequenced. The most important steps of the molecular study protocol are illustrated in figure 9, page 34.

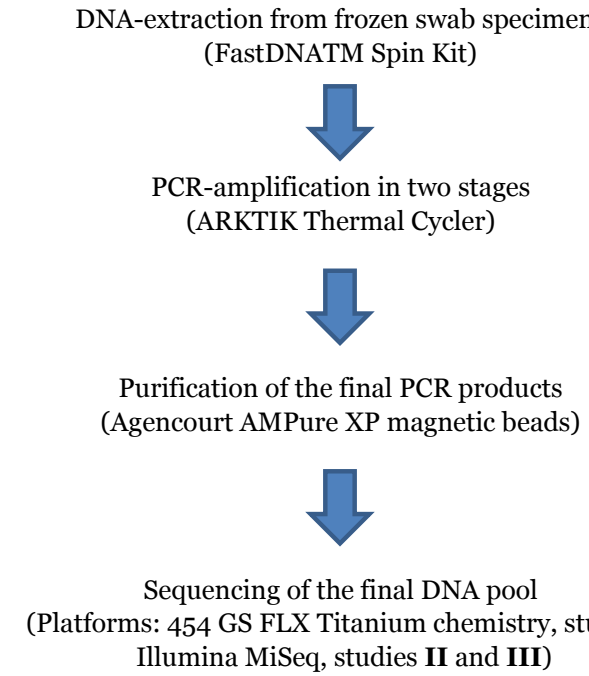

Figure 9 Protocol of DNA-extraction, PCR-amplification and microbiome sequencing

4.4 BIOINFORMATIC AND STATISTICAL ANALYSIS

In studies **I**-**III** the initial quality control and primer removals were carried out with cutadapt *(Martin et al. 2011)*. Sequence data was analyzed using mothur following the Standard Operating Procedures for 454 data (study **I**) *(Schloss et al. 2009)* and MiSeq-sequenced 16S rRNA data (studies **II** and **III**) *(Kozich et al. 2013)*.

In study **I** the following tag and primer sequences and any sequence reads were interpreted to have low quality and removed: any mismatches to the barcode or > 2 mismatches to the forward primer, any ambiguous nucleotides, homopolymers > 8 nucleotides and sequence length of < 200 nucleotides. In addition, the sequences were denoised using mothur's application of the PyroNoise algorithm *(Quince et al. 2009)*. An alignment of the sequences to the SILVA 123 database was carried out, and any sequences that did not align correctly were removed. Chimeric 16S rRNA sequences were removed using UCHIME *(Edgar et al. 2011)*. Finally, based on mothur's naïve Bayesian classifier (the RDP taxonomic outline as the training set against the SILVA 123 database), the good quality sequences were assigned taxonomical classifications. The sequences were grouped at 97 % sequence identity to OTUs (Operational Taxonomic Units). Thus, after all quality control steps, such as trimming, denoising, alignment, and removal of chimeric sequences and non-bacterial reads a sequence rarefication was carried out. Rarefaction is a technique to assess species richness from the results of sampling and allows the calculation microbial diversity based on the construction of rarefaction curves.

In studies **II**-**III** cutadapt was used to trim primers and low-quality ends of sequences from the data, with the parameters -q 30 for both reads and -m 200 for the forward, -m 180 for the reverse read. Pairing the reads, further sequence quality control and taxonomic classification were done with mothur (recommended procedure for MiSeq-sequenced 16S rRNA data) *(Kozich et al. 2013)*. All singleton OTUs were trimmed before further analysis.

The statistical analysis and data visualization were performed in all studies with R (R version 3.2.3, *R Core Team 2015*). In studies **I**-**II** a NMDS ordination was made with phyloseq using the Bray-Curtis distance and rarefied microbiome data *(McMurdie et al. 2013)*. All statistical comparisons were carried out in R using DESeq2 package *(Love et al. 2014)*. Alpha diversity indices were calculated in Vegan *(Oksanen et al. 2013)* package using R with Kruskal-Wallis rank sum (kruskal.test) and Wilcoxon rank sum tests (pairwise.wilcox.test).

In study **II** the staphylococcus sequences were further investigated using oligotyping *(Eren et al. 2013)*. Oligotyping is new a method of improving the accuracy of DNA sequence analysis. It is a computer-based method that may reveal masked bacterial diversity within the final operational units of classification or clustering based DNA sequencing approaches. Oligotyping

data is obtained with biostatistical tools (mainly entropy analysis) of variable sites in the sequences *(Eren et al. 2013)*. Compared with molecular databases or cluster analyses the investigated sequences in oligotyping primarily map to the same taxon *(Ramette et al. 2014)*. Differently from former methods that compare all positions in sequence reads, oligotyping uses only selective sequence information of the 16S rRNA gene variable sites *(Eren et al. 2015)*.

In study **III**, to enable diversity comparisons, an inverse Simpson value > 2 was determined as high diversity and ≤ 2 as low diversity.

4.4.1 ACCESSION NUMBERS OF THE EUROPEAN NUCLEOTIDE ARCHIVE

The sequencing data has been deposited to the European Nucleotide Archive: http://www.ebi.ac.uk/ena (*Toribio et al. 2016*, Human Microbiome Jumpstart Reference Strains Consortium 2010).

Accession numbers:
Study **I**: PRJEB7554, Study **II**: PRJEB15287, Study **III**: PRJEB12986

5 RESULTS

5.1 STUDY I

The microbiome data in study **I** was obtained from patients with cutaneous melanoma and the same patients' healthy skin, and from patients with benign melanocytic nevi and the patients' healthy skin. The data contained 2826 OTUs representing 483 genera, 40 classes and 25 phyla. 99 % of the OTUs belonged to four phyla: Firmicutes (mainly the classes Clostridia and Bacilli), Actinobacteria (class Actinobacteria), Proteobacteria (mainly Alpha-, Gamma-, and Beta-) and Bacteroidetes (class Bacteroidia). On the genus level *Propionibacterium* (50.49 %) was the most common, followed by *Staphylococcus* (13.41 %) and *Corynebacterium* (9.55 %).

The microbiome data of melanomas, melanocytic nevi and the corresponding control samples were similar to each other. There was a notable variation between individual patients. Statistically significant differences were observed in several bacterial taxa and in different body sites but none of these showed a consistent pattern. There were no significant differences in bacterial diversity between lesions and control samples (Wilcoxon rank sum test, $p = 0.99$ for both pairs). Group comparisons (4 groups, Inverse Simpson indices) of melanomas, melanocytic nevi and controls showed no statistically significant differences (Kruskal-Wallis rank sum test, $p = 0.3074$). The comparison of all melanoma samples against all melanocytic nevi was near to statistical significance (2 groups, Kruskal-Wallis rank sum test, $p = 0.0579$).

5.2 STUDY II

The microbiome data was obtained from patients with parapsoriasis and their contralateral healthy skin. It represented a total of 410 genera, 39 classes and 21 phyla. 89 % of the sequences represented four phyla: Actinobacteria (class Actinobacteria, 59.37 %), Firmicutes (mainly the classes Clostridia and Bacilli, 15.02 %), Proteobacteria (mainly Alpha-, Gamma-, and Betaproteobacteria, 12.46 %) and Bacteroidetes (mainly Bacteroidia, 2.16 %). At the phylum level 10.19 % of the sequences remained unclassified. At the genus level the most common were *Propionibacterium* (27.13 %), *Corynebacterium* (21.20 %) and *Staphylococcus* (4.63 %).

Staphylococcus species in the microbiome data were further investigated by oligotyping. This was carried out to more accurately investigate the staphylococcus sequences and to find out if the lesional skin microbiome in parapsoriasis contained *S. aureus*. Oligotyping is used as a novel method of

improving precision in DNA sequencing. The rationale behind it was, that oligotyping could possibly reveal staphylococcal bacterial sequences within the operational taxonomic units that would have been masked by the previously utilized traditional approaches. The sequences could be divided into five oligotypes. The most common were *S. epidermidis* (39.63 %), *Staphylococcus hominis* (33.34 %) and *Staphylococcus capitis* (21.50 %). The remaining two oligotypes (4.61 % and 0.92 %) could not be recognized, but the microbiome data did not contain *S. aureus*.

No significant differences were observed between control and lesional parapsoriasis samples (Kruskal-Wallis rank sum test; $p > 0.34$ for both indices). There were also no significant differences between small and large plaque parapsoriasis ($p > 0.57$ for both indices). Comparable to the results of study **I**, the microbiome data of the same patient's lesional and control skin were similar to each other.

5.3 STUDY III

The microbiome data was acquired from the abdominal skin of 13 preterm, very low birth weight infants. Here, the data showed a high cutaneous microbial diversity in nearly all infants at birth (with the exception of infant number 1). During intensive care treatment the diversity changed substantially in all of the infants. The relative abundances of bacteria in the microbiome data were comparable with previous findings of infant skin *(Capone et al. 2011, Costello et al. 2013)*. The most common phyla at birth were Firmicutes, Actinobacteria, Proteobacteria and Bacteroidetes. At the genus level the most common were *Propionibacterium*, *Staphylococcus* and *Corynebacterium*.

In both septic and non-septic infants a notable decrease in cutaneous bacterial diversity was observed within the first 3 weeks of intensive care. In septic infants this observation was made before antibiotic treatment for neonatal sepsis.

After the decrease in diversity the cutaneous microbiome was dominated by high abundances of one OTU, which was similar to *S. aureus*. (Staphylococcus Otu00007). The exception was infant number 7, where this OTU was not observed. A recovery of higher cutaneous microbial diversity was seen in 6 of the 12 infants (50 %) during intensive care treatment and after the ending of antibiotic treatment. There were no temporal associations between decrease of microbial diversity and sepsis. In septic infants the recovery of high cutaneous diversity was not connected with clinical recovery from sepsis. All infants had received prophylactic antibiotics immediately after birth. The antibiotic treatment given to septic infants showed no effect on the cutaneous microbiome. Inflammatory parameters (e.g. C-reactive protein, blood leucocyte count) were higher in septic infants, but showed no consistent

association with microbial diversity changes. The microbiome of the infants' incubators showed typical environmental bacteria. There were no significant differences between the incubators of septic or non-septic infants.

6 DISCUSSION

All studies of this doctoral dissertation were to our knowledge first original publications concerning the cutaneous microbiome in the investigated patient groups: melanoma and benign melanocytic nevi, parapsoriasis and in preterm very low birth weight infants during the first postnatal weeks of life. In studies **I** and **II** the main goal was to characterize the skin surface microbiome in malignant, premalignant and inflammatory skin changes. The investigated diseases were considered to be appropriate representatives of cutaneous malignant (melanoma) and benign proliferation (melanocytic nevi) and of a chronic inflammatory disorder with potential to progress into malignancy (parapsoriasis). Apart of the possible novel pathogenetic insights microbiome studies would give to our understanding of these disorders, we were also interested whether non-invasive skin swab techniques could offer the potential to be used as new diagnostic tools in the investigated skin diseases.

Study **I** is, to our knowledge, the first microbiome study about skin malignancies in general, and melanocytic nevi. Consequently, there was no former microbiome data to compare our results to. Microbiome studies have been performed in other non-cutaneous malignant diseases, e.g, gastrointestinal and oral cancer, where results have shown possible association of the malignant proliferation to microbiome changes and a bacterial dysbiosis *(Singh et al. 2017, Li et al. 2014)*. Based on reports about a possible association of cancer and the microbiome, it has been under discussion whether the microbiome changes are epiphenomena or play a concrete role in the pathogenesis of the malignant transformation *(Yu et al. 2015, Dmitrieva et al. 2017)*. We decided to investigate the microbiome in skin cancer under the premise that analogously the malignant cutaneous changes would have an impact on the skin microbiome. The skin microbiome has been previously investigated mainly in chronic inflammatory skin diseases, specifically atopic dermatitis, psoriasis and more recently acne *(Malik et al. 2017, Langan et al. 2017, Kelhälä et al. 2018)*. Studies on inflammatory skin diseases have shown, that the underlying inflammation of the skin has profound effects on the surface microbiome and also some disease-characteristic changes have been reported *(Langan et al. 2017, Gonzalez et al. 2017)*. Chronic inflammation has been linked to malignant transformation in a wide range of organ systems *(Chen et al. 2017, Sfanos et al. 2017)*. There have been reports that show, that the microbiome of the gut and the intestine influence cancer risk by promoting chronic inflammation *(Oke et al. 2017, Arthur et al. 2012, Yu et al. 2015)*. Under the rationale, that a chronic inflammatory state or malignant proliferation of the skin would induce microbiome changes, we hoped to gain more insights into the pathogenesis of the investigated skin disorders. Whereas melanoma and melanocytic nevi

were considered representatives for malignant and benign proliferation, respectively, and parapsoriasis was chosen to represent a chronic inflammatory condition with potential to malignant transformation.

Contrary to our expectations, the microbiome data of the disorders investigated in studies **I** and **II** seemed to have minimal or no effects on the surface microbiome. The observed changes were not statistically significant. Altogether the skin microbiome was similar to the results of previous studies on healthy skin *(Baviera et al. 2014, Dréno et al. 2016)*. We also observed a notable variation according to body site and individual patient *(Costello et al. 2009, Grice et al. 2009)*. Our results suggest accordingly, that in melanoma, melanocytic nevi and parapsoriasis, the skin surface microbiome on the investigated lesions (i.e. directly over the melanoma or parapsoriasis skin) does not differ from the same patient's normal healthy skin, even though the investigated skin disorders have strong components of malignant proliferation (melanoma) and inflammation (parapsoriasis) in their pathogenesis *(Mignonga et al. 2017, Sibbald et al. 2016)*. In study **I**, it was intended to concentrate on investigating the impact of malignant and benign proliferation on the skin microbiome. In comparison with melanocytic nevi, the melanoma samples showed a decrease in microbial diversity, but the difference was not statistically significant. Thus, the results of our study suggest that the investigation of the cutaneous microbiome may not be helpful in diagnosing melanoma or melanocytic nevi. Additionally, the results point out, that the proliferation of malignant melanocytic cells in the epidermis and dermis seems to have no significant impact on the surface microbiome. A hypothetical reason why this was not the case is, that in melanoma the malignant cells are mainly located in the dermis and primarily do not change the skin surface. This is mostly the case in superficial and non-ulcerative melanomas like the ones investigated in our study. It could thus be possible, that in deeper and more infiltrative melanomas differences in the skin microbiome are detectable. Additionally, it is possible that melanoma induced inflammation of the epidermal and dermal structures was in our superficial melanomas not sufficient to produce detectable microbiome changes. Skin microbiome changes have been reported mainly in inflammatory skin diseases affecting primarily the epidermis *(Yamazaki et al. 2017, Salava et al. 2014)*. Similar microbiome changes that have been observed in inflammatory skin disorders, may have been absent due to the fact that in superficial melanoma the tumor-induced inflammation is located mainly in deeper parts of the dermis *(Wick 2016)*. The epidermal changes, in comparison, can be marginal and consist of groups of malignant cells in the deeper epidermis *(Damsky et al. 2017)*. To also note, the investigated sampling areas in melanoma and nevi were relatively small (size of the lesions mostly < 1 cm^2) and thus the amount of bacterial DNA was limited. Thus, low bacterial DNA amounts could also have had an effect on the results of the microbiome data.

To our knowledge, study **II** is the first publication about the skin microbiome in parapsoriasis and cutaneous lymphoproliferative diseases. The

objective was to investigate skin microbiome changes in a skin disorder with a chronic inflammatory pathogenesis and a potential for malignant transformation into cutaneous T-cell lymphoma. Here, similarly to study **I**, we did not find statistically significant differences in the skin microbiome between lesional parapsoriasis and control skin. Similar to the results of study **I**, it seems that the inflammation in parapsoriasis, which is located primarily in the dermis, leaves the surface microbiome unaffected from microbiome changes. Immunological factors have been reported to strongly influence the cutaneous microbiome and therefore we expected changes to be visible in parapsoriasis *(Egert et al. 2017)*. The cutaneous microbiome in the patients of study **II** was however comparable to previous studies on healthy skin *(Egert et al. 2016, Oh et al. 2014, Dréno et al. 2016)*. Studies on inflammatory skin disorders have shown marked, and partly specific changes in the skin microbiome *(Yamazaki et al. 2017, Bjerre et al. 2017)*. The role of staphylococci has been particularly under investigation and there have been reports of *S. aureus* colonization during flares in atopic dermatitis *(Kobayashi et al. 2015, Bjerre et al. 2017)*. Based on these recent reports we expected to see likewise in parapsoriasis similar or at least detectable changes in the surface microbiome. In study **II** we could however not detect specific cutaneous bacteria associated with parapsoriasis. The chronic, T-cell dominated inflammation of parapsoriasis did not show to alter the cutaneous bacterial communities on lesional skin.

Parapsoriasis is regarded as a part of the continuum of lymphoproliferative diseases of the skin *(Baderca et al. 2014)*. It has been observed that parapsoriasis and cutaneous T-cell lymphomas are strongly influenced by immunological factors *(Rubio-Gonzalez et al. 2017)*. The possible association to dysfunctions in the immune system was another reason, why we expected to identify microbiome changes in parapsoriasis *(Krejsgaard et al. 2017)*. Also in view of the previously demonstrated associations of the microbiome and cutaneous immune defense *(Grice et al. 2011, Belkaid et al. 2014, 2016)*, we expected that the skin microbiome of the lesional parapsoriasis skin would differ from that of the same patient's normal healthy skin *(Zeeuwen et al. 2013)*. A possible reason why the microbiome data of study **II** showed no significant changes is the character of the T-cell infiltrate of parapsoriasis, which is different from inflammatory skin disorders *(Väkevä et al. 2005)*. Additionally there is an intact epidermal barrier in parapsoriasis, whereas in inflammatory skin disorders, such as atopic dermatitis the barrier is disrupted. The T-cell infiltration in parapsoriasis is mainly located in the dermis, and the epidermis contains only a limited amount of atypical T-lymphocytes *(Sibbald et al. 2016, Väkevä et al 2005)*. This is demonstrated in figure 4, page 23, and figure 5, page 24 (histopathology of normal skin and parapsoriasis). Similarly to the results of study **I**, the microbiome could have remained unaffected because relevant pathogenetic changes take place in deeper structures of the skin. The stratum corneum is known to remain mainly unaffected in parapsoriasis, which differentiates the disease from the formerly studied skin

disorders, where microbiome changes have been demonstrated *(Marrs et al. 2016)*. Microbiome studies of inflammatory skin disorders have been most widely conducted in atopic dermatitis *(Gonzalez et al. 2017)*. The pathogenesis of the disease is believed to be strongly influenced by the host's immune response and immunological factors (e.g. the Th2-shift). Changes in the balance of the microbiome and the host's cutaneous immune response have been shown to aggravate atopic dermatitis *(Yamazaki et al. 2017)*. A colonization of the skin with staphylococci (mostly *S. aureus*) and profound changes of the skin microbiome especially during disease flares have been reported *(Iwamoto et al. 2017)*. From our perspective, parapsoriasis was an ideal condition to expand the knowledge about the skin microbiome in inflammatory skin diseases. Parapsoriasis is a disorder combining inflammatory changes and possible malignant proliferation. Thus, we expected similar microbiome changes as reported in e.g. atopic dermatitis to be present also in parapsoriasis or at least some detectable alteration of the skin microbiome.

There have been reports that Staphylococcus enterotoxins (SAE) could be associated with the immunological dysregulation that is caused by cutaneous T-cell lymphoma *(Willerslev-Olsen et al. 2016, 2013)*. It was shown that Staphylococcal enterotoxin A (SEA) from the affected skin of CTCL patients induced in vitro IL-17 production in primary malignant T-cells of Sezary syndrome patients when cocultured with autologous nonmalignant T-cells (but not in monocultures of malignant T cells) *(Willerslev-Olsen et al. 2016)*. The patients of this study were however not defined in more detail. It is also noteworthy that in these studies *(Willerslev-Olsen et al. 2016, 2013)*, no microbes from healthy skin areas of the CTCL patients were analyzed and the authors did not specify the subtype of CTCL the patients were suffering from. Based on these observations we consequently decided to further investigate the staphylococcus sequences using oligotyping *(Eren et al. 2013)*. Parapsoriasis is known to precede mycosis fungoides, the most common form of CTCL, and we targeted this study to investigate whether the chronic parapsoriasis lesions would have a different skin surface microbiome compared to the individual's healthy (nonlesional) skin sites. The role of staphylococci has, to our knowledge, not been formerly investigated in parapsoriasis. Because of the fact that parapsoriasis is a lymphoproliferative disorder and often precedes cutaneous T-cell lymphoma *(Cerroni 2017, Väkevä et al. 2005)*, we expected to see changes in the skin microbiome. *S. aureus* was however not identified in the microbiome data of study **II**. The results suggest that the role of *S. aureus* or its enterotoxins seem not to be relevant in parapsoriasis. This could be explained by specific differences between cutaneous T-cell lymphoma and parapsoriasis (T-cell infiltrate, localized and systemic disease), but also the metabolomics properties of the skin microbiome *(Brandwein et al. 2016)*. It is also possible that unknown confounders explain our observations and therefore more studies regarding the role of the skin microbiome in parapsoriasis and cutaneous lymphomas

are needed. In future studies if changes in the microbiome in patients with T-cell lymphoma are reproduced, studying large plaque parapsoriasis would be reasonable to associate changes with disease progression as diagnostic tool.

In study **III** we targeted to characterize the cutaneous microbiome in preterm very low birth weight infants. Additionally, based on microbiome research methods we wanted to explore the possible link between skin staphylococcal colonization and neonatal sepsis. Study **III** is to our knowledge the first investigation of the skin microbiome during the first days of life in very low birth weight infants and in intensive care. Microbiome studies have been carried out in low birth weight neonates *(Pammi et al. 2017, Costello et al. 2013)* and the characteristics of the early cutaneous microbiome in healthy infants have been described recently *(Capone et al. 2011, Bokulich et al. 2016)*. We observed in our patient group a high cutaneous microbial diversity during the first days of life regardless of the way of delivery, prematurity causes or perinatal infections. In this regard our results differ from recent reports *(Garite et al. 2017, Dominquez-Bello et al. 2016, Pammi et al. 2017)*, where antibiotics, birth mode and diet were shown to influence the microbiome. Possible reasons could have been differences in the intensive care treatment and use of antibiotics, routine administration of prophylactic antibiotics and skin-to-skin care and feeding habits and possibilities in the intensive care ward. In our patient group bacterial diversity decreased during the first weeks of life independently from diagnosed infections or sepsis. The reasons for this remain unclear, but one possible explanation could be the antibiotic treatment in intensive care which is known to be effective against most of the skin colonizing bacteria *(Underwood et. al. 2017, Grice et al. 2008)*. Especially the primary antibiotics, which are given to most preterm infants in intensive care (and were also given to the patients of study **III**) are known to have high antimicrobial activity against the most bacteria that colonize normal skin *(Sweeney et al. 2017, Butin et al. 2017)*.

Coagulase-negative staphylococci, particularly *S. epidermidis*, are now regarded as the major pathogens in neonatal sepsis *(Shane et al. 2017, Li et al. 2013)*. It has remained unclear which factors change them from a member of the colonizing bacterial community to pathogens *(Tsai et al. 2014)*. Disruption of the skin barrier by medical devices (e.g. venous catheters) and antibiotic treatment have been suggested to initiate these changes *(Bokulich et al. 2013)*. Invasive medical devices could act as an infection port to commensal skin bacteria, whereas antibiotic treatment could cause a selection of virulent strains and suppress non-pathogenic bacteria *(Bradford et al. 2011)*. Other causes that might influence the microbiome are the infants' cutaneous prematurity or environmental factors (intensive care, incubator, mothers' skin) *(Chu et al. 2017, Power Coombs et al. 2013)*. It has been shown that cutaneous microbial diversity is positively correlated with skin-to-skin care and gestational age *(Pammi et al. 2017)*. Similarly to reports on inflammatory skin disorders with disruption and dysfunction of the epidermal barrier *(Byrd et al. 2017)*, it could be postulated that the epidermal prematurity of the

preterm infants has led to the detected microbiome changes (e.g. loss in diversity, staphylococcal domination). In study **III**, there was no association observed between microbial changes and septic infections in the intensive care ward. The microbiome was similar in infants with and without a diagnosed sepsis. Sepsis treatment with antibiotics showed no time-based association with the decrease in microbial diversity. Our results suggest that other factors, such as invasive medical devices and concomitant infections (e.g. gastrointestinal infections) are possible causes *(Shane et al. 2017, Giomerzis et al. 2014)*. In some studies neonatal sepsis has been linked to biofilm-associated catheter infections *(Cheung et al. 2010)*. All patients in the study had a central venous line and were treated according to intensive care guidelines, but there were no clinically detected catheter infections described. There have also been reports of the association of intestinal microbiome and neonatal sepsis *(Madan et al. 2012, Groer et al. 2015)*. We did not investigate this in study **III**, but the skin-intestine axis and its possible links will be interesting and possible research topics in future microbiome studies.

6.1 LIMITATIONS AND CHALLENGES

In studies **I**-**II** the microbiome in lesional and control skin sites were very similar, suggesting that the contralateral body sites did not vary from the skin lesions. However, it is possible that the approaches used in these studies were not suitable for detecting differences. Additionally, the studies were carried out in relatively small and heterogeneous patient cohorts and there was a considerable variation in the microbiome data. There were also no control groups used in the studies and this shortcoming was completely acknowledged.

In study **I** an additional challenge concerned the sample collection. The samples were obtained from patients before the excision of the skin lesion and therefore histologic diagnosis of each lesion as a melanoma or a benign nevus was only confirmed afterwards. Consequently, controlling for subject variables such as age or gender was a difficult task. Skin swab sampling above the lesions with melanoma suspicion had to be carried out carefully because of the possible sampling induced superficial ulceration. In cutaneous melanoma, ulceration is known to be an important prognostic factor *(Woodcock et al. 2017)*. In addition, due to the small lesion size, the sampling areas in melanoma and nevi were small in comparison to studies **II** and **III** (size of the lesions mostly < 1 cm^2). Here, the low amount of bacterial DNA could have affected the microbiome data.

Study **II** was undertaken to investigate the skin microbiome in parapsoriasis skin lesions and contralateral nonlesional skin from the same patients. Firstly, the approach to use the patient's healthy contralateral body sites as control was chosen, because it is known that there is great

interindividual variation in the microbiome of healthy skin and that the skin location is more important than ethnicity *(Ursell et al. 2012, Dréno B, et al. 2016, Perez Perez G et al. 2016)*. Secondly, the patients were by this time already diagnosed with parapsoriasis and our object was to investigate whether skin swab specimens could provide supplementary information to biopsy taking. This could have possibly decreased the need in patient follow up. It could have clinical consequences in the future, due to the fact that successive skin biopsies are often needed in the follow up of parapsoriasis *(Bordignon et al. 2011)*.

In study **III**, recognized limitations were a small patient cohort and irregularly carried out sampling during the intensive care period. A clinical setting such as this, made only a case study possible. Alterations of the guidelines in treatment (e.g. antibiotics) and patient handling (e.g. skin-to-skin care, feeding habits) would have been unethical.

There were many challenges encountered in the studies during sampling. Here the main problems concerned the standardization of skin swab sampling and choosing the different sampling sites. We recognize the microbiome sample analysis also as a challenge, especially the effects of contamination and the low quantities of bacterial DNA. The problem of contamination in skin microbiome studies has been identified frequently in the literature *(Kong et al. 2017, Salter et al. 2014)*. It has been shown that DNA extraction kits can be an important source of contamination *(Salter et al. 2014)*. Other potential sources are the reagents used in the work-flow and the laboratory personnel handling the samples. Even though negative controls (kit controls, PCR blanks) were sequenced in studies **I**-**III**, the removal of the contaminants may not have been enough to neutralize the contamination. It could have also been possible, that during the statistical approach some taxa of the original samples, which were not actual contaminants, were falsely removed.

6.2 FUTURE PERSPECTIVES

A new understanding of the cutaneous microbiome in skin disorders is developing *(Oh et al. 2016)*. Recent microbiome studies have suggested that inflammatory skin disorders could at least partially be connected to a dysbiosis of the microbial community *(Egert et al. 2016, SanMiguel et al. 2015)*. Importantly, several recent and as yet unpublished microbiome studies point to the fact that the complex metabolic properties of the microbiome are apparently more important than the microbiome profile as such *(Blum 2017)*. The metabolomics of the strains of the same bacterial species may vary *(Brandwein et al. 2016, Blum 2017)*. It would be important to consider metabolomics in future investigations of the cutaneous microbiome in skin diseases. Regarding the investigation of the microbiome of malignant skin changes it would be interesting to investigate the cutaneous microbiome in

deeper melanomas and to examine if recognized melanoma risk factors (family history, multiple or dysplastic nevi) have an effect on the microbiome *(Hawkes et al. 2016)*. It would also be important to investigate the microbiome in ulcerated melanomas and whether in these cases the microbiome data differs from our observations.

Microbiome studies are evolving also as an interesting novel method in research of inflammatory skin diseases. In some inflammatory skin disorders the reported microbiome changes have been relatively characteristic *(Langan et al. 2017)*. Therefore they might be useful in diagnosis, e.g. complementary to histopathology *(Powers et al. 2015)*. As a future perspective, exploring the microbiome might answer pathogenetic questions and have a role in diagnostics and treatments *(Kong et al. 2017, Sharon et al. 2014, Zeeuwen et al. 2013)*. Regardless of the results of study **II**, in the course of parapsoriasis a change of the skin microbiome is possible, as this is seen in inflammatory skin disorders like atopic dermatitis *(Bjerre et al. 2017, Shi et al. 2016, Gonzalez et al. 2017)*. Microbiome studies could possibly answer some aspects of the pathogenesis of parapsoriasis and in the future lead to new diagnostic methods. Therefore it would be important to further explore how the microbiome changes during the progression of the disease *(Talpur et al. 2017)*. Furthermore, it would also be interesting to investigate the microbiome in those parapsoriasis patients, where the disease has already developed into cutaneous lymphoma *(Eklund et al. 2016)*.

Microbiome studies have not been conducted largely in neonates and this field of microbiome research is just beginning. We recognize its potential and perhaps future studies will determine the role of the cutaneous microbiome in early skin development and investigate whether there are possible links between skin microbiome changes and developmental or other clinical complications. Comparable to the reported longitudinal differences in the fecal microbiome *(Aujoulat et al. 2014, Singh et al. 2017)*, it would be interesting to further explore how in preterm neonates the cutaneous microbiome changes during early life and whether microbiome changes are linked to problems in early development.

7 CONCLUSIONS

Study I

The microbiome of melanomas and melanocytic nevi was similar to healthy skin. There was a notable variation based on body site and individual patient, which suggests that microbiome studies may not be helpful in diagnosing melanoma or melanocytic nevi. Melanoma samples showed a decreased microbial diversity when compared to melanocytic nevi, but this difference was not statistically significant. It would be important to investigate the microbiome in deeper or ulcerated melanomas and explore if melanoma risk factors alter the microbiome.

Study II

No differences were found between the microbiome of parapsoriasis and the same patients' healthy skin. This suggest that there are no specific associations of cutaneous bacteria, and parapsoriasis does not alter the microbiome of human skin. The role of *S. aureus* seems not to be relevant in parapsoriasis. It would be important to explore microbiome changes during progression of the disease and to investigate the microbiome in cutaneous lymphomas.

Study III

Very low birth weight infants in intensive care showed a high cutaneous microbial diversity during the first days of life regardless of the way of delivery, prematurity causes or perinatal infections. Microbial diversity decreased during first weeks of life independently from sepsis or antibiotic treatment. Other factors such as invasive medical devices are likely to play a role here. It would be important to further explore how the cutaneous microbiome changes during early life and whether microbiome changes are linked to problems in development.

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- vanha suomalainen sanonta
"The beginning is always difficult, in the end stands the thank"
- old Finnish proverb

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Sincerely,
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10 ORIGINAL PUBLICATIONS I-III