Aus der Klinik für Dermatologie, Venerologie und Allergologie

der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

# Entwicklung neuer Methoden für kompartimentspezifische Analysen des Mikrobioms von Haarfollikeln und assoziierten Entzündungsmediatoren.

# Development of new methods for compartment-specific analyses of the hair follicle microbiome and associated inflammatory mediators.

zur Erlangung des akademischen Grades

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## 1. Preface

Some of the results presented in this thesis and reviewed literature on the microbiome of scalp hair follicles have already been published in:

Katarzyna Polak-Witka*, Andria Constantinou*, Rolf Schwarzer, Johannes Helmuth, Alexandra Wiessner, Sabrina Hadam, Varvara Kanti, Fiorenza Rancan, Annette Andruck, Claudia Richter, Annette Moter, Anke Edelmann, Lidia Rudnicka, Ulrike Blume-Peytavi, Annika Vogt. Identification of anti-microbial peptides and traces of microbial DNA in infrainfundibular compartments of human scalp terminal hair follicles. Eur J Dermatol. 2021 Feb; 1;31(1):22-31. doi: 10.1684/ejd.2020.3948.

*****equal contribution

Katarzyna Polak-Witka, Lidia Rudnicka, Ulrike Blume-Peytavi, Annika Vogt. The role of the microbiome in scalp hair follicle biology and disease. Exp Dermatol. 2020 Mar; 29(3):286-294. doi: 10.1111/exd.13935.

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## 2. List of Abbreviations:

HF, hair follicle; C. acnes, Cutibacterium acnes; FISH, fluorescence in situ hybridization; HBD2, human β-defensin 2; IL-17A, interleukin-17A; AMP, antimicrobial peptide; Tregs, T regulatory cells; FD, folliculitis decalvans; AA, alopecia areata; AGA, androgenetic alopecia

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## 5. Abstract (in German and English)

### 5.1 Zusammenfassung (Abstract in German)

**Hintergrund:** Untersuchungen zu regulatorischen Effekten mikrobieller Kolonisation weisen darauf hin, dass Haarfollikel wichtige Nischen für einen Austausch mit dem Immunsystem der Haut sind. Während Besiedlung des oberflächlich gelegenen Haarfollikelinfundibulums weitreichend beschrieben ist, bilden Nachweise bakteriellen Materials auf der Ebene der Dermis die Grundlage für diese Arbeit. Im Fokus stehen die Wulstregion und der Bulbus, deren immun-priviligierter Status Voraussetzung für einen regulären Haarzyklus ist. Aufgrund der Komplexität sind Möglichkeiten zur nicht-invasiven Gewinnung von repräsentativem in vivo Material eine wesentliche Voraussetzung für weitergehende Untersuchungen.

**Ziel:** Ziel dieser Arbeit war daher die Entwicklung von Protokollen zur Probengewinnung für zukünftige klinische Studien an Patienten. Es ging insbesondere um die Extraktion und Sequenzierung von bakterieller DNA aus infra-infundibulären Kompartimenten von Anagenhaarfollikeln der Kopfhaut und ergänzende Biomarker-Analysen.

**Methoden:** 16S rRNA Sequenzierung erfolgte nach Anreicherung von bakterieller DNA aus Anagenhaarfollikeln, welche zwölf gesunden Probanden (6 weiblich, 6 männlich) sowohl frontal als auch okzipital durch Zupfen von Haarwurzeln entnommen wurden. Ergänzend erfolgte Fluoreszenz in situ Hybridisierung (FISH) an Schnitten von gezogenen Haarwurzeln. Basierend auf immunhistochemischen Färbungen von Immunzellen und Entzündungsmarkern erfolgten ELISA Quantifizierungen für IL-17A und humanes beta-Defensin 2 (HBD2) aus Proteinextrakten. Zusätzlich wurden Standard-Abstrichuntersuchungen und nicht-invasive Messmethoden, darunter Fluoreszenzfotografie als Maß für die Besiedlung mit *Propionibacterium* (*Cutibacterium*), pH- und Sebum-Messungen, durchgeführt.

**Ergebnisse**: Obwohl es sich um infra-infundibuläre Kompartimente handelt, konnte bakterielle DNA reproduzierbar aus gezogenen Haarfollikeln angereichert werden. *Lawsonella clevelandensis*, *Staphylococcaceae* und *Propionibacteriaceae* wurden als häufigste Stämme identifiziert. Mittels FISH konnten Biofilme von *Cutibacterium acnes* (früher: *Propionibacterium acnes*) und *Staphylococcus* sp. Kolonien nachgewiesen werden, auch wenn diese zur Tiefe hin deutlich abnahmen. Immunohistochemie und ELISA zeigten IL17A, und noch deutlicher HBD2 in tiefen Kompartimenten.

**Schlussfolgerung:** Das Ziehen von Haarwurzeln kann eine Alternative zur Kopfhautbiopsie sein. Das Vorgehen ist kaum invasiv und kann auch wiederholt am selben Individuum durchgeführt werden kann. Die reproduzierbare Isolierung von bakterieller DNA aus infra-infundibulären

Anteilen deutet darauf hin, dass Kolonisierung tiefer reicht als weithin angenommen. Auch wenn die Daten zur Expression von anti-mikrobiellen Peptiden wie HBD2 keinen kausalen Zusammenhang beweisen, könnten Störungen der mikrobiellen Besiedlung und Antworten des daruntergelegenen Gewebes Einfluss auf Haarwuchs und den Verlauf einiger chronisch entzündlicher Haarerkrankungen haben.

### 5.2 Abstract in English

**Background:** Basic scientific studies on the regulatory effects of microbial colonization on immune cell responses suggest that hair follicle openings provide a unique niche for continuous exchange with the cutaneous immune system. While the presence of microbiota in the upper follicle is acknowledged, recent bacterial findings at the dermal level encourage a closer look at lower compartments encompassing the bulge and the bulb - two structures featuring a relative immune-privileged status that is indispensable for physiological cycling. Yet, further investigations on host-microbiome interactions in health and disease strongly depend on the availability of *in vivo* material, indicating a high need for low-invasive sampling methods.

**Objective:** The aim was to establish protocols for extraction and sequencing of bacterial DNA from infrainfundibular compartments of anagen hair follicles, and to add biomarker analyses in order to establish a clinical study set-up suitable for larger studies on patients.

**Methods:** 16S rRNA sequencing was performed on DNA extracts enriched for bacterial DNA from anagen hair follicles plucked from frontal and occipital sites of 12 healthy volunteers (6 females, 6 males) and complemented by fluorescence in situ hybridization (FISH). Based on immunohistochemical stainings for a range of immune cells and inflammatory markers, IL-17A and human β-defensin 2 (HBD2) were selected for confirmation by ELISA on protein extracts from plucked hair follicles. Standard swab analyses and non-invasive readouts including *in vivo* fluorescence imaging of porphyrins as readout for *Propionibacterium* (*Cutibacterium*), scalp surface pH and sebum measurements were added.

**Results**: Enrichment for bacterial DNA from plucked hair follicles gave reproducible results, even though the material was obtained from infrainfundibular portions. *Lawsonella clevelandensis*, *Staphylococcaceae* and *Propionibacteriaceae* were identified as the most abundant bacteria. Accordingly, FISH captured biofilm structures formed by *Cutibacterium acnes* (formerly *Propionibacterium acnes*) and *Staphylococcus* sp. colonies below the infundibulum, although a distinct gradient towards minimal traces in very deep compartments around the bulb was observed. Immunohistochemistry and ELISA revealed the presence of IL-17A, and even more distinctly, of HBD2 in deeper portions of the hair follicles.

**Conclusion:** Hair pulling may be an alternative method to scalp biopsies. Its low invasiveness may help to collect material from larger study groups and even repetitively from the same individual. Reproducible isolation of bacterial DNA from infrainfundibular portions indicates that colonization may reach deeper than expected. A causal relationship to defense molecules like HBD2 remains to be established, but the presence of microbiota in proximity to vital follicular

structures may be of relevance for physiological follicular cycling and may also influence the course of some chronic inflammatory hair diseases.

## 6. Introduction

### 6.1 Anatomy and physiology of the hair follicle

More than 100,000 hair follicles developed during embryogenesis undergo life-long cyclic hair growth on the human scalp (1). Tubular invaginations of the epidermis create follicular canals that greatly increase the skin surface area. With respect to the aspect of microbial colonization, the formation of pockets that reach deeper skin layers is of special relevance (2). While sebum excretion contributes to upward flow transporting lipophilic compounds like vitamin E or cell detritus towards the skin surface, the transfollicular pathway significantly contributes to downward penetration processes. Here, the infundibulum represents an important reservoir for compounds entering from the outside, where they remain for prolonged time periods, e.g., up to several days in the case of 320 nm nanoparticles (3-5). This is especially relevant for particles in the micro- and nanometer range, which preferentially aggregate in hair follicle openings and frequently show a capacity for deeper penetration, depending on the size and physicochemical properties (3).

Thus, the upper follicular portion, the infundibulum (Figure 1), is not only filled with debris and sebum, but also with various microbiota encompassing bacteria, yeasts/fungi, viruses and mites (2, 6). Dense infiltration of antigen-presenting cells (Langerhans cells (LCs)) within its epithelium points toward a key strategic role of the HF part in the crosstalk between the local immune system and the external environment. The infundibulum along with structures located below, the isthmus and bulge, comprise the permanent parts of the HF, which do not undergo significant changes during the cyclic hair growth (2, 7).

Situated at the lower levels of this compartment, the bulge is a key structure, providing a niche for epithelial and neuroectodermal stem cells, and different immature cell populations including Langerhans cells and mast cells. Destruction of this compartment of the outer root sheath typically leads to hair loss, as observed in cicatricial alopecia. The isthmus is the portion located between the sebaceous gland duct opening and the bulge, relatively devoid of distinctive features (1).

The transient cycling component of the HF can be divided into the suprabulbar area and the bulb, which determines the thickness, length and pigmentation of the hair shaft by proliferation of matrix keratinocytes and melanin synthesis. Matrix keratinocytes are located on the dermal papilla, which is a composition of specialized mesenchymal cells of special inductive properties. A capillary loop going into the papilla of terminal hair follicles provides nutrition for the papilla and the overlying matrix cells. Dermal papillae of vellus hair follicles are much smaller and do not have capillaries (1).

The hair follicle is composed of concentric mesenchymal and epithelial cell layers, ensheathed by the fibrous sheath. The epithelial compartments of the hair follicle consist of the matrix cells, the inner root sheath and the outer root sheath. The inner root sheath, extending from the base of the bulb to the isthmus level, includes three keratinizing layers, the Henle, Huxley and cuticle. The inner sheath is thought to determine the cross-sectional and longitudinal shape of the hair. The outer root sheath extends higher, reaching the entry of sebaceous duct. Its cells release a large variety of mediators and hormones. The outer and inner root sheaths surround the hair shaft. The mesenchymal sheath stays separate from the epithelial sheath by a vitreous or basal membrane. A dense vascular network and free nerve endings encompass the hair follicle, which together with the sebaceous gland and the arrector pili muscle is called the pilosebaceous unit (1, 2).

The overall anatomical structure between different types of hair follicles, i.e., terminal, intermediate and vellus hair follicles, is rather similar. However, in the aspect of reservoir function and hosting skin microbiota, the capacity of large terminal hair is significantly larger than the vellus follicle. The anagen bulb of the terminal hair follicle can reach deep subcutaneous tissue, in contrast to the vellus hair follicle only reaching the upper reticular dermis. Furthermore, the relation between the size and activity of the sebaceous gland in the pilosebaceous unit differs in the different body regions (1). On human scalp, terminal follicles produce long, thick, pigmented hair, whereas only 6.15 ± 4.6% are vellus follicles, which form short, thin, unpigmented hair (1, 8).

HFs exhibit cyclic growth, which can be divided into the growth phase (anagen) lasting for several years on the scalp, a transitional period of a few weeks (catagen), and finally the resting phase (telogen), which on average lasts a few months and ends with the shedding of the old hair (3). While the upper follicular portion, being in direct contact with the external environment and skin microbiota, features high immune cell trafficking, the bulge along with the anagen bulb are considered as sites of a relative immune-privileged status. This milieu features low number of immune cells and the presence of mediators of immunosuppressive characteristics such as proopiomelanocortin-derived neuropeptide (α-MSH) and “no danger” signals, CD200. Furthermore, it is characterized by low or absent expression of major histocompatibility complex (MHC) class Ia/β2 microglobulin, making self-peptide presentation ineffective or impossible, and by secretion of immunosuppressants such as transforming growth factor β. In addition, the suppression of intrafollicular antigen-presenting cell, perifollicular natural killer (NK) cell and mast cell functions by downregulation of MHC class II on HF Langerhans cells, downregulation of NKD2G ligands and upregulation of macrophage migration inhibitory factor, respectively, can

be observed (9). A disruption of the immune-privileged milieu within either the bulb or the bulge seems crucial in the pathogenesis of alopecia areata or scarring alopecias, respectively (10, 11).

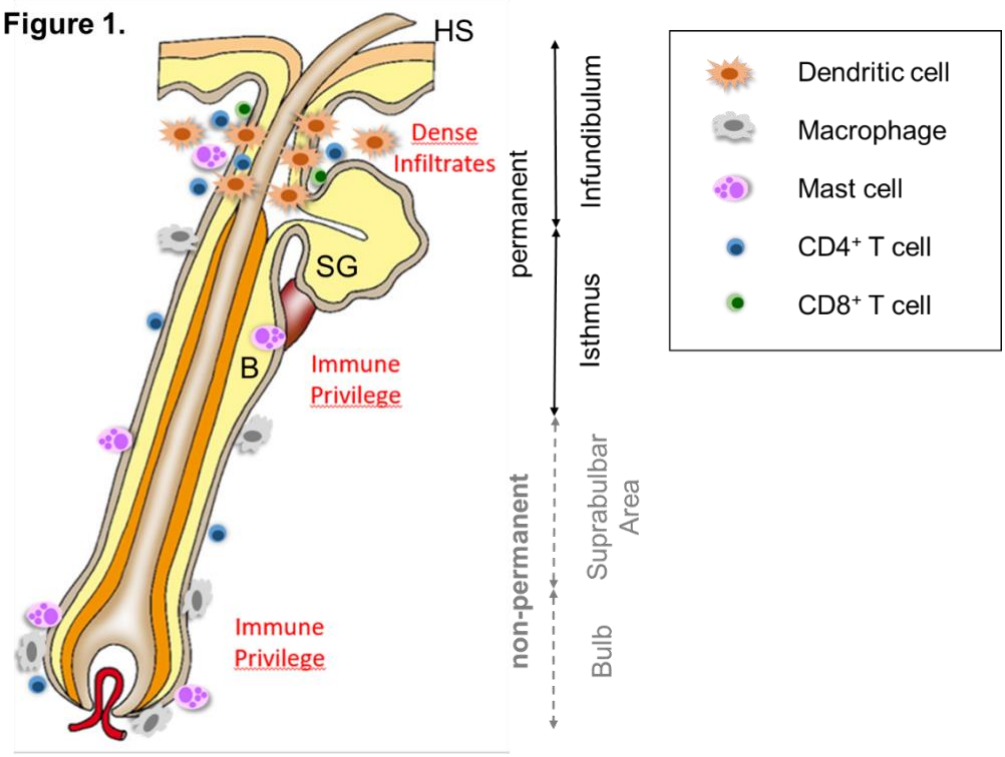



*Figure 1.*

*Architecture and immune infiltrates of the pilosebaceous unit*

*The upper part of the pilosebaceous unit, i.e., infundibulum, isthmus, bulge, and sebaceous gland are the permanent structures, while the deeper portions including the bulb and the suprabulbar area are non-permanent during the hair cycle and start regressing in the catagen phase. High immune cell trafficking with dense infiltrate of antigen-presenting cells (dendritic cells) and CD4+ cells is found within the infundibulum. Mast cells and macrophages are rather evenly distributed over the entire length of the follicle in the connective tissue sheath, outside the outer root sheath. CD8+ cells are present mainly at the infundibulum level. The infundibulum hosting rich microbiota is densely infiltrated by immune cells, in contrast to the bulge and bulb featuring a relatively immune-privileged status. HS, hair shaft; B, bulge; SG, sebaceous gland. Reproduced with kind permission of the publisher John Wiley and Sons (12).*

## 6.2 Relevance of the skin microbiome

### 6.2.1 Composition of skin microbiome; intra- and interindividual variabilities

It has been estimated that one square centimetre of human skin including its appendages hosts around one billion microbiota, i.e., various bacteria, fungi, viruses and mites, forming a community called the skin microbiome (6, 13). There are significant differences in microbiome composition between particular individuals and even in the same individual across skin areas, which have been attributed to variations in moisture, temperature, pH and sebum excretion (14, 15). A wide variety of exogenous factors affect the skin microbiome, including air temperature and humidity, sun exposure, antibiotic intake, lifestyle behaviours such as smoking cigarettes and the application of skin cosmetics, and vaginal or caesarean type of birth. A presumed genetic susceptibility to some microorganisms, along with the above-mentioned factors, yields distinctive interpersonal variations that may even be used in criminal detection in the future (5-7).

The vast majority of skin bacteria is assigned to four phyla: Actinobacteria, Firmicutes, Proteobacteria and Bacteroidetes. *Corynebacteria*, *Propionibacteria* and *Staphylococci* are among the most abundant genera. Detailing different body sites, *Propionibacteria spp.* and *Staphylococci spp.* predominate in sebaceous sites, while *Corynebacteria spp.* predominate in moist sites, although *Staphylococci spp.* are also represented. A mixed population of bacteria inhabits dry sites, with a greater prevalence of β-Proteobacteria and Flavobacteriales (16). Considering bacterial community membership and community structure, the most consistent sites over time are the sites that are at least partially occluded, e.g., the external auditory canal or the nare, whereas sites exposed to external factors are of a greater diversity and tend to be less stable over time, e.g., the forearm and the plantar heel. Compared to the microbiome of the mouth and the gut, the skin microbiome has the greatest variability over time (17). Besides bacteria, fungal organisms also inhabit human skin, with *Malassezia spp* being predominant. (18). *Demodex* mites (such as *Demodex folliculorum* and *Demodex brevis*) are also considered part of the normal skin microflora (19). Furthermore, viruses are likely to be an important part of the skin ecosystem; however, techniques allowing their identification are only just being developed (20).

### 6.2.2 Bacterial capability of deeper penetration

Epithelial surfaces are colonized by planktonic bacteria, as well as by bacteria forming complex communities named biofilm. Biofilms are matrix-encased cell aggregations, ranging in size from small aggregates of ten cells to large structures encompassing hundreds of thousands of bacteria.

There are many advantages of thriving in biofilm formation, including the protection against the innate immune system and the normal shedding of skin cells, and therefore it enables survival in a more hostile environment and the occupation of lower skin compartments (18). It has been identified that *Staphylococcus epidermidis* (*S. epidermidis*) can form biofilm between the three to ten outer layers in the stratified epithelium in normal skin (18). Macrocolony or biofilm built by *Cutibacterium acnes* (*C. acnes*) was found in the deeper parts of sebaceous follicle adhering to the hair shaft in the skin of patients with acne and less frequently in normal skin (9). In addition, microbes may be armed with special enzymes that facilitate invasion of the tissue and deeper penetration (3).

To learn about how and where microbes get in touch with viable cells, various aspects have to be taken into consideration. Since bacteria are in the low micrometer range, their outreach along the hair follicle canal should be limited to the lower portions of infundibulum and sebaceous glands. This is a typical site of *Cutibacterium* colonization, not only in facial acne but also in the scalp hair follicle (21-23). Yet, whereas the upper parts of the infundibulum are lined with thick stratum corneum, its lower portions are overall perceived as more permeable areas (24).

As we have learned from studies on particle penetration in the skin, microparticles mainly remain on its surface, but they may also reach the deep portions of the infundibulum in the scalp hair follicle. Deeper penetration of nanoparticles is also observed to a limited extent and is mainly determined by their physicochemical characteristics (25-27). However, a slight disruption of the skin barrier may lead to translocation of particles into the viable cells and cellular uptake of particles followed by immune reaction (28). Although the skin features a strong barrier function and largely limits the translocation of larger particles and particulate compounds, such events take place regularly and are mainly observed in skin furrows and hair follicles. Dense infiltrates of Langerhans cells within the infundibulum may point toward a crucial role of this structure (29). Furthermore, new observations have been made based on studies on transcutaneous vaccinations. The results have shown that a mild inflammatory stimulation of Langerhans cells switches them to an active mode, and may lead to an increased uptake of larger molecules through the barrier of the skin (30).

### 6.2.3 Host and microbiome cross-talk

Skin surface and hair follicles are areas of an intensive antigen presentation, inhabited by a rich microbiome changing with age, and concomitantly exposed to pathogen invasion. How exactly immunity discriminates between pathogens and normal residential flora remains to be elucidated. The reason why a common commensal such as *S. epidermidis* may cause inflammation, whereas a pathogen such as *Staphylococcus aureus* (*S. aureus*) colonizes the skin asymptomatically, also remains to be addressed. Currently it is known that the skin microbiota interacts with the local immune system and supports its proper function (31).

A vast range of immune cells, i.e., both innate cells (such as macrophages, dendritic cells including Langerhans cells, mast cells, and innate lymphoid cells) and adaptive lymphocytes (including CD4+ and CD8+ T cells), are distributed in the skin and around skin adnexa, close to microbial organisms (Figure 2) (32). The complex interaction networks between residential commensals and the immune system are currently only known to a small extent. Langerhans cells (LCs), which densely infiltrate the epidermal layer and the outer root sheath (ORS) of the upper follicular part, are able to protrude dendrites between keratinocytes and further to capture antigens presented on microbes (33). Under normal conditions, LCs interact with skin-resident memory T regulatory cells and promote their activated state and proliferation. The T cells presumably feature the memory for antigens that they encounter through epithelium and seem responsible for the determination of peripheral tolerance to microbiota. Contact of LCs with a pathogen, on the other hand, may induce pathogen-specific resident effector memory T cells to proliferate (34). Furthermore, keratinocytes or other antigen presenting cells can also engulf microbial antigens, or the antigens may diffuse across the epithelium in a passive way, especially in the deeper portion of the infundibulum. A higher permeability of this region results from the shift of differentiation from epidermal towards trichilemmal (32). Shifts in the composition of microbiome may be sensed by skin-resident CD103+ dendritic cells (DCs), which subsequently promote the inflow of IL-17A+CD8+T cells into the epidermal layer and the release of cytokines such as IL-17a and IFN-y, giving no overt signs of inflammation. These T cells can further be tuned by IL-1-producing CD11b+ DCs to release cytokines and enhance the defense capabilities of keratinocytes by production of antimicrobial peptides (AMPs). Recent studies have shown that colonization of murine skin with different commensals results in marked differences in the profile of produced cytokines, e.g. *S. epidermidis* caused the strongest activation of 17A+CD8+T cells (35). Hence, the skin immunology may be affected by microbiome composition (7, 35). Decreased expression of IL-17a in a germ-free murine model and restoration of its normal production by skin

colonization with *S. epidermidis* highlighted an association of this cytokine with cutaneous commensals (35).

Skin microbiome has a profound and multifaceted impact on host defense against pathogens. Colonization of the skin by commensals protects it from the occupation of the same niche by pathogenic species. The diminution of nutrients and an influence on skin pH decrease the attractiveness of human skin for pathogens (17). Furthermore, microbiota stimulate the production of cytokines participating in immune response, both its initiation and further maintenance, and upregulate the expression of components of the complement system. They promote IL-1 signaling, and in consequence, effector T cell function. The role of residential microbes was presented in a model of skin infection caused by *Leishmania major*. Infected germ-free mice had severely impaired effector responses, and their protective immunity along with IL-1α production were restored by colonization of the skin with *S. epidermidis* (31, 35). In addition, commensals help reduce the extent of inflammation and promote repairing of tissue damage. Lipotechoic acid, which is a part of the cell wall of *S. epidermidis*, inhibits the release of pro-inflammatory cytokines by binding to toll-like receptor 2 (TLR2) located on keratinocytes (36). The connection of skin microbiome with host immune response has been shown by the analysis of gene expression in germ free mice compared to mice colonized with normal skin microbiota. Meisel et al. found upregulated expression of genes encoding innate immune response proteins (e.g., TLRs, AMPs, and the complement cascade) and genes linked to cytokine expression (e.g., involved in IL-1 signaling) in mice reared with normal microbiome. In addition, a relationship was found between the presence of microbiota and expression of genes associated with proteins involved in keratinocyte differentiation and cornification (37).

Antimicrobial peptides released by keratinocytes and sebocytes defend the host against pathogens and also supervise the skin microbiome composition. These peptides are directed not only against bacteria but also against fungi, parasites and viruses. They can directly kill microorganisms and exert chemotactic activity. Some AMPs are constitutively expressed, while the others are released upon stimulation by microbial components and pro-inflammatory cytokines (e.g. HBD2) (38). Mast cells found as hair growth regulators and immunomodulating cells can also be attracted by HBD2 (39-41). Toll like receptors (TLRs) on keratinocytes, belonging to pattern recognition receptors (PRRs), link to pathogen-associated molecular patterns (PAMPs), including peptidoglycan and the above-mentioned lipotechoic acid of Gram-positive bacteria, lipopolysaccharide (LPS) of Gram-negative bacteria or mannan from fungal cell walls. Activated receptors may either induce the immune reaction, or the release of cytokines, chemokines and AMPs, or conversely, may suppress the inflammatory response (17).

Expression of diverse AMPs has been found in the hair follicle structures. Some of the AMPs are constantly released, while the release of others is induced via inflammation. Human β-defensin 1 and 2 are released in a steady state by all epidermal suprabasal strata, the sebaceous gland and by the distal and medial portion (including the area of the bulge) of the outer root sheath of the follicle. These AMPs are also weakly expressed within the proximal part of the ORS, as well as the inner root sheath (IRS) (42). Specific microbiota-derived agonists (e.g. LPS) induce the production of β-defensin 2 via stimulation of TLR2, TLR4 and TLR5 in murine follicles (43). Psoriasin, directed against *Escherichia coli* and *Candida albicans*, was found within the epidermal layers and the distal ORS of the scalp HF, while RNase7, with a broad-spectrum antimicrobial activity, was expressed throughout the entire ORS. In addition, both Psoriasin and RNase7 (which are proteins, but according to their function are classified as AMPs) are identified in sebaceous glands and can be produced in response to classic microbial antigens (44). LPS activates TLR4 in the scalp HFs, which results in the release of psoriasin and RNase7, while flagellin (a Gram-negative bacteria component) stimulates TLR5 and additionally the release of RNase7 (44). In the IRS, the presence of bactericidal/permeability-increasing protein, an AMP with a cytotoxic activity against Gram-negative bacteria, has been identified (45). To summarize, the upper follicular portions, being in close proximity to the external environment and microbiota, feature the highest expression of AMPs. These peptides protect the host against pathogen invasion, including the penetration down the HF canal, and presumably participate in shaping the skin and hair follicle microbiome niche (44).

Interestingly, AMPs can also be released by skin colonizing microbiota. As reported, *S. hominis* and *S. epidermidis* produce lantibiotics, working synergistically with human AMP LL-37 to prevent *S. aureus* colonization (40). Furthermore, some strains of *S. epidermidis* may express serine protease glutamyl endopeptidase (Esp), which causes dissolution of proteins crucial for *S. aureus* to form biofilm structures. However, the commensal microbiota can also favor the colonization of the skin by pathogenic microorganisms. *Cutibacterium* spp. have been noted to be able to stimulate the formation of bacterial aggregates by *S. aureus* in specific circumstances (14).

Morphogenesis of the HF and microbial colonization of the murine skin in the neonatal period were found to be critical for the influx of CD4+Foxp3+ regulatory T cells (Tregs) to the HF, and thus for the determination of immune tolerance to bacterial commensals. Tregs are a special subtype of T cells that have the capability to suppress autoimmune response and maintain immune equilibrium in peripheral tissues. Recent research has shown that, based on a murine model and also human skin explants, skin microbiota can induce chemokine Ccl20 expression in the infundibulum of a forming HF. The receptor for this chemokine (Ccr6) was found expressed

on Tregs both in the thymus gland and neonatal skin, and the Ccl20-Ccr6 pathway turned out to be crucial for the migration of the thymic Tregs to the HF (7, 46). A temporary interruption of the Treg inflow in the neonatal murine skin or postponing of the skin colonization by bacterial commensals for adulthood resulted in inflammatory response and a failure to determine immune tolerance to the commensals (46). Correlation between Tregs and microbes after the neonatal period is obscure. It remains unknown whether these cells recognize antigens derived from commensal microbes in later life and whether microbiota influence their action in the skin (47). In addition, studies conducted on mice may not entirely reflect human conditions. In humans, the immune system and HF develop to a large extent in utero prior to microbial exposure (1, 48). However, if there is also a time-restricted process of determination of immune tolerance to bacterial commensals in humans, disturbance of this process or the introduction of antigens in later life may cause inflammatory response (6).

Tregs have also been associated with the regulation of hair follicle cycling. As recently reported, this subset of T cells seems involved in the fostering of follicle regeneration. Tregs gather adjacent to the stem cell-bearing bulge of the follicle in the telogen phase and promote stem cells to proliferate and differentiate, and thus promote the entrance of the HF into the anagen stage. Absence of these cells resulted in a disruption of the follicle regeneration process during the normal growth cycle as well as after depilation (49).

### 6.2.4 Scalp microbiome

The scalp surface environment is largely influenced by the external environment and sebum produced by the sebaceous gland and transported via the sebaceous duct into the infundibulum and further outside the HF ostium. Sebum constitutes a composition of cell debris and lipids, and it features strong antimicrobial activity. However, there are some microbiota that are able to survive in this harsh habitat. Hydrolysis of triglicerides and the release of free fatty acids by *Cutibacterium spp.* favour their adherence. In addition, *Cutibacterium spp.* and *Malassezia spp.* benefit from sebum lipids as they do not produce the lipids themselves. While the rich microbiota in the upper follicle niche is considered to be mainly affected by fluctuation of oxygen tension and properties of sebum, the knowledge of the habitat conditions of the lower follicle is very limited (17).

The vast majority of studies on scalp microbiome rely on the analysis of scalp swabs. *Cutibacterium spp*. (with the predominance of *C. acnes*) and *Staphylococcus spp.* (with the majority of *S. epidermidis*) comprised ca. 90% of the total gene sequences recovered from scalp swabs of healthy individuals. *Streptococcus spp.*, *Corynebacterium spp., Prevotella spp.*, and

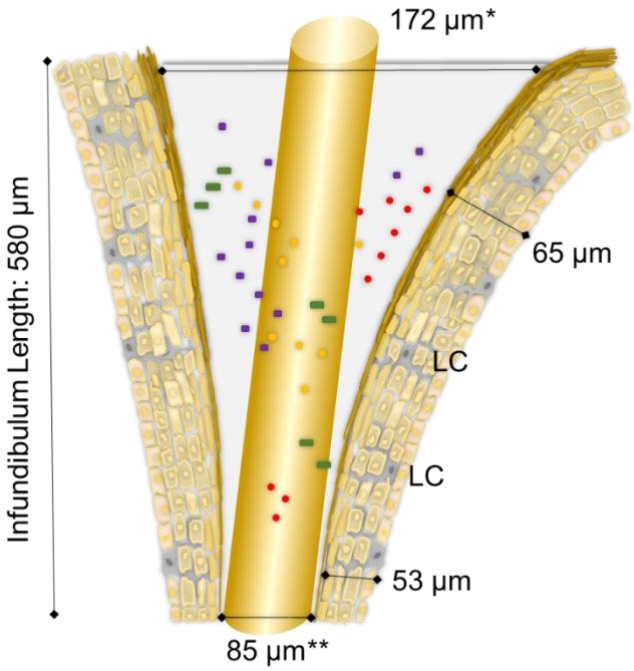
*Acinetobacter spp.* have also been found in the swab material but far less numerously (50-52). Not all the studies have reported uniform data, e.g. Perez-Perez et al. presented results with a significantly lower representation of *Cutibacterium spp.* These inconsistent findings can be partially explained by usage of different primers or different sequencing techniques (53). Furthermore, Shibagaki et al. found shifts in the scalp microbiome composition with age. The older group (60-76 years) that was analysed was characterized by a higher richness of species and also a significant increase of the number of minor species in contrast to the younger group (21-37 years). In addition, a relationship between the changes of the composition of the scalp microbiome with age and shifts in the microbiome within the oral cavity has been observed (51).

Microbial presence below the follicular ostium, and thus not collected by scalp swabbing, is currently becoming a subject of special interest. The first reports on Gram-positive bacteria colonizing the deep infundibulum, depicted in biopsy samples by light and electron microscopy, date back to the 1970s (22, 23). In a more recent study, Matard et al. found structures that might correspond to bacterial biofilm formed below the infundibulum. Images of those structures, located in the supramatrical zone of plucked HFs from two out of three examined healthy volunteers were captured by confocal laser scanning microscopy and field emission scanning electron microscopy. They consisted of bacilliform bacteria that were morphologically compatible with *C. acnes* (54). *C. acnes* was further found, by using genomic identification (IRIDICA BAC Spectrum SF assay-Abbott), as the most abundant bacterial species recovered from the subepidermal part of scalp biopsy specimens. However, using the culture technique, coagulase negative staphylococci predominated in the same location (55). The latter finding may be consistent with the bacterial DNA sequencing results of Pinto el. al, who reported Firmicutes, Actinobacteria and Proteobacteria predominance (in order of decreasing abundance) in the dermis and subcutis of the scalp specimens encompassing hair follicles (56). Unlike these reports, the study of Ho et al., sequencing the results of the material recovered from the follicular unit extracted from scalp biopsy specimens, revealed the predominance of bacterial species assigned to Proteobacteria. The middle portion of the hair follicle was predominantly colonized by *Burkholderia contaminans* (*B. contaminans*) and *B. cepacia*, which along with *B. cenocepacia*, and *B. kururiensis* were mutually exclusive in each hair follicle. A more diverse microbiome was found in the lower part with the predominant genera of *Brevibacterium*, *Methylobacterium komagatae* and *Sphingomonas* (57).

Similarly to the bacterial microbiome of the human scalp, most studies on the fungal community (mycobiome) rely on material collected by swab method. *Malassezia spp.* are definitely the most abundant species, among which *Malassezia restricta* (*M. restricta)* and M. *globosa* predominate (52, 58-60). *Coniochaeta spp.*, *Ascomycota* (*Didymella bryoniae,*

*Acremonium spp.*), other *Basidiomycota* (*C. diffluens, Cryptococcus liquefaciens*), *Rhodotorula spp.* were also found on the scalp of healthy individuals but far less numerously (61). It has been reported that mycobiome in children's scalp (age <14) was more diverse, with a relatively lower portion of *Malassezia spp.* in contrast to the scalp of adults (age 20s-30s). Lower activity of sebaceous glands and a different sebum composition before puberty may explain such differences (59). Yeasts colonizing the infundibulum were also found in biopsy samples using light and electron microscopy as well as *in vivo* as assessed by confocal laser scanning microscopy (22, 62).

Species of bacteria and fungi constituting the scalp microbiome are currently of special interest due to their supposed connections with different diseases. However, there are other organisms, namely viruses and mites, that also inhabit the human scalp, but of which our knowledge is still scarce. Boxman et al. identified human papillomavirus in plucked hairs and in desquamated keratinocytes collected from the scalp of healthy individuals. (63). Among mites, *Demodex brevis* (*D. brevis*) *and D. folliculorum* are commonly found in pilosebaceous units, *D. brevis* in sebaceous glands and *D. folliculorum* in the infundibulum of HF (64). In the material recovered by vacuuming the normal scalp, Naspitz et al. detected *Euroglyphus spp. and Dermatophagoides spp.* (65).




| Bacteria | Size [µm] |
|---|---|
| *Cutibacterium acnes* | 0.8-0.9 × 0.4-0.5 |
| *Staphylococcus spp.* | 0.5-1.0 |
| *Corynebacterium spp.* | 2-6 × 0.5 |
| *Streptococcus spp.* | 0.5-2.0 |
| **Yeasts** | |
| *Malassezia globosa* | 2.5-8 |
| *Malassezia restricta* | 2.5–4 × 1.5–3 |
| **Mites** | |
| *Demodex folliculorum* | 300-400 |

*Figure 2.*

*Diverse microbiota inhabit the follicular infundibulum*

*The infundibulum, which is a tubular invagination of epidermis, extends from the follicular ostium to the opening of sebaceous duct and hosts rich microbiota including bacteria, fungi, viruses and mites. The vast majority of studies on scalp microbiome have relied on the analysis of scalp swabs so far, and thus has been limited to follicular openings and the scalp surface of the interfollicular area. Although the presence of microorganisms in the infundibulum has been proven, their organization level as well as the interactions between particular microbiota and the local immune system remain vague. LC, Langerhans cell.*

* *Diameter of the infundibulum at the level of follicular ostium*

** *Diameter of the infundibulum at the level of sebaceous duct opening*

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### 6.2.5 Microbial imbalance and diseases of the scalp

The most convincing data linking a microbial imbalance to the development or exacerbation of scalp diseases has been collected for folliculitis decalvans (a type of scarring alopecia) and dandruff/seborrheic dermatitis (Figure 3). Administration of treatment targeting microbiota usually yields a temporary improvement in these disease entities. However, frequently occurring

relapses, treatment resistance concerning some patients and the necessity to apply anti-inflammatory treatment in flare-ups suggest a complex etiopathogenesis of these diseases (54, 66).

- Folliculitis decalvans

Folliculitis decalvans (FD) belongs to the group of primary scarring alopecias. Its pathophysiology to date remains obscure. There is quite strong evidence linking bacteria with the development of FD. *Staphylococcus aureus* was recovered from swabs collected from lesional and non-lesional scalp areas, and identified by both culturing and genomic identification methods in lesional subepidermal skin in patients suffering from FD (54, 55). A good clinical response to antibiotics targeting staphylococci was accompanied by almost complete disappearance of *S. aureus* in the examined material (55). In another study, structures corresponding to biofilm and composed of bacteria consistent with *C. acnes* were found in HFs of biopsy specimens (species verified by in situ hybridization) as well as in pulled HFs (bacteria morphologically corresponding to *C. acnes*) in some patients suffering from FD (54, 55). Despite the fact that these structures were also identified in HFs pulled from the scalp of healthy volunteers, the theory about biofilm formations underlying FD seems plausible. Only the transient efficacy of antibiotic therapy and infiltrates of polymorphonuclear neutrophils in histopathology, which cause the destruction of the HF but do not manage to liquidate biofilms, along with the normal immune system of patients, have been listed to support the hypothesis. A biofilm formation of nonpathogenic properties may expand and become pathogenetic under some circumstances. Although antibiotics can combat planktonic bacteria released from biofilm and provisionally alleviate the symptoms, they typically do not manage to kill all biofilm bacteria that remain the source of chronic inflammation (54, 55).

- Dandruff/seborrheic dermatitis of the scalp

Seborrheic dermatitis develops in areas rich in sebaceous glands and is a kind of inflammatory disease. Its milder type, limited to the scalp, is called dandruff. The etiopathogenesis of seborrheic dermatitis has been linked to an inflammatory immune response to yeasts for years (66). Lipases produced by *Malassezia* hydrolyze lipids such as triglycerides release free fatty acids that are connected with the induction of inflammation and hyperproliferation of the epidermis (66). A correlation between seborrheic dermatitis/dandruff and imbalance in yeasts communities has been investigated in many studies. However, not all the studies provided consistent results. The abundance of *Malassezia* is increased by 1.5 to 2 times compared to normal scalp according to McGinley et al. and Park et al.; however, Soares et al. noted no difference in relation to the microbiome of normal scalp (61, 67, 68). It was also reported by Xu et al. that in dandruff,

particular strains of *M. restricta* may have either positive or negative correlation with the disease (52). Furthermore, not only yeasts but also other fungi, i.e., *Filobasidium spp.*, have recently been linked to dandruff (61, 68).

Shifts in bacterial communities may also underlie the development of dandruff. The severity of the disease seems strongly associated with a higher abundance of *Staphylococcus spp.* as well as the underrepresentation of *Cutibacterium spp.* (52, 60, 69). In addition, symptoms experienced by patients suffering from seborrheic dermatitis and dandruff, e.g., burning or itching, may be correlated with the presence of *Deinococcus* and *Hymenobacter* (69). There is also a new postulated hypothesis, stating that particular bacterial or fungal species do not contribute to the disease state, but rather there are some more complex shifts in microbial communities leading to dandruff formation (50, 60, 68, 69).

- Scalp psoriasis

Psoriasis is a chronic immune cell-mediated inflammatory skin disease with a strong genetic predisposition and often triggered by external factors such as microorganisms, stress and drugs (70, 71). *Malassezia spp.* positive cultures were more often found in psoriatic lesions in contrast to normal scalp (70, 72, 73). *M. furfur*, *M. restricta* or *M. globose* were the predominant fungal species detected in swabs collected from psoriatic scalp lesions according to the published studies (70, 73-76). Shifts in the scalp mycobiome composition were correlated with the severity of psoriasis. *M. globosa* was found more frequently in moderate and severe psoriasis, whereas *M. restricta* was identified in mild cases. *M. globosa* was also the most frequently isolated species in patients with a history of disease flare in the last month (70). A role of *Malassezia* in the exacerbation of psoriasis is confirmed by cases of successful treatment of refractory scalp psoriasis with imidazoles associated with a decrease in yeast population (77). Upregulation of transforming growth factor beta 1 in keratinocytes, the release of cytokines through TLR2, stimulation of the complement system and mobilization of neutrophils are linked to the action of yeasts that may induce exacerbation of scalp psoriasis (70).

A higher diversity in the bacterial community along with an increased abundance of *S. aureus* were reported in psoriatic lesions, including those located on the scalp, in contrast to control samples. As shown in a murine model, *S. aureus* can lead to increased Th17 activation. Th17 immune polarization seems crucial in the pathogenesis of psoriasis. Thus, it has been speculated that *S. aureus* colonization may influence the development of psoriasis (78).

In addition, the analysis of gut microbiome in patients with psoriasis compared to controls has shown dissimilarities in microbiota composition. The ratio of Bacteroidetes and Firmicutes was disturbed in patients. *Bifidobacterium species*, belonging to Actinobacteria and known of immunosuppressive properties, were relatively underrepresented in psoriasis patients in contrast to healthy individuals. These findings and recent reports on increased gut permeability in psoriatic patients may point towards a role in the pathogenesis of psoriasis (79-81).

- Androgenetic alopecia

Androgenetic alopecia (AGA) is characterized by a progressive miniaturization of HFs with a transition of terminal follicles into vellus follicles, shortening of the anagen phase and an increased telogen/anagen ratio (82). Lymphocyte and mononuclear cell infiltrates are identified in ca. 50% of scalp biopsy specimens. The micro-inflammations are localized within the infundibula, and thus in the area harbouring rich microbiota. Furthermore, porphyrins that are released by *Cutibacterium spp.* and promote complement cascade were found significantly more often in the pilosebaceous duct in patients suffering from AGA in contrast to healthy individuals (58% vs 12%, respectively) (83). An increased abundance of *Cutibacterium acnes* in the middle and lower compartments of miniaturized HFs was lately reported in the study of Ho et al. (57). Moreover, the concomitance of AGA and seborrheic dermatitis of the scalp is often observed in patients (84). The presented findings, and the fact that antimicrobial treatment often brings a marked improvement, point toward a link between scalp microbiome and the pathogenesis of androgenetic alopecia (83).

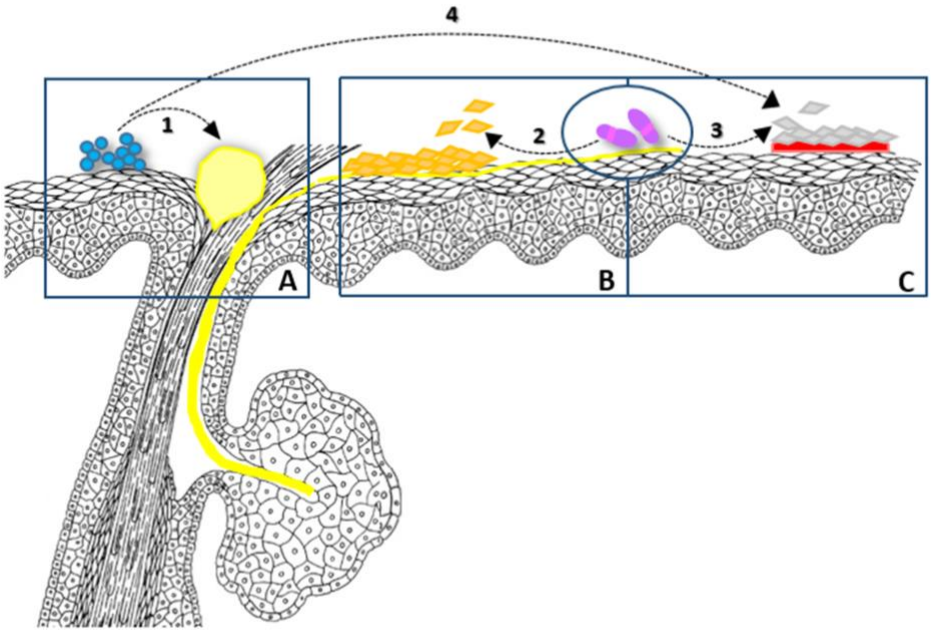


*Figure 3.*

*Microbiota connected with scalp diseases*

*S. aureus is often cultured from pustules and erosions in folliculitis decalvans (1); unknown pathomechanism of the disease [A]; Lipases of yeasts release free fatty acids from sebaceous triglycerides (2). Unsaturated free fatty acids are of a strong pro-inflammatory potential and induce hyperproliferation of the epidermis in dandruff/seborrheic dermatitis [B]; Yeasts may contribute to the exacerbation of psoriasis by stimulation of the release of cytokines, activation of the complement system and mobilization of neutrophils (3); S. aureus may trigger Th17 immune response in psoriasis (4) [C]. Adapted from Fig.4 of publication "The role of the microbiome in scalp hair follicle biology and disease". Reproduced with kind permission of the publisher John Wiley and Sons (12)*

- Alopecia areata

Alopecia areata (AA) is a non-scarring autoimmune hair loss disorder. Unlike scarring alopecia, the lower part of the anagen HF, namely its bulb, serves as a target for inflammation. The inflammation and the influx of cytotoxic T-cells lead to the breakdown of the immune-privileged status of the bulb at the growth phase and further to the premature transition of the HF into catagen and consequently to the hair loss. As the stem cells remain intact, when the inflammatory reaction

subsides, HF can return to the normal hair cycle and hair regrowth may be observed. The triggers of this reaction are still undetermined (11). There was a hypothesis of a role of viruses in the induction of inflammation. DNA sequences of cytomegalovirus were identified in biopsy specimens taken from AA lesions (85). However, further studies did not support these results (86).

Fungal communities have also been analysed. *Alternaria spp.* were cultured from scrapings of the scalp from 20% of AA patients versus 13.3% of healthy individuals (87, 88). Studies on the scalp bacterial microbiome in AA have also provided differences compared to controls. Pinto et al. found a smaller proportion of *S. epidermidis* and a larger proportion of *C. acnes* in scalp swabs recovered from subjects suffering from AA (56). Furthermore, the analysis of biopsy specimens showed a bacterial shift in the subepidermal compartments in patients compared to controls. A decrease of *S. epidermidis* and an increase in the *Neisseria genus* at the deep epidermis and dermis level were reported. However, no statistically significant differences in the bacterial composition were noted in hypodermis layers of AA and healthy subjects (89).

Recent studies have also linked the gut microbiome to AA pathogenesis. An increased number of Firmicutes and decreased number of Bacteroides were found in stool samples of patients with AA in contrast to healthy controls (90). Local dysbiosis and/or inflammation leading to increased permeability of intestines may consequently trigger inflammatory response in HFs in individuals of a genetic predisposition. A low-residue diet causing a lower release of short-chain fatty acids by colonic bacteria may disadvantage the integrity of intestine mucosa and also the function of local Tregs considered as crucial immunoregulators (91). A good response to the transplantation of faecal microbiome in two cases of patients suffering from refractory AA remains in line with the hypothesis of a gut-skin axis in the pathogenesis of AA (92).

### 6.3 Methods of microbiome analysis

Our current knowledge of skin microbiome composition is mainly based on sampling of the outermost layers of the epidermis. Swabbing and scraping of the scalp recover material from the scalp surface and follicular openings, which are the entrance to the upper part of the infundibulum (slightly deeper when sampled by using the scraping method), and also of the hair shaft just above the scalp surface, and of the stratum corneum which covers the interfollicular skin surface area (14). The tape-stripping method with cyanoacrylate glue flowing into infundibula allows for sampling of infundibular material (93). Skin biopsy is a method providing an insight into the deepest parts of the HF, at the level of the dermis and subcutaneous tissue. In biopsy samples, the whole hair follicle together with the sebaceous gland can be examined (54, 94). As has been lately reported, the human dermal microbiota differs from the microbiota colonizing the epidermis - to

be precise, the dermis contains a specific subset of the epidermal microbiota. Furthermore, the dermal microbiome seems more homologous among individuals than the epidermal microbiome, which is widely influenced by a wide range of environmental factors. Thus, the bacteria inhabiting at the dermal level may more accurately reflect the genetic and immunological status of the host. It is hypothesized that the dermal microbiota may be of importance for human skin health and diseases arising in the dermis (95, 96).

Although biopsy of the scalp serves as a better method for skin microbiome studies than the so far commonly used swabbing, it has some significant limitations. The invasiveness of the method, which heals with scarring, resulting in permanent hair loss at the biopsy spot, and thus, often small study groups providing unrepresentative results, provides encouragement to look for some alternatives (96-98). Only a few studies investigating bacterial composition in the subepidermis encompassing HFs of healthy scalp have been published so far (55-57). However, based on recent findings, the analysis of the microbiome extending beneath the scalp surface may be of relevance for our better understanding of HF physiology and disease status in some chronic inflammatory diseases (95).

Traditional culture-based *in vitro* techniques for microbiota analysis have by now been largely replaced or complemented by sequencing methods based on proceeding with the entire complement of genetic material without a targeted amplification step (shotgun metagenomic sequencing), or on sequence variation in conserved taxonomic markers (amplicon sequencing), with 16S ribosomal RNA gene employed for bacteria, and the internal transcribed spacer 1 regions for fungal identification (14). Viruses have no conserved regions analogous to bacterial 16S and fungal ITS regions and, therefore, metagenomic shotgun sequencing is mainly used for human virome analysis (99).

## 6.4 Objectives

In order to further investigate preliminary immunohistochemical screening results on the expression of selected immunoregulatory cytokines and antimicrobial peptides in the anagen scalp hair follicle, we aimed to develop a non-invasive method for the collection and further processing of material from scalp and hair follicles. Specifically, the aim was to create an experimental set-up for the plucking of hair and subsequently splitting it into different compartments that would provide suitable material for the assessment of biomarkers as well as for the extraction of microbial DNA. The suitability of this set-up was assessed in a first clinical pilot study on human volunteers.

## 7. Materials and Methods

Ethical approval for this exploratory study was provided by the Charité Ethics Committee. All procedures were conducted in accordance with the Declaration of Helsinki and ICH-GCP. After obtaining written informed consent and conducting a thorough examination, 12 healthy individuals (6 male and 6 female) aged between 18-47 years were enrolled in the study. The inclusion criteria comprised there being no manifestation (confirmed with trichoscopy) and personal history of any scalp disease. Volunteers with known hormonal imbalance, chronic viral infection, diabetes mellitus, with a history of antibiotic intake within the last 3 months, who underwent systemic therapy with retinoids, immunosuppressive and immunomodulatory agents in the last four weeks or topical use of corticosteroids in the last 3 weeks, were excluded from the study. As detergent washing was reported to largely not affect bacteria colonizing the hair, no restrictions were put on hair washing before the sampling (18).

### 7.1 Demographic data and non-invasive measurements

The following data of the enrolled subjects were collected: gender, age, height, weight, Fitzpatrick skin type, known atopic dermatitis history. Two skin physiological parameters, sebum secretion and pH-value, were recorded using the Multi Probe Adapter® 5 (Courage + Khazaka Electronic GmbH, Köln, Germany) from two scalp areas, i.e., frontal and occipital. Swabs were collected from a ~2cm$^2$ surface from the frontal and occipital scalp regions. DNA-free, EtO-sterilized, self-drying cotton swabs (Sarstedt, Nümbrecht, Germany) were moistened in sterile sodium chloride 0.9% solution. The collected swabs were subsequently frozen at −25°C until further sequencing procedures.

### 7.2 Standardized scalp photography and trichoscopy

Standardized scalp photography of frontal and occipital sides was conducted using a VISIA-CR® (Canfield Scientific Inc., Fairfield, NJ, USA) facial imaging booth with a Nikon D90 digital camera body. The captured images included the following lighting modalities: Coproporphyrin III (CpIII) and Protoporphyrin IX (PpIX) Fluorescence Image, presenting molecules produced by *C. acnes*, Coproporphyrin III and Protoporphyrin IX on scalp as green and red spots, respectively (100). The expression of porphyrins was analyzed based on the number of spots, irrespective of their size and fluorescence intensity. The area of the scalp for analysis was delineated to include

approximately 5 cm of the scalp middle part in the front-view and occipital-view images. Trichoscopy was performed using a videodermoscope (FotoFinder Dermoscope, TeachScreen). Images from frontal and occipital sites were taken at 20× and 70× magnification.

### 7.3 Hair sample collection

The subjects had approximately 50 hairs plucked from the frontal and occipital region of the scalp, using tightly closing epilation forceps and following the standard trichogram procedure. Only hair follicles in the anagen phase were chosen for further processing (Figure 4). Proximal ends of the plucked hairs were cut and placed in a sterile Petri dish kept on ice. ~20 anagen hair follicles cut at the level of the outer root sheath were stored at -80°C for cytokine extraction, then the next ~ 20 anagen hair follicles were further cut into two parts, namely the bulb and the infrainfundibular portion (HF w/o bulb), and stored in sterile, DNA-free sample tubes (Molzym, Bremen, Germany) at –25°C until further processing for microbiome analysis. Anagen hair follicles from 6 subjects were stored at 4°C in FISH-FIX (MoKi Analytics, Berlin, Germany) fixation liquid for fluorescence in situ hybridization (FISH). Follicles for microbial rRNA and FISH analyses were cut using sterile instruments. A DNA decontamination solution, DNA-ExcitusPlus$^{TM}$ (PanReac, AppliChem, ITW Reagents, Darmstadt), was used to remove DNA from surfaces, instruments and a microscope.

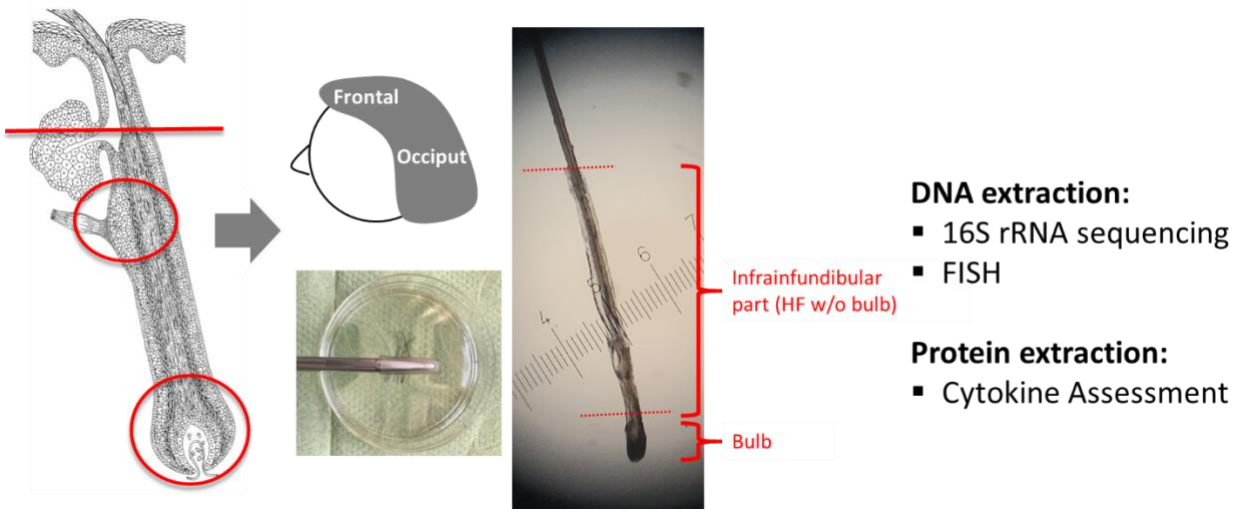


*Figure 4.*

*In vivo sampling of anagen hair follicles*

*Approximately 50 hairs were plucked from the frontal and occipital sides of the scalp according to the standard trichogram procedure. The proximal ends of the plucked hairs in the anagen phase were cut at the level of the outer root sheath, ~20 of which were stored at -80°C for cytokine extraction, then the next ~20 anagen hair follicles were further cut into two parts, namely the bulb and the infrainfundibular portion (HF w/o bulb), until further processing for microbial DNA extraction and 16S rRNA sequencing. Anagen hair follicles from 6 subjects were stored at 4°C in a fixation liquid for fluorescence in situ hybridization (FISH). Hair follicles were cut using sterile instruments.*

### 7.4 Removal of human DNA and extraction of enriched microbial DNA from plucked hair follicles and scalp swabs

An Ultra-Deep Microbiome Prep Kit (Molzym, Bremen, Germany) was used according to the manufacturer's protocol with minor changes (β-mercaptoethanol was replaced with Dithiothreitol (DTT)) to remove human DNA and isolate enriched microbiome DNA from pulled hair follicles for further 16S rRNA sequencing. Each set of extracted samples included one negative control sample (only reagents). Total extracted DNA concentrations were quantified using a DS-11 spectrometer (DeNovix, Wilmington, DE, USA). Samples were stored at -25°C until further processing. Microbial DNA from scalp swabs was isolated using a QIAamp DNA Mini Kit (QUIAGEN, Hilden, Germany) following the manufacturer's protocol. The usage of two different kits was caused by financial restrictions.

### 7.5 16S rRNA sequencing performed on microbial DNA extracted from plucked hair follicles and scalp swabs

The bacterial 16S ribosomal RNA genes were amplified by PCR using the contaminant-purified UCP Multiplex PCR Mastermix (QIAGEN, Hilden, Germany), with primers covering the V3-V4 hypervariable regions (V3V4_Fwd: CCTACGGGNGGCWGCAG, V3V4_Rev: GACTACHVGGGTATCTAATCC according to Klindworth et al.) (101). Index sequences were attached in a second limited-cycle PCR. The samples were pooled and sequencing was performed on an Illumina MiSeq sequencer with the Illumina paired-end MiSeq Reagent Kit v2 (500 cycle) following Illumina's standard protocol. After demultiplexing, paired-end reads were merged and the merged amplicon sequences were clustered into operational taxonomic units with a sequence similarity threshold of 97%. Bacterial taxonomic assignment was carried out at species level using BLAST against the NCBI 16S database.

### 7.6 Fluorescence in situ hybridization (FISH)

Plucked HFs from 6 healthy individuals and biopsy specimens of healthy scalp from plastic surgery patients were fixed in 3.7% formaldehyde in PBS fixation solution (FISH FIX, MoKi Analytics, Berlin, Germany) and embedded utilizing cold polymerizing resin (Technovit 8100; Kulzer, Wehrheim, Germany) as described before (102). Briefly, samples were washed in PBS containing 6.8% (w/v) sucrose, dehydrated with acetone for 1 h and embedded in methacrylate according to the manufacturer´s instruction. After polymerization, sections of 5 µm were straightened on sterile water and placed on polysine slides (Menzel, Braunschweig, Germany). FISH analysis was performed according to Mallmann et al. (103). Briefly, sections were hybridized in 20 µL of a pre-warmed hybridization solution containing 0.9 M NaCl, 20 mM Tris HCl (pH 7.3) and 20 pmol of the pan-bacterial probe $EUB338_{CY5}$ (104) to visualize the entire bacterial population, the *Staphylococcus* genus-specific probe $STAPHY_{FITC}$ (105), and the species-specific probe $PRAC_{CY3}$ (106, 107) for visualization of *Cutibacterium acnes*. DAPI (4′,6-Diamidine-2′-phenylindole dihydrochloride) was used for the visualization of nucleic acids in host cell nuclei and bacteria. After incubation in a dark humid chamber at 50°C for 1.5 h, slides were rinsed with sterile double-distilled water, air dried, and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). For microscopy, an epifluorescence microscope (AxioImager Z2; Carl Zeiss, Jena, Germany) equipped with narrow band filter sets (AHF

Analysentechnik, Tübingen, Germany) was used. Digital images were generated using the ZEN software. As the sectioning of samples was technically difficult and only a small number of representative pictures of stained HFs were received, no quantitative analysis was performed.

### 7.7 Protein extraction from plucked hair follicles

Mechanical (steel beads, Bertin Corp., Rockville, MD, USA) and chemical (1ml EDTA, 1% Triton-x-100 Buffer, 150 ml NaCl, 10 ml Tris HCl) protein extraction using the TissueLyser system (Qiagen Retsch GmbH, Hannover, Germany) (for 1 min at 30 Hz) were followed by an 2-hour incubation in ice at 600 rpm and ultrasonic extraction (SONOREX SUPER RK 102 H, BANDELIN, Berlin, Germany). Subsequently, the samples were centrifuged at 2,500 rpm at 4°C for 7 minutes and the collected protein supernatant was frozen at −80 °C until ELISA cytokine analysis was performed.

### 7.8 Enzyme-linked immunosorbent assay (ELISA)

ELISA was employed to evaluate concentrations of IL-17A and human β-defensin 2 (HBD2) in plucked anagen follicles from all protein-extracted samples. ABTS sandwich ELISA development kits (PeproTech, Rocky Hill, New Jersey) for IL-17A and HBD2 were used following the manufacturer's protocol. Total extracted protein concentration was assessed in each sample by Pierce™ 660 nm Protein Assay (Thermo Scientific, Waltham, Massachusetts). To standardize and further compare the values of IL-17A and HBD2 between samples, their concentrations were divided by the total protein concentrations.

### 7.9 Hematoxylin and eosin, Gram and Giemsa staining, Immunohistochemistry

Scalp specimens were obtained from 3 subjects who underwent plastic surgery and had provided written informed consent. On routine histology, none of the examined scalp sections had any obvious pathological abnormality. All tissues were fixed in buffered 10% formalin and embedded in paraffin using standard pathology protocols. Sectioning was performed perpendicularly to HFs to minimize the risk of contamination of the lower follicle with the material of the upper follicle. Hematoxylin and eosin and Giemsa staining was performed on cut paraffin sections of 2-4 μm thickness following standard protocols. Sections for Gram staining were cooked at 60 °C for 20 minutes, cooled at room temperature, and further deparaffinized and rehydrated through graded

alcohols to distilled water. A ready-to-use Gram staining Kit (Sigma-Aldrich, St. Luis, MO, USA) was used following the protocol recommended by the manufacturer.

Seven different primary antibodies were used to determine the distribution of selected immune cells and molecules. Immunohistochemical staining against CD3, CD4, CD8, FOXP3, IL-17A, and HBD1 and 2 (Table 1) was performed according to standard procedures. Sections were dewaxed in xylene, rehydrated, and then cooked for 20 minutes in antigen retrieval solution pH 6 (for anti-IL-17a antibody) or pH 9 (for anti-CD3, -CD4, -CD8, -FOXP3, -hBD1 and –hBD2 antibodies) using an electric steam cooker. Endogenous peroxidase activity was blocked with 0.45% hydrogen peroxide (for anti-CD4 antibody with 0.15% hydrogen peroxide) for 15 minutes. Non-specific binding was blocked using normal horse serum (R.T.U Vectastain KIT, VECTOR, Burlingame, CA) for 30 minutes followed by an avidin/biotin blocking kit (each for 15 minutes) at room temperature (AVIDIN/BIOTIN Blocking Kit, VECTOR, Burlingame, CA). Primary antibodies (anti-CD4, -CD8 and –FOXP3 antibodies of a 1:50 dilution, anti-hBD2 antibody of a 1:100 dilution, and anti-hBD1, -IL-17a, -CD3 antibodies of a 1:200 dilution) were incubated for 1 hour at room temperature (only anti-CD3 and –CD8 antibodies) or overnight at 4 °C. Subsequently, the slides were treated with a biotinylated secondary antibody (R.T.U Vectastain KIT, VECTOR, Burlingame, CA) for 30 minutes at room temperature (for anti-CD3,-CD8, -hBD1 and –hBD2 antibodies) or for 1 hour at 37°C (for anti-CD4 and –FOXP3 antibodies). After developing the color with chromogenic peroxidase substrate (AEC Peroxidase Substrate Kit, VECTOR; Burlingame, CA), counterstaining with Meyer's hematoxylin was performed.

| Antibody | Type | Vendor | Catalog No. | Working Dilution | Heat induced epitope retrieval | Peroxide Blocking |
|---|---|---|---|---|---|---|
| **HBD-1** | Rabbit polyclonal | abcam | ab115813 | 1:200 | pH9 | 3% H2O2, Before Normal Horse serum |
| **HBD-2** | Rabbit polyclonal | abcam | ab63982 | 1:100 | pH9 | 3% H2O2, Before Normal Horse serum |
| **Anti-FOXP3** | Mouse monoclonal | abcam | ab20034 | 1:50 | pH9 | 3% H2O2, After secondary antibody |
| **Anti-IL-17A** | Rabbit polyclonal | abcam | ab217359 | 1:200 | pH6 | 1.5% H2O2, Before secondary antibody |
| **Anti-CD3** | Rabbit polyclonal | DAKO | A0452 | 1:200 | pH9 | - |
| **Anti-CD4** | Mouse monoclonal | Leica | NCL-L-CD4-368 | 1:50 | pH9 | 0.5% H2O2, After secondary antibody |
| **Anti-CD8** | Mouse monoclonal | DAKO | M7103 | 1:100 | pH6 | - |

*Table 1.*

*Antibodies used for Immunohistochemistry*

*Antibodies used for IMHC, along with the antibody type, brand, catalog number, working dilution used for staining, pH of citrite buffer used for the heat induced epitope retrieval step, and the concentration (in %) of $H_2O_2$ used, when needed, for peroxide blocking. Reproduced with kind permission of the publisher John Libbey (108)*

### 7.10 Histomorphometry

Positively stained cells were counted and analysed by histomorphometry following the techniques described by Christoph et al. (109). At least 10 anagen follicles from scalp regions, which were cut in a standardized, precisely longitudinal orientation, were scanned per antibody in immunohistochemistry and the presence of mast cells in Giemsa staining. The hair follicle was divided into anatomically distinct compartments: infundibulum, central part (including bulge area), proximal part of the hair follicle and bulb. Three pictures of each compartment of each hair follicle were taken (magnification 400 x) and analyzed with Image J. Positive cells were counted with Cell Counter Plugin-Image J for Giemsa staining and all antibodies except for HBD1 and 2, for which semi-quantitative analysis (0 negative, +/- irregular staining, ++ positive and +++ strong positive staining) was conducted.

### 7.11 Statistical analysis

The Mann-Whitney U test was applied to compare selected data between independent individuals (men vs women), while the Wilcoxon signed-rank test was used to compare intraindividual parameters (data collected from frontal vs occipital region in the same individual). To present the

association between cytokines concentration (HBD2 and IL-17A), pH and sebum value, and relative abundance of selected bacteria, the Spearman's rank correlation coefficient was applied. Calculations and graphs were made using the R statistical package.

This is a retrospective study with an exploratory character and the p-values therefore do not have a confirmatory character and no adjustment for multiple testing (e.g. Bonferroni correction) has been made.

## 8. Results

### 8.1 Distribution of immune cells and immune-regulatory molecules at different levels of the scalp HF

The highest density of immune cells in anagen phase hair follicles was observed at the level of infundibulum (Figures 5 and 9). CD3+ (a marker of T cells), CD4+ (expressed on the surface of T helper cells, monocytes, macrophages, and dendritic cells) and IL17A+ cells (associated with commensal microbiota under steady-state conditions but also released for host defense against pathogens (32)) were numerous in the perifollicular connective tissue sheath, and much less numerous in the epithelium of the upper follicular portion (11). The expression of these antigens dropped distinctly below the sebaceous duct opening (Figures 6, 7 and 8). However, they were all present along the whole length of the follicle, including the bulbar region. CD8+ cells (predominantly found on cytotoxic T cells) were less abundant but similarly distributed as the above-mentioned cells, with the highest density in the infundibulum and single cells below. In contrast to Paus et al., we found much fewer CD4+ and CD8+ cells within the outer root sheath (11). Only a few Foxp3+ cells (of immunoregulatory role) were identified, dispersed in different follicular portions. Mast cells detected with Giemsa staining were evenly spread along follicles in the connective tissue sheath.

Strong expression of antimicrobial peptides, i.e., both HBD1 and HBD2, was detected within the upper follicular compartment (38). Marked immunoreactivity was further found within the bulge region for anti-HBD1 antibody. Quite interestingly though, strong signals for anti-HBD2, a leading epithelial defense protein produced in response to bacterial stimuli, were also noted below the infundibulum, in the peribulbar region (Figures 8 and 9).

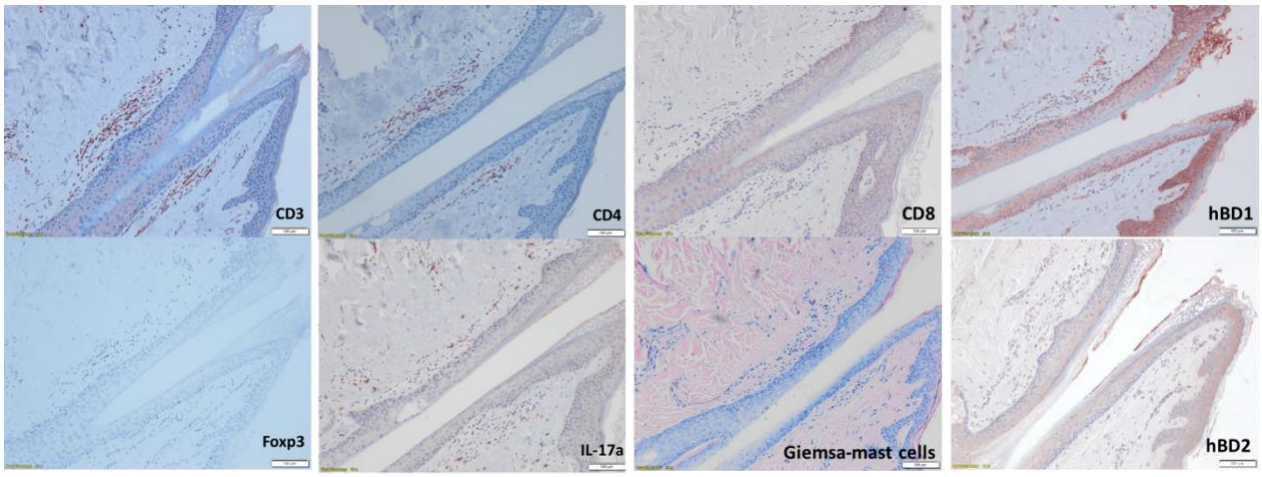


*Figure 5.*

*Immune cell infiltrate of the upper follicle (infundibulum) in healthy scalp.*

*Immunohistochemical staining for the detection of immune cells CD3+, CD4+, CD8+ and FOXP3, and immune mediators HBD-1, HBD-2, IL-17A, and Giemsa staining for mast cell detection. Magnification x100 (scale bar = 100μm).*

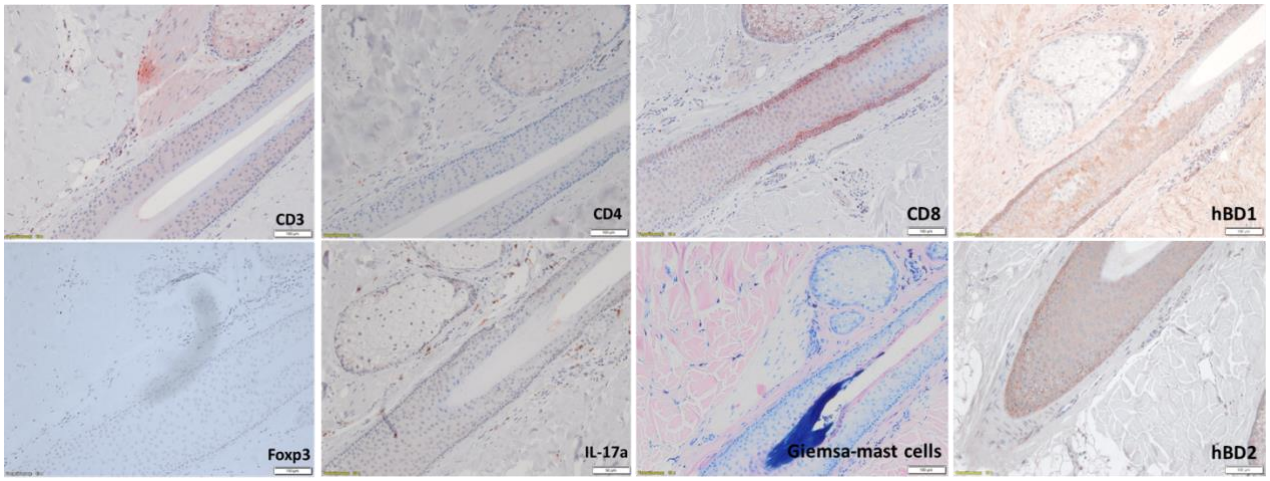


*Figure 6.*

*Immune cell infiltrate of the infrainfundibular portion of the hair follicle (including the bulge area) in healthy scalp.*

*Immunohistochemical staining for the detection of immune cells CD3+, CD4+, CD8+ and FOXP3, and immune mediators HBD-1, HBD-2, IL-17A, and Giemsa staining for mast cell detection. Magnification x100 (scale bar = 100 μm).*

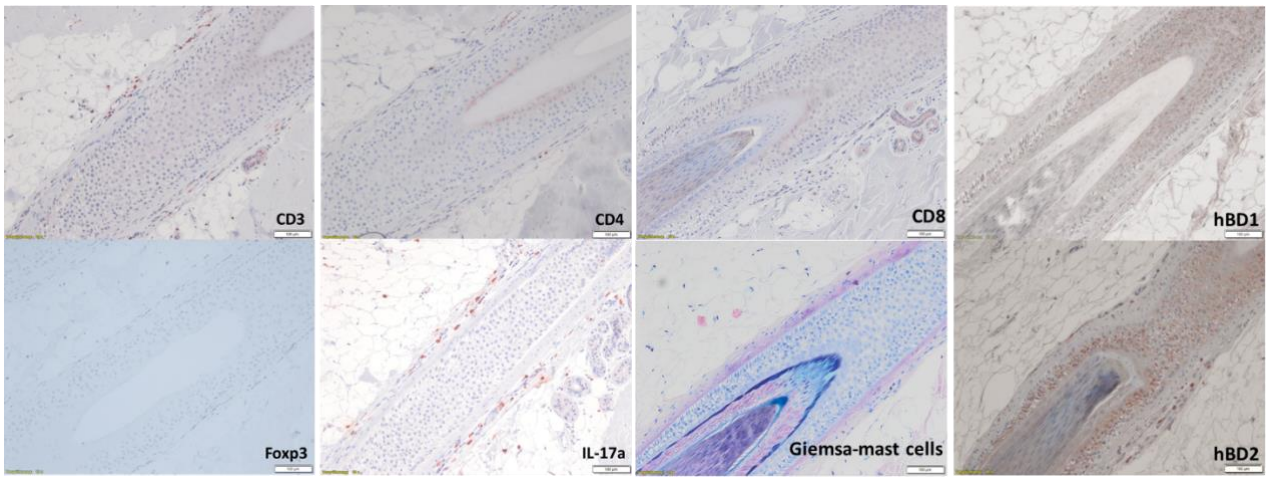


*Figure 7.*

*Immune cell infiltrate of the proximal part of the hair follicle (distally to the bulge area and proximally to the bulb) in healthy scalp.*

*Immunohistochemical staining for the detection of immune cells CD3+, CD4+, CD8+ and FOXP3, and immune mediators HBD-1, HBD-2, IL-17A, and Giemsa staining for mast cell detection. Magnification x100 (scale bar = 100 μm).*

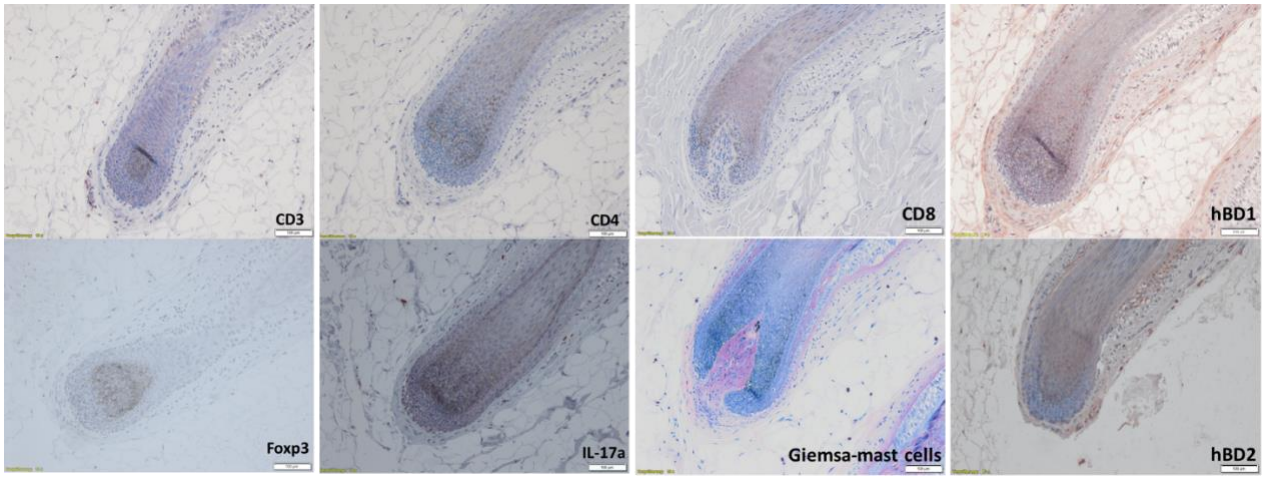


*Figure 8.*

*Immune cell infiltrate at the level of the bulb of the hair follicle in healthy scalp.*

*Immunohistochemical staining for the detection of immune cells CD3+, CD4+, CD8+ and FOXP3, and immune mediators HBD-1, HBD-2, IL-17A, and Giemsa staining for mast cell detection. Magnification x100 (scale bar = 100 μm).*

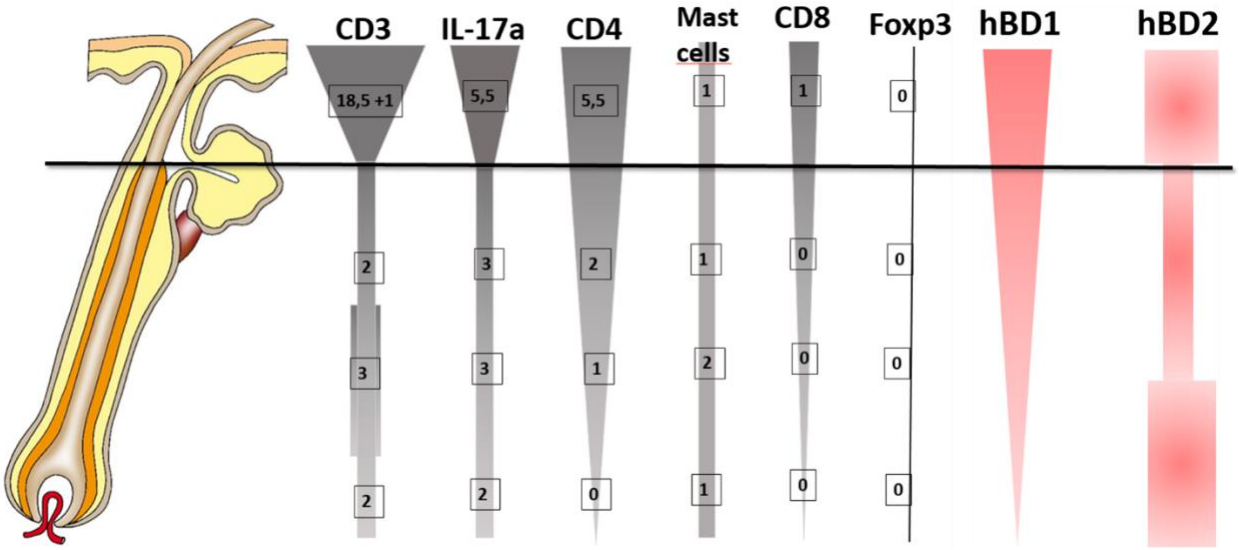


*Figure 9.*

*Graphic illustration of gradients of positive cells*

*The numbers in the box are mean numbers of positive cells within the perifollicular connective tissue sheath (first value) and the epithelium (second value). Rich infiltration of immune cells, mainly CD3+, CD4+, and IL-17A+, as well as high expression of beta defensin 1 and 2 were identified within the infundibulum. The number of cells distinctly dropped below the infundibulum, however not completely, especially for CD3+, CD4+, and IL-17A+, whereas distribution of CD8+ and FOXP3+ cells is sparse, with only single cells below the infundibulum. Marked expression of HBD1 within the bulge and HBD2 within the peribulbar area was observed.*

The expression of HBD2 and IL-17A below the infundibulum was further confirmed by ELISA measurements (Figures 10 and 11) of protein extracts from freshly plucked lower HFs; these were higher for hBD2 than IL-17a (2.68 and 1.16 pg/µg total protein concentration, respectively). Hence, the findings raised the question as to whether microbial material is able to reach deeper HF compartments, even though the anagen HF contains immune-privileged sites.

No statistically significant differences in the concentrations of HBD2 and IL-17A between sexes (application of Mann-Whitney U test) or scalp regions (frontal and occipital side; Wilcoxon signed-rank test) were found. There was also no association between the concentration of HBD2 and IL-17a (application of Spearman's rank correlation coefficient) (Figure 12).

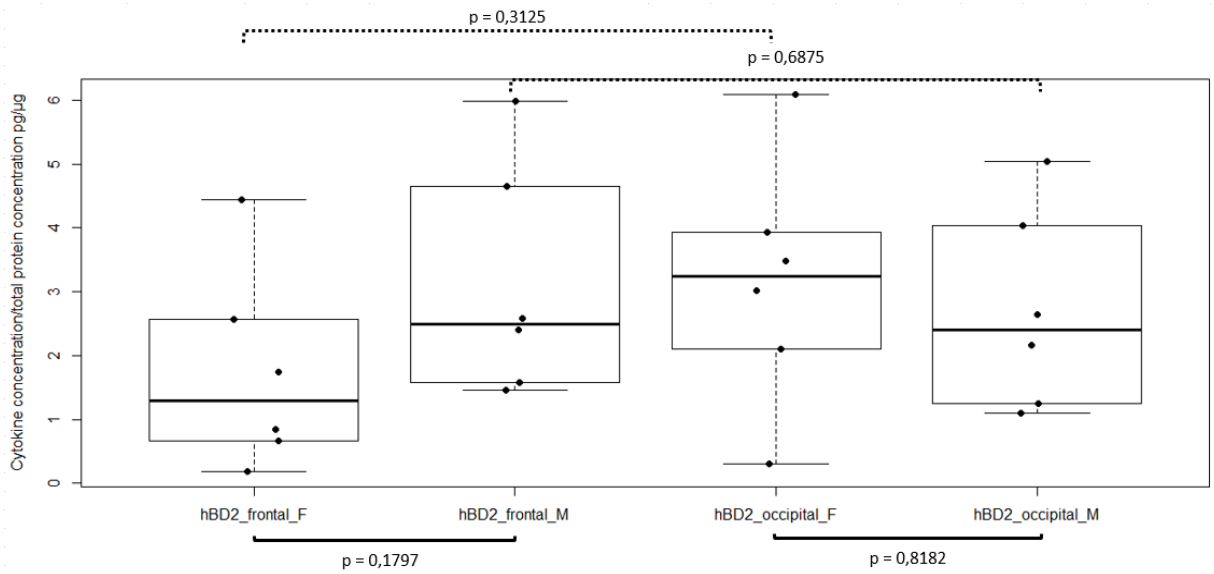


*Figure 10.*

*Expression of HBD2 in the hair follicle below the infundibulum*

*HBD2 expression in lower follicle was confirmed by protein extraction from plucked follicles and ELISA measurements. Concentrations were normalized to total protein content. The Mann-Whitney U test was applied for a correlation of cytokine concentrations between men and women (solid line); the Wilcoxon signed-rank test was applied for a correlation between frontal and occipital sites (dashed line). No statistically significant correlations were found. hBD2_frontal_F, concentration of HBD2 in frontal site in women; hBD2_frontal_M, concentration of HBD2 in frontal site in men; hBD2_occipital_F, concentration of HBD2 in occipital site in women; hBD2_occipital_M, concentration of HBD2 in occipital site in men.*

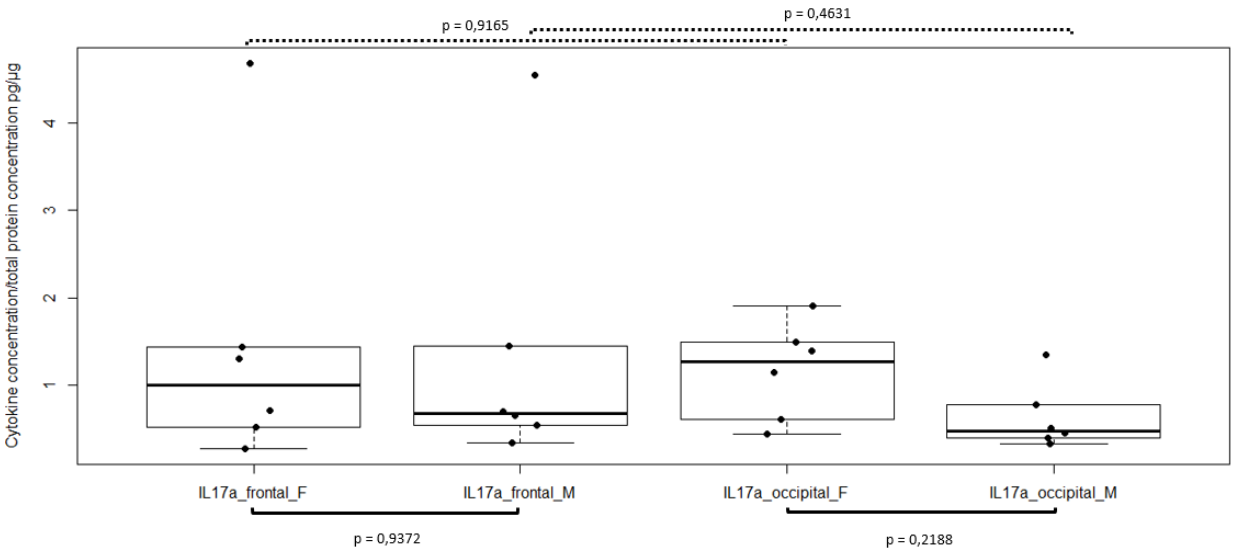


*Figure 11.*

*Expression of IL-17A in the hair follicle below the infundibulum*

*IL-17A expression in lower follicle was confirmed by protein extraction from plucked follicles and ELISA measurements. Concentrations were normalized to total protein content. The Mann-Whitney U test was applied for a correlation of cytokine concentrations between men and women (solid line); the Wilcoxon signed-rank test was applied for a correlation between frontal and occipital sites (dashed line). No statistically significant correlations were found. IL17a_frontal_F, concentration of IL-17A in frontal site in women; IL17a_frontal_M, concentration of IL-17A in frontal site in men; IL17a_occipital_F, concentration of IL-17A in occipital site in women; IL17a_occipital_M, concentration of IL-17A in occipital site in men.*

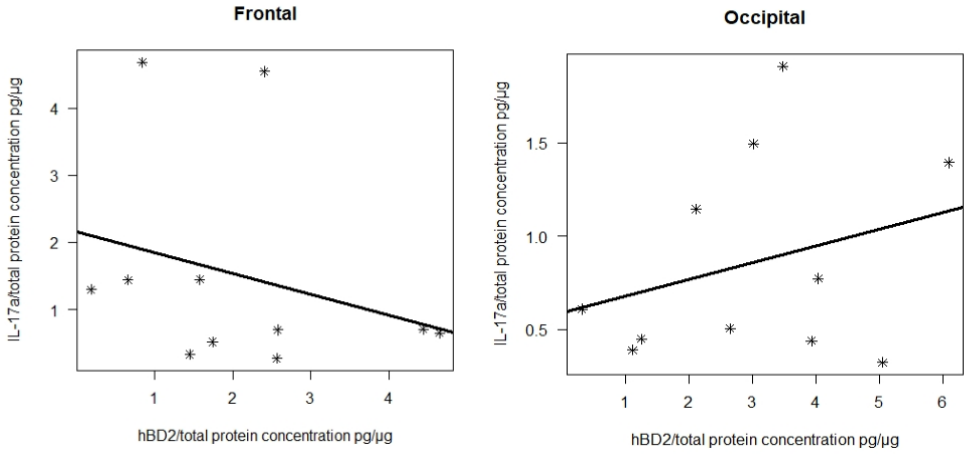


*Figure 12.*

*Correlation of IL-17A and HBD2 concentrations*

*There was no association between the concentrations of IL-17A and HBD2 in both the frontal and occipital scalp region (Spearman's rank correlation coefficient for the frontal site $R_S$ = -0,3818182; Spearman's rank correlation coefficient for the occipital site $R_S$ = 0,1454545).*

### 8.2 Scalp surface characteristics and profile of bacteria colonizing the upper follicular portions and the interfollicular area

Sample characteristics and clinical measurements are shown in Table 2. Beginning with the characterisation of the scalp surface niche in healthy individuals, pH and sebum secretion were measured in the frontal and occipital region. The pH value was comparable in both areas in both sexes and was around 6 (ranging between 4.3 to 8; mean 5.9). The secretion of sebum was higher in women than in men (mean 83.3 vs. 52.5) and at frontal site in general (mean 80.9 vs. 54.9). There was a statistically significant correlation between pH and sebum; lower pH values were correlated with a higher production of sebum (p= 0.0004976; $R_S$= -0.6562042, according to Spearman's rank correlation).

Porphyrins (Protoporphyrin IX and Coproporphyrin III) produced by *Cutibacterium acnes* (110) showed a high variability among individuals. In women, however, more fluorescent spots were found at the frontal site, a region with higher sebum levels, in contrast to the occiput (Figure 13, Table 2). Expression of porphyrins did not always correspond to the subsequently presented swab findings (Figures 14 and 15). For example, some individuals with a high number of fluorescence spots, either red or green, had no or a very small number of reads of *Cutibacterium acnes* in the swab material.

| Number of subjects | Men | | Women | | Total | |
|---|---|---|---|---|---|---|
| | n=6 | % | n=6 | % | n=12 | % |
| **Mean age, years (SD)** | 25.8 (2.6) | | 31.8 (9.4) | | 28.7 (7.2) | |
| **Mean Weight, kg (SD)** | 74.3 (2.7) | | 58.8 (11.1) | | 67.1 (10.8) | |
| **Mean Height, cm (SD)** | 179.2 (6.6) | | 161.4 (4.1) | | 170.8 (10.2) | |
| **Fitzpatrick phototype** | | | | | | |
| **Type II** | 3 | 50 | 2 | 33 | 5 | 42 |
| **Type III** | 2 | 33 | 1 | 17 | 3 | 25 |
| **Type IV** | 0 | 0 | 3 | 50 | 3 | 25 |
| **Type V** | 1 | 17 | 0 | 0 | 1 | 8 |
| **Atopic Dermatitis** | 0 | 0 | 0 | 0 | 0 | 0 |
| **Mean scalp pH (SD)** | | | | | | |
| **frontal** | 6 (1.5) | | 6.3 (1.6) | | 6 (1.5) | |
| **occipital** | 5.6 (1.1) | | 6 (1.4) | | 5.7 (1.2) | |
| **Mean scalp sebum, µg/cm$^2$ (SD)** | | | | | | |
| **frontal** | 69.8 (60.9) | | 107.4 (96.5) | | 80.9 (77.8) | |
| **occipital** | 31.5 (31.1) | | 78.8 (73.6) | | 54.9 (57.7) | |
| **Mean PpIX presence** | | | | | | |
| **frontal** | ++ | | ++ | | ++ | |
| **occipital** | + | | + | | + | |
| **Mean CpIII presence** | | | | | | |
| **frontal** | + | | ++ | | ++ | |
| **occipital** | + | | + | | + | |

*Table 2.*

*Subject demographics and collected clinical data*

*Mean age, mean weight, mean height, Fitzpatrick skin type, known atopic dermatitis, scalp pH and sebum, Protoporhyrin (PpIX) and Coproporphyrin (CpIII) fluorescence spots presence on the scalp, using VISIA CR images, fluorescence dots x field of view: +.50 – 150; ++.150 – 250; +++, >250. (n = 12) Reproduced with kind permission of the publisher John Libbey (108)*

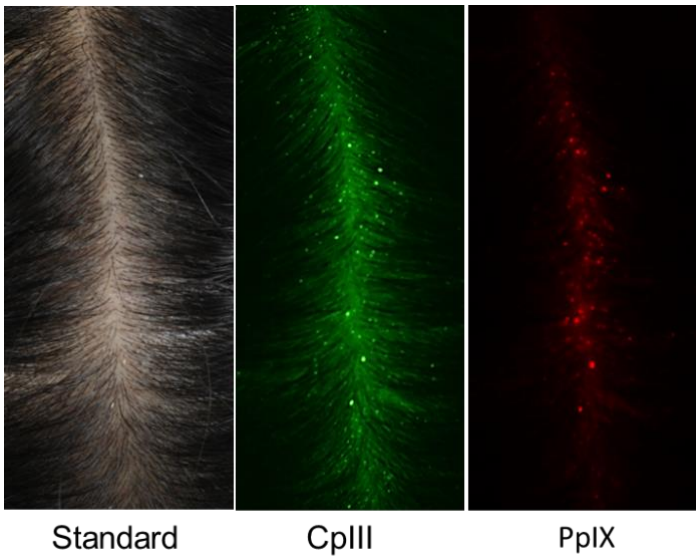


*Figure 13.*

*Fluorescence of porphyrins on the scalp surface of healthy subjects.*

*Visia-CR images. From left to right, Standard White Light Image (Clinical Photograph) of the scalp surface (follicular openings and the interfollicular area) showing the area of interest; Coproporphyrin (CpIII) Fluorescence Image and Protoporphyrin (PpIX) Fluorescence Image. Green fluorescence spots correspond to Coproporphyrin III; red fluorescence spots correspond to Protoporphyrin IX. Porphyrins released by Cutibacterium acnes were more numerous on the frontal scalp site compared to occipital, and in women compared to men. Adapted from Fig.3 of the publication "Identification of anti-microbial peptides and traces of microbial DNA in infrainfundibular compartments of human scalp terminal hair follicles". Reproduced with kind permission of the publisher John Libbey (108)*

The composition of the bacterial microbiome within the outermost portion of the scalp HF (i.e., follicular openings) and the interfollicular area was investigated by analysis of scalp swabs. The most abundant bacteria recovered from the frontal region (n=12 samples) were *Staphylococcaceae*, *Lawsonella clevelandensis* and *Propionibacteriaceae*, with a mean relative abundance of 44.7%. 19.6% and 7.5%, respectively (Figures 14 and 15). This was similar to the occipital region (n=12 samples), which had a mean relative abundance of 36.5%. 15.2% and 18.6%, respectively. Gram-positive cocci and rod-shaped bacteria extending to the deepest parts of the infundibulum were found numerously in scalp follicles of paraffin sections (Figure 16B). The presence of abundant bacteria in this area was subsequently displayed by fluorescence in situ

hybridization (FISH), displaying DAPI positive microorganisms forming complex colonies and biofilms attached to the lining epithelium in depths not covered by swab techniques (Figure 16A).

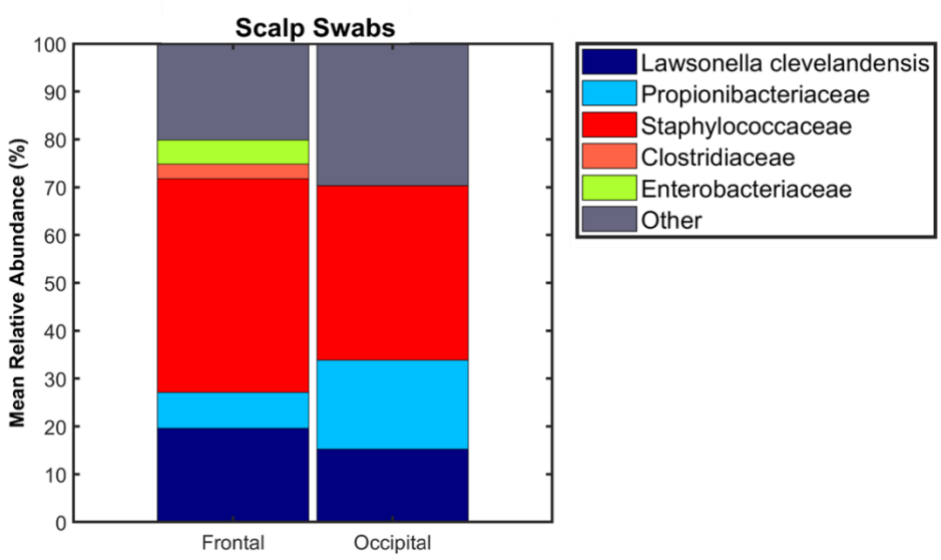


*Figure 14.*

*Bacterial profile of the scalp surface of healthy subjects.*

*Mean relative abundance (%) of the most abundant bacteria recovered from scalp swabs, collected from frontal and occipital scalp regions. Data are presented in the taxonomic rank family, with the exception of the species Lawsonella clevelandensis, which does not fall within an existing family or genus. Lower abundances are classified as "Others". Staphylococcaceae mostly include coagulase-negative staphylococci, such as S. epidermidis, S. capitis and Staphylococcus sp. strains, but in a few samples S. aureus cannot be excluded. Propionibacteriaceae mostly include Cutibacterium acnes. Adapted from Fig.3 of the publication "Identification of anti-microbial peptides and traces of microbial DNA in infrainfundibular compartments of human scalp terminal hair follicles". Reproduced with kind permission of the publisher John Libbey (108)*

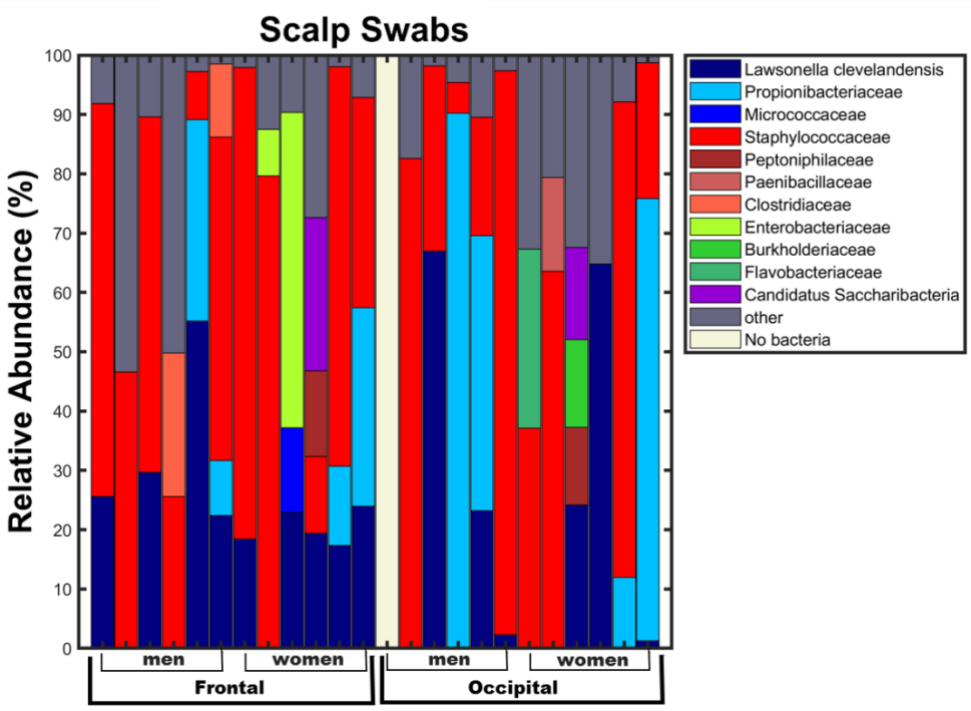


*Figure 15.*

*Bacterial abundances in scalp swabs from healthy subjects.*

*Relative abundance (%) of bacteria collected from frontal and occipital regions of 12 healthy subjects, 6 men and 6 women. Data are presented in the taxonomic rank family, with the exception of the species Lawsonella clevelandensis, which does not fall within an existing family or genus. Families are shown in color panels according to their phylum level: blue, Actinobacteria; red, Firmicutes; green, Proteobacteria; violet, Bacteroidetes. Lower abundances are classified as "Others". One sample is considered negative for bacterial material.* Reproduced with kind permission of the publisher John Libbey (108)

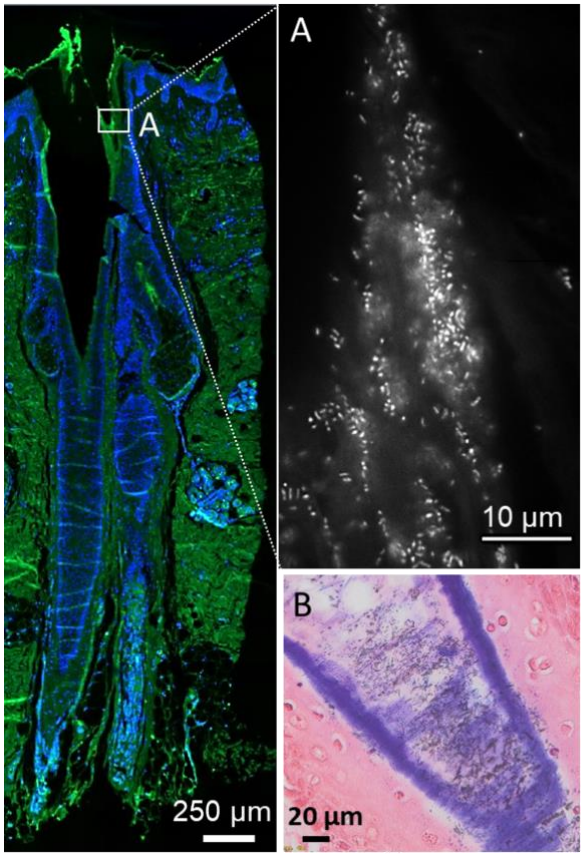


*Figure 16.*

*Bacterial colonization of the upper follicular portion (infundibulum).*

*A high density of bacteria colonizing the upper follicular compartment displayed by fluorescence in situ hybridization (FISH) and Gram staining, performed on a scalp cross-section biopsy sample from a healthy control. Image A, Higher magnification, depicts DAPI positive microorganisms in greyscale. Image B, Gram staining shows Gram-positive cocci- and rod-shaped bacterial structures. Adapted from Fig.1, of the publication "Identification of anti-microbial peptides and traces of microbial DNA in infrainfundibular compartments of human scalp terminal hair follicles". Reproduced with kind permission of the publisher John Libbey.*

(108)

8.3 Presence of bacteria below the infundibulum, in the vicinity of vital follicular structures

Structures localised below the infundibulum that might correspond to Gram-positive bacteria were first visualized by Gram staining (Figure 17). The initial findings were further verified by using more objective research methods. The extraction of microbial DNA was performed from 48 samples of plucked anagen HFs. The average number of anagen HFs per sample was 17. 16S rRNA sequencing conducted on the material extracted from the infrainfundibular portion of plucked scalp follicles yielded robust and reproducible read numbers in all samples (mean 52310 ± 91610 reads for HF w/o bulb and 2622 ± 6787.5 reads for bulbs; six samples containing bulbs were considered to be negative for follicular bacterial material, in contrast to one negative HF w/o bulb sample) (Figure 18). *Lawsonella clevelandensis*, *Staphylococcaceae* and

*Propionibacteriaceae* were the most abundant in the infrainfundibular part (HF w/o bulb) (mean relative abundances 69.9%, 17.3%, 7.1% from frontal and 63.6%, 28.1%, 5% from occipital sites, respectively) (Figures 19 and 20). Similarly, in bulbs *Lawsonella clevelandensis* and *Staphylococcaceae* constituted the majority of bacteria, accounting for 33.9%, 15.6% from frontal and 40.5%, 10.9% from occipital sites, respectively (Figures 19 and 21). There were no *Propionibacteriace* recovered from the frontal bulbs and only 3% from the occipital. *Staphylococcaceae* mostly included coagulase-negative staphylococci, such as *S. epidermidis*, *S. capitis* and *Staphylococcus* spp. strains, but in a few samples *S. aureus* cannot be excluded. *Propionibacteriaceae* mostly included *C. acnes*. A strong negative correlation between the relative abundance of *Lawsonella clevelandensis* and *Staphylococcaceae* in the material extracted from the infrainfundibular portion (HF w/o bulb) of plucked HFs from 12 healthy subjects was observed (Spearman's rank correlation coefficient, $R_S$ = -0.8886957) (Figure 22). There was no correlation between the relative abundance of *Lawsonella clevelandensis* and *Propionibacteriaceae,* and between *Propionibacteriaceae* and *Staphylococcaceae* in this material.

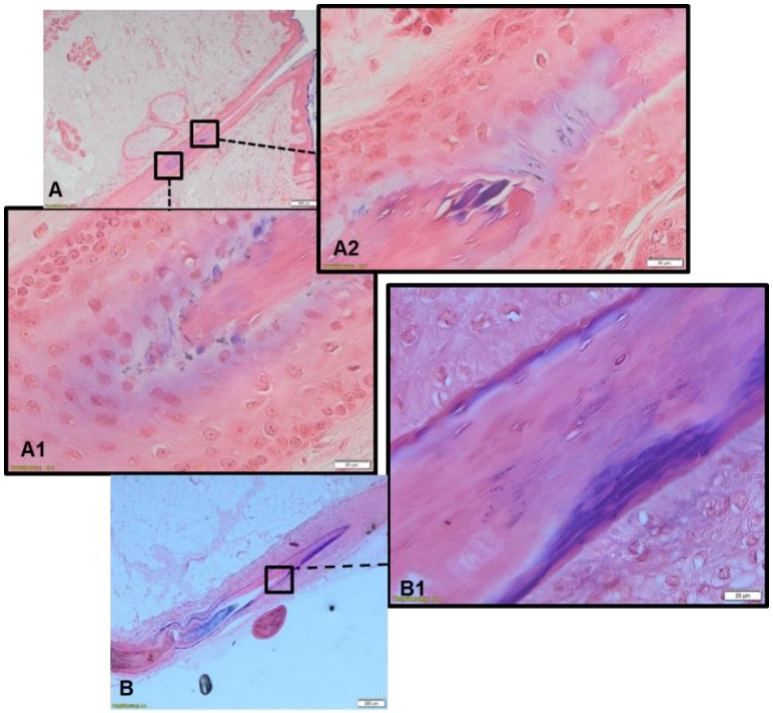


*Figure 17.*
*Gram-positive structures below the infundibulum.*
*Structures that might correspond to bacteria were found in the lower follicle, close to the hair shaft. For **A**, **B** magnification x100 (scale bar = 100 μm), for **A1**, **A2**, **B1** magnification x400 (scale bar = 20 μm).*

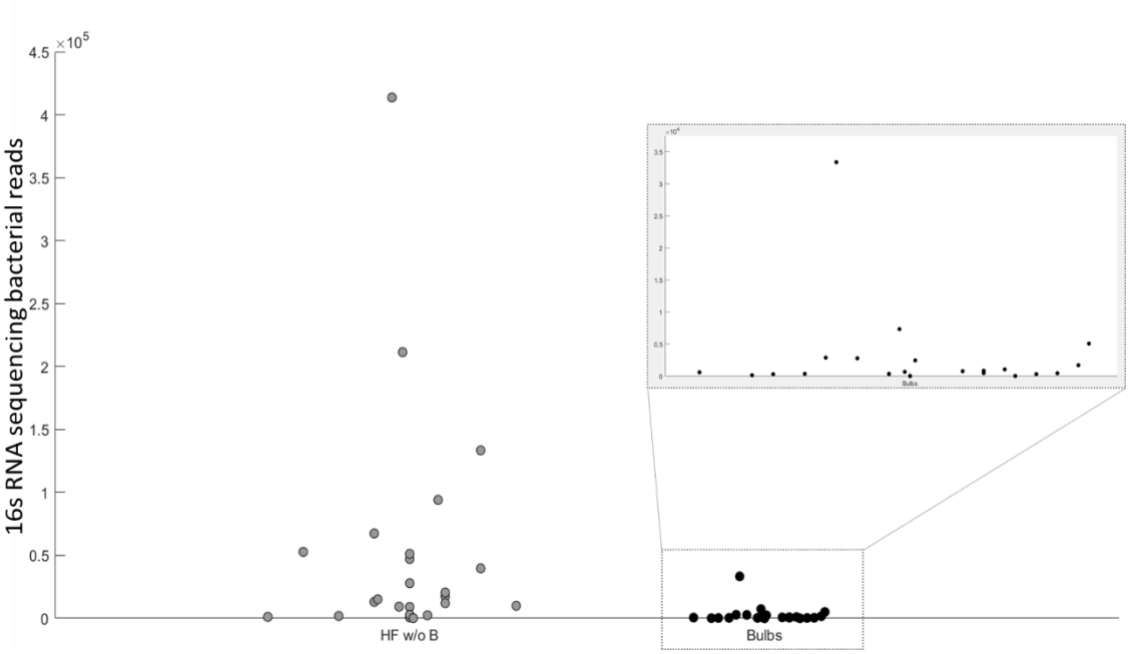


*Figure 18.*

*Number of 16S rRNA sequencing reads recovered from the lower scalp hair follicle.*

*From left to right, robust number of bacterial reads from the material extracted from the infrainfundibular portion of the hair follicle (HF w/o B) and smaller numbers of reads of the bulbs material (six bulb samples considered negative for bacterial material; one negative HF w/o bulb sample).*

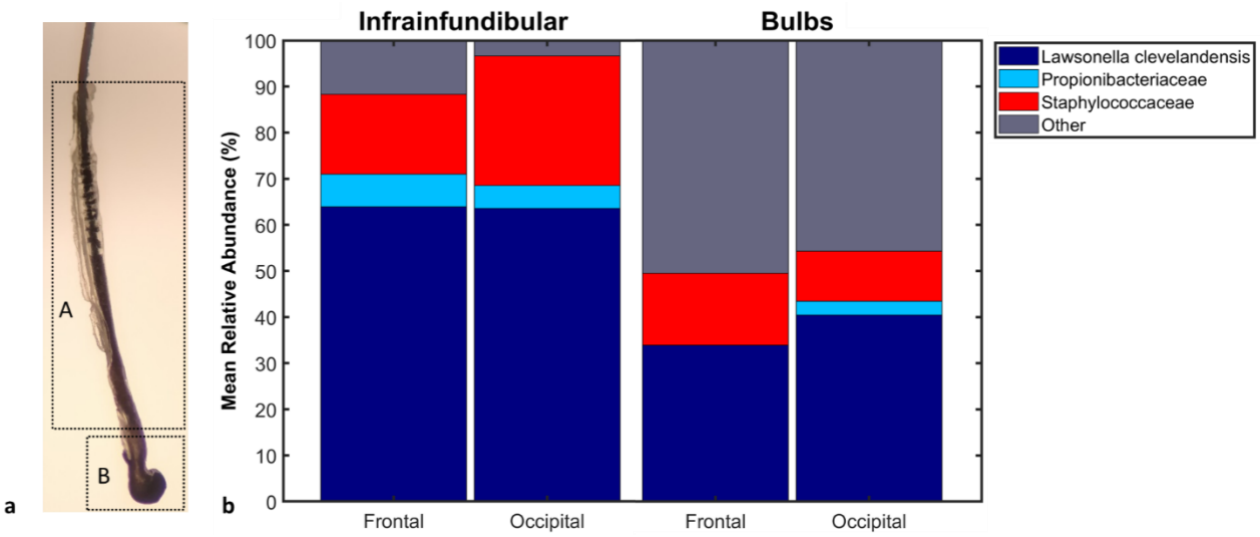


*Figure 19.*

*Profile of bacteria recovered from the lower follicle of healthy scalp.*

***(a)*** *Plucked hair follicle at the anagen stage. Samples collected from the frontal and occipital region, divided into (A) the infrainfundibular part (HF w/o bulb) and (B) the bulb.*

***(b)*** *Mean relative abundance (%) of the most abundant bacteria, identified in (A) the infrainfundibular part and (B) the bulb, from frontal and occipital site. Results are presented as a mean relative abundance percentage (%) of all samples, in the taxonomic rank family, with the exception of the species Lawsonella clevelandensis, which does not fall within an existing family or genus. Families are shown in color panels according to their phylum level: blue, Actinobacteria; red, Firmicutes; green, Proteobacteria. Lower abundances are classified as "Others". Staphylococcaceae mostly include coagulase-negative staphylococci, such as S. epidermidis, S. capitis and Staphylococcus sp. strains, but in a few samples S. aureus cannot be excluded. Propionibacteriaceae mostly include Cutibacterium acnes. Reproduced with kind permission of the publisher John Libbey (108)*

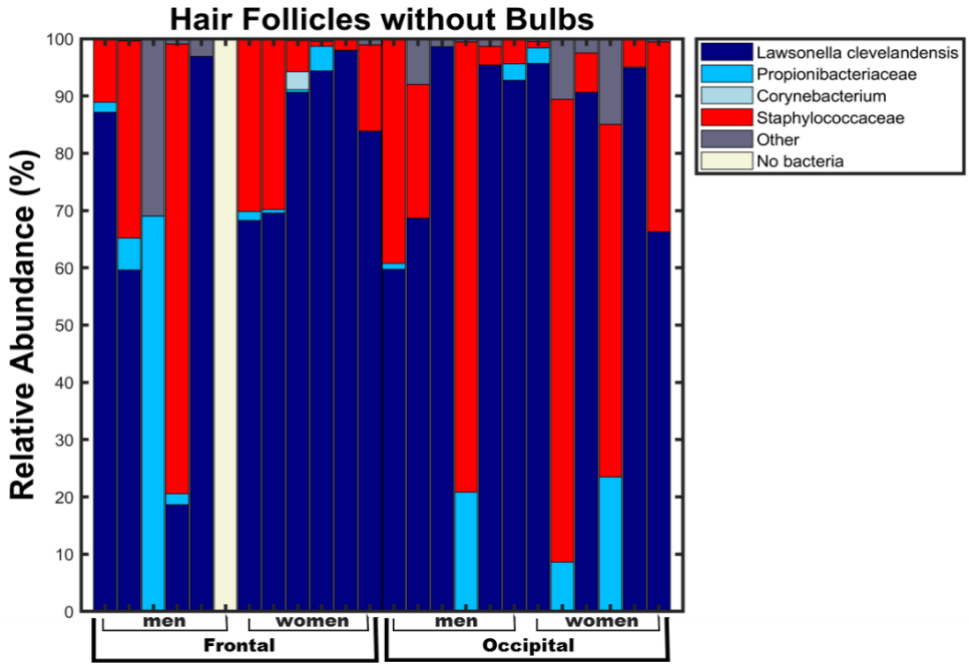


*Figure 20.*

*Bacterial abundance of the infrainfundibular portion of the hair follicle plucked from healthy subjects.*

*Relative abundance (%) of bacteria extracted from the infrainfundibular portion of hair follicles (HF w/o bulb, Figure 19 **(a.)** A) plucked from 12 healthy subjects (6 men, 6 women), from two scalp regions, frontal and occipital, presented in the taxonomic rank family, with the exception of the species Lawsonella clevelandensis, which does not fall within an existing family or genus. Families are shown in color panels according to their phylum level: blue, Actinobacteria and red, Firmicutes. Lower abundances are classified as "Others". One sample is considered negative for bacterial material. From the same individual, two more samples containing bulbs are negative as well. Reproduced with kind permission of the publisher John Libbey (108)*

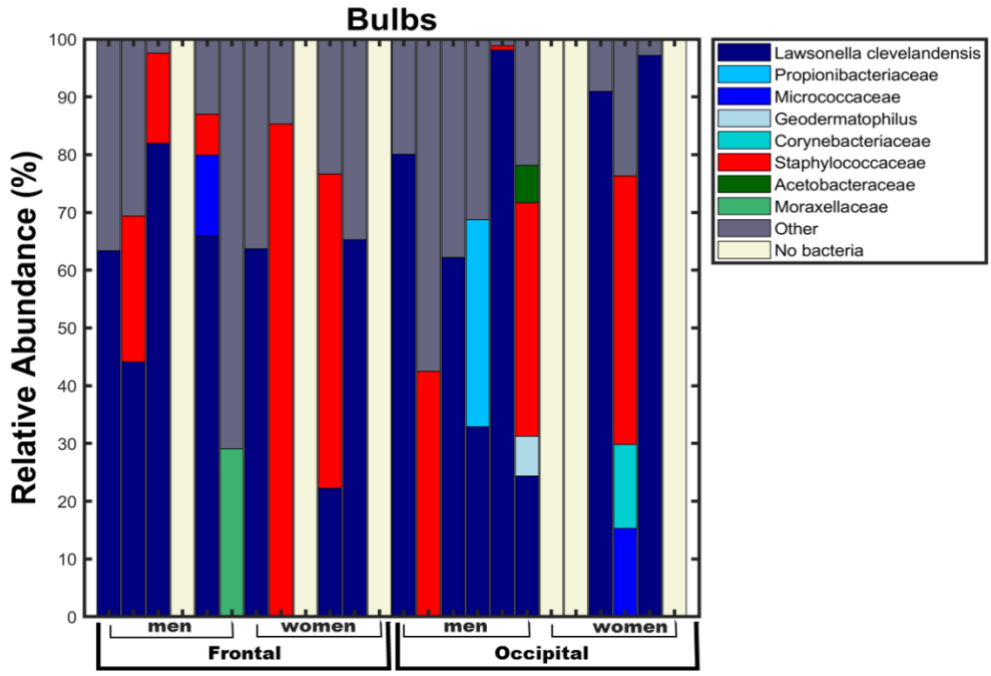


*Figure 21.*

*Bacterial abundance identified in the bulb of the hair follicle plucked from healthy subjects. Relative abundance (%) of bacteria extracted from bulbs (Bulbs, Figure 19 (**a**) B) plucked from 12 healthy subjects (6 men, 6 women), from two scalp regions, frontal and occipital, presented in the taxonomic rank family, with the exception of the species Lawsonella clevelandensis, which does not fall within an existing family or genus. Families are shown in color panels according to their phylum level; blue, Actinobacteria; red, Firmicutes and green, Proteobacteria. Lower abundances are classified as "Others". Six samples, two from the same individual (frontal and occipital) mentioned in Figure 20, are considered negative for bacterial material. Reproduced with kind permission of the publisher John Libbey (108)*

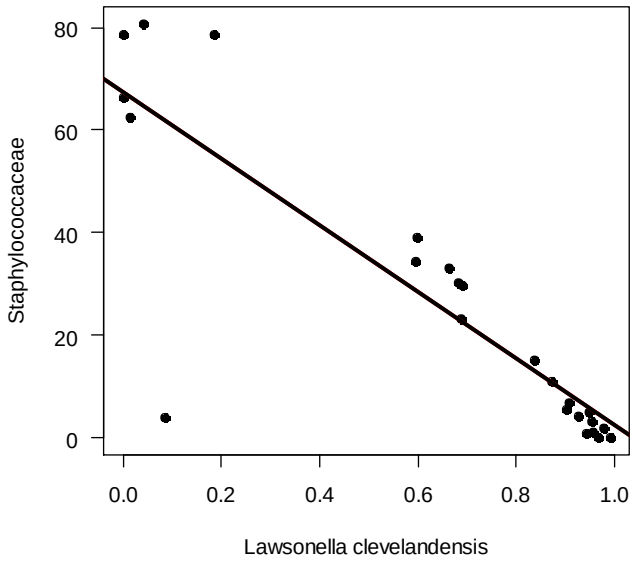


*Figure 22.*
*A negative correlation between the relative abundance of Lawsonella clevelandensis and Staphylococcaceae in the infrainfundibular portion (HF w/o bulb) of plucked HFs from 12 healthy subjects (application of Spearman's rank correlation coefficient).*

The sequencing results were further supported by FISH performed on plucked scalp follicles, showing *C. acnes* biofilm formations (Figure 23) between the inner root sheath and the hair shaft, presumably closely below the infundibulum. As the inner root sheath extends from the bulbar area to the isthmus level, it was indisputable that the detected bacteria were found below the infundibulum; however, their exact location could not be determined. Furthermore, structures corresponding to *Staphylococcus sp.* colonies were identified in this area. FISH visualizations were only produced in plucked, freshly prepared HFs, but not in sectioned tissue samples. We assume that routine processes of paraffin embedding and subsequent staining, connected with several de- and rehydration and washing steps, might have affected the negative results.

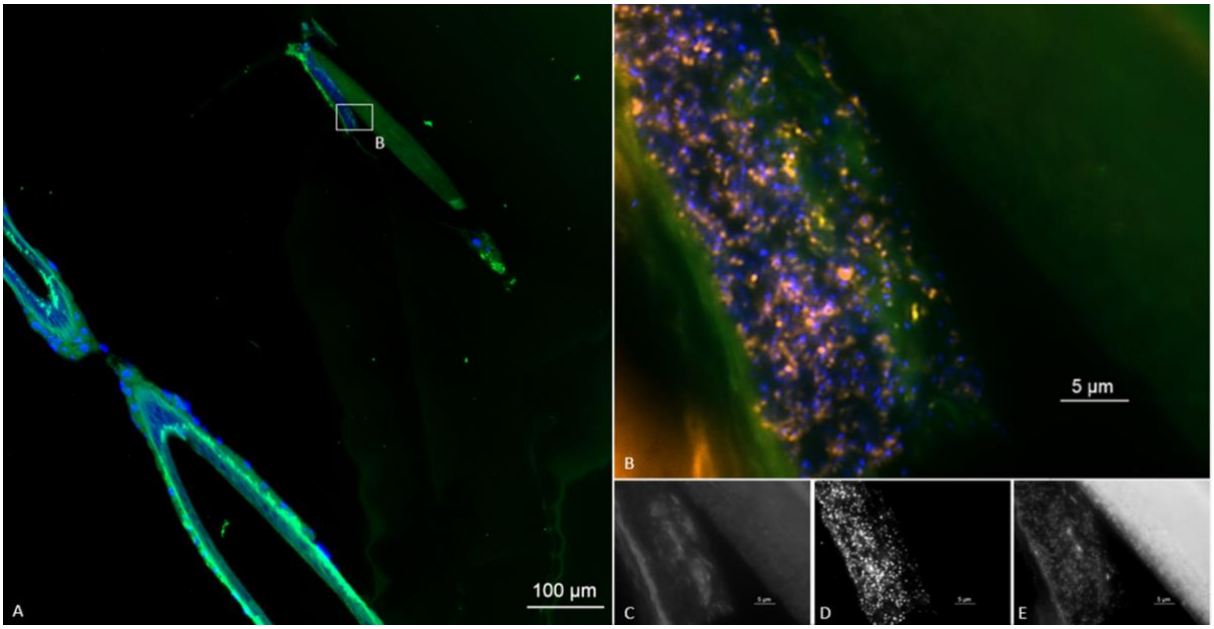


*Figure 23.*

*Identification of biofilm on a plucked hair follicle depicted by fluorescence in situ hybridization (FISH).*

***(A)** Overview of a longitudinal section of a lower portion of a plucked anagen hair follicle from the scalp of a healthy subject, hybridized with the species-specific Cutibacterium acnes probe $PRAC_{Cy3}$ (orange) and stained with DAPI (blue). Background autofluorescence is shown in green.* ***(B)** A higher magnification shows the overlay of the Cy3 and DAPI channels, visualizing FISH-positive Cutibacterium acnes biofilm below the infundibulum level.* ***(C)** Autofluorescence of the tissue background. (**D**) Biofilm stained with nucleic acid-specific stain 4',6-diamidino-2-phenylindole (DAPI). (**E**) Active Cutibacterium acnes bacteria in a biofilm. Reproduced with kind permission of the publisher John Libbey (108)*

## 9. Discussion

Our immunohistochemistry screening analysis of the expression of regulatory cytokines and host defense molecules in the upper part of the scalp hair follicle were mostly in line with current knowledge. We found dense infiltration of immune cells and high expression of antimicrobial peptides (HBD1 and HBD2) at the infundibulum level. Aside from the previously described high-abundance of CD4+ cells and lower abundance of CD8+ cells and mast cells, we also identified IL-17A+ cells densely infiltrating the connective tissue sheath outside the infundibular epithelium (11). On an immune-regulatory level, T helper 17 cells (CD4 and IL-17 positive cells) and IL-17-secreting CD8+ T cells (CD8 and IL-17 positive cells) are calibrated to produce IL-17, not only during infection, but also at steady state, in connection with cutaneous commensals (32, 35). An increased expression of IL-17A in a germ-free murine skin in response to *S. epidermidis* colonization provided evidence of an association of this cytokine with skin commensal microbiota (35). The overall enhanced immune cell trafficking and high expression of antimicrobial peptides within the upper follicular portion reflect a constant interplay between the local immune system and the external environment including resident microbial communities (98).

In accordance with Christoph et al., we found a significant drop in the number of CD4+ and CD8+ cells below the infundibulum (109). However, we made the observation that CD4+ cells, mast cells and also IL-17A+ cells were still present over the entire length of the follicle, even in deeper compartments. Unexpectedly, we also found a marked immunoreactivity of HBD2 in the lower follicle, in the peribulbar region. HBD2 belongs to antimicrobial peptides and is typically released in response to microbial stimulation (e.g., by *C. acnes* or by microbial components such as lipopolysaccharides) and also by pro-inflammatory cytokines (IL-1α, IL-1β and TNF-α) (111). Aside from its direct antimicrobial activity, HBD2 may act chemotactically for immune cells including mast cells, which are considered to play a role in hair growth control (111, 112), or activate cells, such as neutrophils and dendritic cells (113). The expression of IL-17A and HBD2 in the lower follicle was further confirmed by protein extraction from plucked follicles and subsequent ELISA analysis. We found the findings remarkable and pointing toward a possible presence of microbiota below the infundibulum, in the area so far widely considered as microbial-free.

To verify whether microbiota colonize the scalp HF below the infundibulum under normal circumstances, we applied several techniques in our exploratory study. We performed Gram staining, FISH and next generation sequencing of microbial material extracted from the lower portion of plucked HFs and scalp swabs. In line with current knowledge, rich bacterial microbiome

inhabiting the scalp surface and the upper follicular portion was identified (98). Bacteria forming colonies and biofilms extended from the follicular openings to the deepest parts of the infundibulum. Intriguingly, using the Gram staining technique, we found structures that might correspond to Gram-positive bacteria in the lower follicle. As the sensitivity and specificity of Gram staining is rather low, we decided to use 16S rRNA sequencing and FISH. As a material for microbiome analysis, we chose pulled terminal HFs at the anagen phase, whose bulbs are typically placed in the deep dermis or even deeper, i.e., in the subcutaneous tissue of the scalp. Bacterial DNA was recovered from the majority of plucked HFs. The method brought reproducible results and yielded a significant number of bacterial reads, suggesting that bacteria may penetrate deep along the HF canal. Considering the fact that the lower portion of HF contains structures of a relative immune-privileged status, our findings may have significant implications for the cycle of HF.

To our knowledge, we are the first to report biofilm structures below the infundibulum of the terminal HF of healthy scalp identified by FISH method. The visualization of *C. acnes* biofilms and *Staphylococcus sp.* colonies was consistent with recent results of Matard et al. (55). Similarly, Ho et al. and Pinto et al. recently isolated bacterial DNA from the subcutaneous tissue encompassing HFs (56, 57). The presented results also provide evidence for the complexity of biofilm structures. The knowledge of interactions within such formations as well as between biofilm and the local immune system still remains obscure (114).

The shift from the epidermal towards trichilemmal differentiation in the deeper parts of the infundibulum, connecting with its higher permeability and tubular downgrowth of the upper epithelial compartment during anagen onset, may enable microbes to translocate to the deeper follicular portions and partially explain our findings (24). Furthermore, the normal movements of hair, working like a pumping mechanism, may push small particles, possibly including bacteria, in the direction opposite to sebum excretion, down the HF canal (115). Taking a lesson from nanoparticle studies, we know that reservoirs such as HF orifices and skin furrows favor deeper enetration of particles (29). We also know that a mild inflammatory stimulation of Langerhans cells, switching them in an active mode, may lead to an increased uptake of larger molecules through the barrier of the skin (30). Bacterial factors such as the capability of forming biofilms and releasing different molecules such as enzymes or toxins, may help them reach the deeper follicular compartments.

Compared to previous studies, we found a significantly lower abundance of *Propionibacteriaceae* in scalp swabs. Similarly to Perez Perez et al., *Staphylococcaceae* constituted the dominant family in the investigated material (50-53). The differences in sequencing

results between studies may result from various factors such as differences in material collection techniques, material processing, methods of DNA sequencing and finally in data analysis procedures. The lack of unification of methods of microbiome analysis brings discrepancies in results between studies, and thus, hinders further data comparisons.

The most abundant bacterial species isolated from both the infraindibular part and the bulb of plucked terminal HFs was *Lawsonella clevelandensis*. This is an anaerobic bacterial species added recently to genomic libraries (in a new genus in the *Corynebacterineae* suborder), reported as a common inhabitant of healthy skin, nostrils and scalp hair shafts (116-119). Interestingly, the component of its cell wall is mycolic acid of a potential to exacerbate skin inflammation along with a higher production of IL-17A (120). *Lawsonella clevelandensis* was also abundantly present in our scalp swab material. In addition, we found a strong negative correlation of *Lawsonella clevelandensis and Staphylococcaceae* in the infrainfundibular portion of HFs.

If bacteria inhabit the whole infundibulum, including its deepest parts, and extend even below the infundibulum level, scalp microbiome analysis methods such as swabbing, scraping and tape-stripping do not give an insight into the real composition of microbiota of the scalp HF. As the microbes colonizing the most superficial parts of the HF are particularly susceptible to the external environment and altered quite easily, microbiota inhabiting the dermis - and possibly hypodermis - seem more specific for particular individuals and better reflect the immunological status of the host and the HF (95). The performance of scalp biopsy, including follicle-targeted biopsy, is a more accurate method for microbiome studies (97). However, its invasiveness hampers recruitments of larger groups of subjects, and thus, the collection of reliable results. Herein, we developed a new, non-invasive method for the investigation of the lower portion of scalp HF microbiome, using pulled hairs sampled according to the standard trichogram procedure and further processed, as reported in the materials and methods section. All the steps were performed with sterile instruments decontaminated with DNA decontamination liquid. Through processing of the follicle right below the outer root sheath level, we specifically targeted the infrainfundibular portion of the HF. Biopsy of the scalp and hair pulling can both provide the whole length of the HF for analysis, including the bulb. In contrast to a biopsy sample, the pulled HF does not include the sebaceous gland and the whole outer root sheath forming the bulge. The pulled hair only consists of the bulb and the hair shaft encased in the root sheaths. These arguments may be considered as a disadvantage of hair pulling; however, on the other hand, the less non-bacterial cells in a sample undergo microbial extraction, the more accurate bacterial sequencing results may be received afterwards. Each step of material preparation for 16S rRNA sequencing, both for a biopsy sample and a pulled hair, may be affected by an external contamination. Therefore, all

possible measures should be taken to maintain sterile, DNA-free conditions. During the hair plucking, a risk of the contamination of lower follicular compartments with microbial material also cannot be excluded.

We assume that our sequencing results, in combination with the immunohistochemical findings and ELISA measurements of biomarkers, point toward a practical relevance of our data, which we would like to discuss in separate sections below.

We would also like to emphasize that the results found in this exploratory study need to be confirmed by further independent studies.

### 9.1 Hair physiology

The epithelium of the terminal scalp HF, which reaches deep down in the dermis and even the subcutaneous tissue to an average of 3864 µm, may constitute a quite large interface for microbial-immune system interactions (121). While microbes on the skin surface are constantly exposed to unfavorable environmental factors and challenged by stratum corneum shedding, follicular skin invagination provides a sort of a shelter for microbiota (98). Furthermore, from the bulge region downward, there is a relatively immune-privileged portion of the HF, where keratinocytes actively suppress antigen presentation by downregulation of MHC class Ia molecules and the release of immunoinhibitors such as TGFβ, IL-10, α-MSH and CD200. This milieu may favor microbial colonization and survival (10, 11, 98).

Production of antimicrobial peptides is probably one of the most crucial instruments used by HFs to manage and control their microbiome. Not only defensins but also cathelicidins, psoriasin and RNase7 are released in response to bacterial products and metabolites (44, 122, 123). In addition, the specific composition and metabolic activity of commensal bacteria and fungi likely prevent pathogens from colonizing the HF niche. In the case of an excessive pathogen invasion and microbiome balance disturbance, outer root sheath keratinocytes of murine HFs can produce chemokines, interleukins, double-stranded RNA and short chain fatty acids, promoting efficient immune responses and host defense (51, 124).

Crucial interactions between microbes and HFs probably start very early in life, as depicted in a murine model and reported by Scharschmidt et al. Concomitant HF morphogenesis and colonization of the skin with commensal bacteria were indispensable to the determination of immune tolerance to bacterial skin commensals (7, 46). The leading immune cells determining this tolerance were Treg cells, whose presence close to the bulge promotes anagen entry and thus, HF regeneration (49). Microbial ligands can also activate toll-like receptors on mast cells and

stimulate keratinocytes to produce mast cell growth factors (125, 126). As mast cells are known for their broad anti-bacterial activity, they may help to keep the microbiota colonizing the HF in surveillance (127, 128). Therefore, mast cell and microbe interactions *were considered herein as* a form of negative feedback. Furthermore, mast cells modulate the activation and differentiation of HF stem cells (129, 130). A high level of their degranulation during late telogen to early anagen transition and late anagen to early catagen transition has been reported (131). Interestingly, overrepresentation and higher degranulation activity of mast cells have been described in some scalp diseases such as alopecia areata, androgenetic alopecia and telogen effluvium (129, 132). Therefore, we speculate that microbiota may affect the HF cycle via mast cell stimulation. Going a step further, we also ask the question of whether microbiota may influence the intrafollicular production of hair growth factors such as IGF-1 and TGF-β2. Olfactory receptors (OR2AT4) recently found on the outer root sheath could be the receptors through which such interactions may happen (133).

Interestingly, roxithromycin, a macrolide antibiotic, was found to modulate hair growth in the absence of bacterial folliculitis. As reported by Ito et al., roxithromycin increases hair elongation and inhibits catagen-like changes induced *in vitro* with IFN-ɣ in murine and human HFs. It was reported that roxithromycin solution is able to effectively restore hair growth in ca. 50% of individuals with androgenetic alopecia. This raises the intriguing question of whether the impact of roxithromycin on hair may be associated with its direct antibacterial action and microbiome modification (98, 134).

### 9.2 Hair diseases

Disturbances in the composition of the follicular microbiome have been reported in several skin diseases. Acne patients showed a higher prevalence of *Cutibacterium acnes* macrocolonies/biofilms, colonizing deeper portions of vellus follicles (21). *Demodex* mites were more numerous in follicular units in rosacea (135). Biopsies including HFs in hidradenitis suppurativa revealed a higher abundance of *Corynebacterium*, *Porphyromonas* and *Peptoniphilus* species compared to controls (136). In addition, recent studies on the microbiome of deeper skin compartments encompassing scalp HFs have recorded a higher abundance of Proteobacteria, Bacteroidetes and Firmicutes in alopecia areata, *Staphylococcus aureus* in folliculitis decalvans, and *C. acnes* in miniaturized follicles in androgenetic alopecia (55-57). The main disputable point of the aforementioned findings is that it remains obscure whether HF dysbiosis is a primary driver

of hair pathology or is only a secondary phenomenon to inflammation and metabolic or endocrine changes in the HF niche.

Considering that bacteria may colonize deeper follicular compartments under physiological conditions, we assume that some alterations of the microbiome, either its composition or metabolic activity, might have an impact on the immunology and/or physiology of the HF. We hypothesize that the breakdown of the immune-privileged status of the lower follicle and premature HF regression may follow inflammatory reactions caused by microbiome disturbances. Depending on whether such disturbances take place at the bulge or the bulb level, different clinical manifestations may be presented, namely the destruction of the bulge results in a permanent hair loss (cicatricial alopecia), whereas destruction of the bulb results in a reversible alopecia with a preserved HF (alopecia areata).

The most recent hypothesis about the pathogenesis of alopecia areata is the immune privilege collapse of the anagen bulb. Yet, its triggering factors still remain unclear. IFN-γ induced upregulation of MHC class I and II, decrease of TGF-β2 and MIF (macrophage migration inhibitory factor) expression, increase of MICA (MHC class I chain-related A gene) expression, mast cell degranulation and facilitated autoimmune response by CD4+, CD8+ T cells and NK cells are suggested mechanisms leading to the breakdown of the hair follicle immune privilege (137). A possible role of stress-induced mast cell degranulation (possibly via CRH, substance P or nerve growth factor release) and neurogenic inflammation is also currently being studied. Microbiome dysbiosis is enumerated among possible factors stimulating IFN-γ and/or substance P release by perifollicular sensory nerves (138). Some pathological interactions between mast cells and CD8+ T cells may cause the switch of mast cell function. To be precise, the cells lose their immuno-inhibitory function and acquire a pro-inflammatory phenotype, possibly contributing to the pathogenesis of alopecia areata. In addition, Treg cell regulatory properties may also be deficient in diseased individuals. Both mast cells and Treg cells have already been associated with microbiota. In particular, mast cells may be considered as a potential linkage between microbiota and the arrest of anagen, resulting in hair loss observed in alopecia areata (137).

There is a higher incidence of atopic dermatitis in alopecia areata patients than in the general population. Furthermore, atopic dermatitis is known as a predictor of a more severe course of hair loss. These data may point toward common pathomechanisms of the diseases (139). As recently reported, the severity of atopic dermatitis may be associated with the imbalance of skin microbiome. An overrepresentation of *S. aureus* along with a decrease in the number of commensal bacteria, including *Corynebacterium*, *Cutibacterium* and *Streptococcus*, have been identified in atopic dermatitis patients. The peculiarities of *S. aureus*, including proteases, toxins

and superantigens, and the direct interactions of *S. aureus* with the local immune system, may lead to an inflammatory response and disadvantage the skin barrier function (140). Therefore, we speculate that microbial dysbiosis, especially at the bulbar level, may be present in patients with alopecia areata. In addition, defects in the skin barrier integrity in atopic dermatitis may facilitate translocation of microbes colonizing the skin surface to the deeper follicular compartments and further change the composition or metabolic activity of follicular microbiota.

A possible role of *S. aureus* in the pathogenesis of folliculitis decalvans has been widely discussed so far (54, 55). However, we would also debate whether microbial dysbiosis may contribute to the course of other scarring alopecias such as lichen planopilaris or frontal fibrosing alopecia. In both lichen planopilaris and frontal fibrosing alopecia, the bulge immune privilege collapse and inflammation-induced epithelial hair follicle stem cell destruction are thought to be key components in their pathogenesis (10, 141). Aside from an increased expression of MHC class I and II, and the decrease of TGFβ2 and CD200 concentrations as well as Th1-biased cytotoxic T cell response, rich mast cell infiltration has been identified within diseased HFs in these disease entities. The mast cells were found positive for IL-17A in biopsy material from patients with lichen planopilaris (10, 142). Considering these results, our findings and the connection between mast cells and IL-17A release with microbial components, we further speculate about a role of microbiome in the pathogenesis of lichen planopilaris and frontal fibrosing alopecia. Furthermore, dysbiosis of microbiome has been recently reported in the saliva of patients suffering from a mucosal variant of lichen planus (oral lichen planus) (143). Increased numbers of mast cells and/or their degranulation activity have also been reported in androgenetic alopecia and telogen effluvium, which may point toward some associations with the scalp and follicular microbiota (129, 132).

In addition to the above-mentioned immunohistochemistry findings in patients with lichen planopilaris, a higher concentration of IL-17A in serum compared to controls and IL17RA gene polymorphism were identified in patients with alopecia areata (144-146). IL-17A is a cytokine strongly associated with microbiota, which is reported to, for example, induce epithelial antimicrobial peptide production in response to bacterial stimuli (147, 148). Considering that the component of the wall of *Lawsonella clevelandensis* may promote IL-17A production (120), we hypothesize about the connection of this bacteria and inflammation taking place in the lower follicle in inflammatory hair diseases. Interestingly, however, antibodies targeting IL-17 did not bring the desired results in the treatment of alopecia areata (149). Furthermore, there were reported cases of alopecia areata development in patients with psoriasis vulgaris treated with IL-17 inhibitors (150). We hypothesize that the release of IL-17A is not pathogenic in these diseases, but

may rather be a consequence of microbial dysbiosis and an attempt of the host to limit the pathologic event and restore the microbial balance.

### 9.3 Drug delivery to and via hair follicles

A better understanding of how microorganisms are organized within the hair follicle could contribute to the development of treatments bringing more satisfactory results for patients dealing with a chronic inflammatory hair disease. Advanced technologies rely on targeted delivery of therapeutic agents via specific particles in hair follicles and allow for the gradual release of actives with time (3). Controlled release from such drug delivery systems can be achieved by the introduction of elements responsive to physical or chemical stimuli (151), and even biofilm penetration properties have recently been proposed (152, 153). Hence, particles loaded with active agents targeting shifts in microbial composition and inflammation may provide a novel treatment option for patients suffering from the diseases, who have largely unmet clinical needs and a heavy psychosocial burden (154). Furthermore, we presume that the transport of topical agents to the HF and/or via the HF may be influenced by microbes residing in the HF. In fact, the effective delivery of topicals to deeper compartments of the hair follicles remains a challenge. There is little known about how the environment of the infundibulum and the infrainfundibular follicular portion affects the transferred particles. Lots of factors may play a role, such as the size of microbial colony, its consistency and permeability for particles, and also the complexity of biofilm structures (155). In addition, microbial peculiarities such as released toxins, enzymes, and direct interplay between the transferred molecules and microbiota along with the role of released antimicrobial peptides may foster or hinder penetration processes. We postulate that a better understanding of these interactions is worth investigating to further optimize the methods of transfollicular drug delivery (40).

A great reservoir in HFs for bacterial flora is considered responsible for rapid recontamination of the skin after antiseptic application and ineffective decolonization treatment with, for example, mupirocin in some patients colonized by *Staphylococcus aureus*. Common antiseptic- and topical antibiotic agents remain incapable of reaching bacteria deep in the HF canal (156, 157). Such increased attention on the HF reservoir function encourages researchers to work on new formulations of topical antibiotics, chemotherapeutics and antiseptics, ameliorating follicle targeting (158, 159).

## 10. Conclusion

In conclusion, the herein presented results show that bacteria may penetrate deeper along the hair follicle canal than has been believed so far. The presence of microbiota below the infundibulum, in proximity to the immunocompromised areas, being crucial for hair cycling, may have implications for the physiology and pathology of the hair follicle. Microbiota may also influence the delivery of drug particles to and via hair follicles. The presented set-up of clinical and experimental studies on non-invasively collected material from healthy individuals provides foundations for further studies including patients. Utilizing the hair pulling method instead of performing a biopsy of the scalp should enable the recruitment of larger and thus more representative study groups. As we have already learnt that microbiome imbalance may be associated with a wide range of human diseases (160), and that the dermal microbiome may better reflect the host immunological status (95), we believe that a deeper insight into the microbiome of the scalp hair follicle may help the understanding of the pathophysiology of some chronic inflammatory hair diseases, whose management is challenging and often brings unsatisfactory results. A deeper knowledge of the immunological processes occurring at the transition area of the hair follicle may help the elucidation of the role of external factors, e.g., stimuli provided by microbial colonization in hair diseases characterized by immune privilege collapse. The profiling of microbiota and biomarker expression on a larger data set may yield valuable information on the complex interplay and microbiome-host interactions in those niches.

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## 12. Statutory Declaration

I, Katarzyna Polak-Witka, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "Development of new methods for compartment-specific analyses of the hair follicle microbiome and associated inflammatory mediators.", "Entwicklung neuer Methoden für kompartimentspezifische Analysen des Mikrobioms von Haarfollikeln und assoziierten Entzündungsmediatoren.", independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.

Date Signature

_____

**Declaration of my own contribution to publications**

**Publication 1:** Katarzyna Polak-Witka*, Andria Constantinou*, Rolf Schwarzer, Johannes Helmuth, Alexandra Wiessner, Sabrina Hadam, Varvara Kanti, Fiorenza Rancan, Annette Andruck, Claudia Richter, Annette Moter, Anke Edelmann, Lidia Rudnicka, Ulrike Blume-Peytavi, Annika Vogt, Identification of anti-microbial peptides and traces of microbial DNA in infrainfundibular compartments of human scalp terminal hair follicles, Eur J Dermatol. 2021
*****equal contribution

Contributions: K. PW's contribution was mainly focused, but not limited, on the lower compartments of the scalp hair follicle. A.C's contribution was mainly focused on the scalp surface.
Polak-Witka's contributions:

- formulation of the hypothesis that bacteria may penetrate deeper compartments of the scalp hair follicle, what may be of relevance for scalp health and chronic scalp diseases;
- development of new protocols or adjustment of existing protocols concerning following methods: pulling scalp hair follicles and their subsequent splitting in distinct compartments, removal of human DNA and extraction of enriched microbial DNA from plucked hair follicles, protein extraction from plucked hair follicles and further ELISA performance on the recovered protein extracts, haematoxylin and eosin, Gram and Giemsa staining, Immunohistochemistry on scalp biopsy specimens;
- hair sample collection and its subsequent splitting and further processing i.e. microbial DNA extraction, protein extraction, ELISA performance for IL-17A, analysis of the results including statistics, drafting preliminary graphs in R;
- immunohistochemistry staining of biopsy specimens and further analysis, creation of the corresponding graphs;
- literature review;
- writing – original draft preparation;
- partial contribution in subjects' recruitment and non-invasive measurements collection, also in the analysis of microbial material recovered from scalp swabs.

**Publication 2:** Katarzyna Polak-Witka, Lidia Rudnicka, Ulrike Blume-Peytavi, Annika Vogt. Polak-Witka, K, Rudnicka, L, Blume-Peytavi, U, Vogt, A, The role of the microbiome in scalp hair follicle biology and disease, Exp Dermatol, 2020

Contributions: K. PW's contribution was topic conceptualization, literature review, writing an original manuscript draft, creation of figures. The other co-authors supervised the work and made revision of the original draft.

**Publication 3:** Andria Constantinou, Katarzyna Polak-Witka, Marios Tomazou, Anastasis Oulas, Varvara Kanti, Rolf Schwarzer, Johannes Helmuth, Anke Edelmann, Ulrike Blume-Peytavi, George M Spyrou, Annika Vogt, Dysbiosis and Enhanced Beta-Defensin Production in Hair Follicles of Patients with Lichen Planopilaris and Frontal Fibrosing Alopecia, Biomedicines, 2021

Contributions: K. PW's contribution was mainly focused, but not limited, on the delivery of healthy control data as well as the development of protocols further applied for patients with cicatricial alopecia (these data will not be part of the monograph itself). A.C's contribution was mainly focused on the collection of material from patients with cicatricial alopecias and further data analysis.

Polak-Witka's contributions:

- development of protocols concerning compartment-specific dissection of pulled hair follicles for microbial DNA analysis and protein extraction;
- delivery of data on microbiome of deeper compartments of the scalp hair follicle in healthy subjects serving as a control group;
- commentary and revision of the original draft;
- partial contribution in patients' recruitment and hair sample collection from patients with scarring alopecias.

_____

Signature, date and stamp of first supervising university professor / lecturer

_____

Signature of doctoral candidate

## 13. Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

## 14. List of publications

2021 Polak-Witka K, Constantinou A, Schwarzer R, Helmuth J, Wiessner A, Hadam S, Kanti V, Rancan F, Andruck A, Richter C, Moter A, Edelmann A, Rudnicka L, Blume Peytavi U, Vogt A. Identification of anti-microbial peptides and traces of microbial DNA in infrainfundibular compartments of human scalp terminal hair follicles. Eur J Dermatol. 2021 Feb 1;31(1):22-31.

2021 Constantinou A, Polak-Witka K, Tomazou M, Oulas A, Kanti V, Schwarzer R, Helmuth J, Edelmann A, Blume-Peytavi U, Spyrou GM, Vogt A. Dysbiosis and Enhanced Beta-Defensin Production in Hair Follicles of Patients with Lichen Planopilaris and Frontal Fibrosing Alopecia. Biomedicines. 2021 Mar 7;9(3):266

2021 Constantinou A, Kanti V, Polak-Witka K, Blume-Peytavi U, Spyrou GM, Vogt A. The Potential Relevance of the Microbiome to Hair Physiology and Regeneration: The Emerging Role of Metagenomics. Biomedicines. 2021 Feb 26;9(3):236.

2020 Polak-Witka K, Rudnicka L, Blume-Peytavi U, Vogt A. The role of the microbiome in scalp hair follicle biology and disease. Exp Dermatol. 2020 Mar;29(3):286-294.

2017 Waskiel-Burnat A, Dyttus-Cebulok K, Polak-Witka K, Pawlowska-Kisiel M, Szutkowski Z, Rudnicka L, Olszewska M. Stevens-Johnson syndrome induced by combined treatment: carbamazepine and cranial radiation therapy. A case of EMDART?, 1/2017, Przeglad Dermatologiczny

2015 Rudnicka L, Polak-Witka K. The diagnostics of hair disorders in children, Analiza Przypadków w Pediatrii, 1/2015, PZWL

## 15. Acknowledgements

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## 16. Confirmation by a statistician

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### Bescheinigung

Hiermit bescheinige ich, dass Frau Katarzyna Polak-Witka innerhalb der Service Unit Biometrie des Instituts für Biometrie und klinische Epidemiologie (iBikE) bei mir eine statistische Beratung zu ihrem Promotionsvorhaben „Entwicklung neuer Methoden für kompartimentspezifische Analysen des Mikrobioms der Haarfollikel und der Entzündungsmediatoren“ eng. „Development of new methods for compartment-specific analyses of the hair follicle microbiome and inflammatory mediators.“ wahrgenommen hat.

Folgende Beratungstermine wurden wahrgenommen:

- Termin 1: 08.01.2019
- Termin 1: 21.09.2021

Folgende wesentliche Ratschläge hinsichtlich einer sinnvolle Auswertung und Interpretation der Daten wurden während der Beratung erteilt:

- Empfehlung zur adäquaten Deskription der der beiden Gruppen (gesunde Männer und Frauen) je nach Art und Verteilung der Variablen
- Empfehlungen zur korrekten Anwendung geeigneter statistischer Tests in Abhängigkeit von den Fragestellungen (Mann-Whitney-U-Test zum Vergleich ausgewählter Daten zwischen unabhängigen Individuen (Männer vs. Frauen), Wilcoxon Signed-Rank-Test zum Vergleich intraindividueller Parameter (für Daten, die von der Frontal- vs. Okzipitalregion desselben Individuums erhoben wurden), Spearman-Rangkorrelationskoeffizient für die Assoziation zwischen Zytokinkonzentration (HBD2 und IL-17A), pH- und Talgwert und relativer Abundanz von ausgewählten Bakterien)
- Empfehlung eines prozentualen Ansatzes für die Darstellung bestimmter Bakterienarten
- Hinweise zur Interpretation der Ergebnisse

- Bitte explizit erwähnen, dass dies eine retrospektive Studie mit explorativem Charakter ist und die p-Werte entsprechend keinen konfirmatorischen Charakter haben und dass keine Adjustierung bzgl. multiplen Testens (z.B. Bonferroni-Korrektur) vorgenommen wird.
- Bitte in der Diskussion aufgreifen, dass die in dieser explorativen Arbeit gefundenen Resultate durch weitere unabhängige Studien bestätigt werden müssen.

Diese Bescheinigung garantiert nicht die richtige Umsetzung der in der Beratung gemachten Vorschläge, die korrekte Durchführung der empfohlenen statistischen Verfahren und die richtige Darstellung und Interpretation der Ergebnisse. Die Verantwortung hierfür obliegt allein dem Promovierenden. Das Institut für Biometrie und klinische Epidemiologie übernimmt hierfür keine Haftung.

Datum: 28.09.2021 Name des Beraters/ der Beraterin:

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Unterschrift BeraterIn, Institutsstempel

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