Aus der Klinik für Dermatologie, Venerologie und Allergologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Hair follicle bacterial dysbiosis: a distinct finding in patients with inflammatory hair diseases.

Bakterielle Dysbiose der Haarfollikel: ein deutlicher Befund bei Patienten mit entzündlichen Haarerkrankungen.

zur Erlangung des akademischen Grades
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von

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List of abbreviations

4',6-diamidino-2-phenylindole	DAPI
Alopecia areata	AA
Alopecia areata circumsripta	AAc
Entimicrobial peptides	AMP
Cutibacterium acnes	C. acnes
Enzyme-linked immunosorbent assay	ELISA
Epithelial HF stem cells	eHFSCs
Fibroblast growth factor	FGF
Fluorescent in situ hybridization	FISH
Folliculitis decalvans	FD
Frontal fibrosing alopecia	FFA
Hair follicle	HF
Hidradenitis suppurativa	HS
Human beta defensin 1	HβD1
Human beta defensin 2	HβD2
Immune privilege	IP
Integrative Human Microbiome Project	iHMP
Intercellular Adhesion Molecule	ICAM-1
Interleukin-1	IL-1
Interleukin-17A	IL-17A
Interleukin-8	IL-8
Lichen planopilaris	LPP
National Institute of Health	NIH
Ribosomal ribonucleic acid	rRNA
Staphylococcus aureus	S. aureus
Stratum corneum	SC
T helper 2	TH2
Transforming growth factor beta	TGF-β

Abstract

Introduction: A diverse community of microorganisms, usually non-pathogenic, resides on human skin and in hair follicle (HF) openings. Their role in promoting the innate and adaptive immune system to help maintain homeostasis has received increasing attention. While cutaneous dysbiosis associations with inflammatory skin diseases like acne and atopic dermatitis are well-studied in recent studies, the follicular microbiome of the scalp and its relation to hair inflammatory disorders like lichen planopilaris (LPP), frontal fibrosing alopecia (LPP) or alopecia areata (AA), remained mostly underexplored. We hypothesized that exposure to external triggers such as microbial components or metabolites, may elicit dynamic immune responses, potentially initiating or perpetuating inflammatory reactions like alopecia. Our objective was to investigate the bacterial composition and the production of selected inflammatory markers within this area.

Methods: In an observational, cross-sectional, descriptive study, data from FFA patients were collected via patient chart review or by active patient interview, to identify demographic, health characteristics, comorbidities, cosmetics used and treatments associated with the severity of FFA. In an exploratory experimental study, we further investigated the bacterial composition of the scalp and plucked HF of patients with FFA, LPP and AA, compared with healthy individuals, in relation to cellular infiltrates and the expression of defense mediators.

Results: The most common comorbidity found in FFA patients was thyroid disease, whereas abnormal testosterone and estrogen values were associated with a lower disease activity. Our correlation analyses did not support the previously hypothesized association between leave-on cosmetic products and the pathophysiology of frontal.

Our microbiome analyses confirmed the presence of bacteria below the infundibulum, both in healthy and patients. FISH revealed biofilm structures formed by Cutibacterium acnes and Staphylococcus sp. below the infundibulum in heathy plucked HFs. Metagenomic analyses revealed Staphylococcus to be the most abundant genus in lesional and non-lesional areas of LPP and FFA patients, while Lawsonella dominated in healthy individuals and in AA patients. We also found statistically significant differences in the ratio of Firmicutes to Actinobacteria between healthy scalp, lesional, and non-lesional samples of FFA and LPP patients. An enhanced beta-defensin production was observed in HFs from LPP and FFA patients.

Conclusion: Our findings represent the first report of dysbiosis in LPP and FFA. However, the possible link between bacterial shifts and the inflammatory infiltrate along the HF is yet to be explored. Larger-scale studies, employing advanced metagenomic technologies, are needed to identify the potential functions and metabolic activities of the microbial community within the follicular microenvironment.

Zusammenfassung

Hintergrund: In menschlicher Haut sind besonders die Haarfollikel (HF) dicht von Mikroorganismen besiedelt. Ihre vielfältige Rolle bei der Formung des angeborenen und des adaptiven Immunsystems findet zunehmend Anerkennung. Obwohl Assoziationen von Dysbiose mit entzündlichen Hauterkrankungen wie Akne und atopischer Dermatitis, intensiv erforscht werden, liegen kaum Daten zum Mikrobiom von Terminalhaarfollikeln und Beziehungen zu entzündlichen Haarerkrankungen, wie Lichen planopilaris (LPP), frontal fibrosierende Alopezie (FFA) oder Alopecia areata (AA), vor. Wir stellen die Hypothese auf, dass die Exposition gegenüber mikrobiellen Stimuli über dynamische Immunantworten zur Entzündung bei LPP und FFA beitragen kann. Das Ziel dieser Arbeit war es daher, bakterielle Besiedlung und ausgewählte Entzündungsmarker entlang von menschlichen Kopfhaarfollikeln zu untersuchen.

Methodik: In einer beobachtenden, deskriptiven Studie wurden Daten von FFA-Patienten/Patientinnen gesammelt, um demographische, gesundheitliche Merkmale, Komorbiditäten, verwendete Kosmetika und Behandlungen zu identifizieren, die mit dem Schweregrad der FFA in Verbindung stehen. Darüber hinaus untersuchten wir in einer explorativen experimentellen Studie anhand von Extrakten aus unterschiedlichen Kompartimenten gezogener Haare die Verteilung von Bakterien und die Expression von Abwehrmediatoren bei Patientinnen mit FFA, LPP und AA, im Vergleich zu gesunden Personen.

Ergebnisse: Die häufigste Komorbidität in FFA-Patienten/Patientinnen waren Schilddrüsenerkrankungen, während abnormale Testosteron- und Östrogenwerte mit einer geringeren Krankheitsaktivität verbunden waren. Unsere Korrelationsanalysen sprechen nicht für eine Rolle von Leave-on-Kosmetikprodukten in der Pathophysiologie der FFA. Unsere Mikrobiom-Analysen bestätigten dagegen das Vorhandensein von Bakterien unterhalb des Infundibulums. FISH-Analysen zeigten Biofilmstrukturen, die von Cutibacterium acnes und Staphylococcus sp. infrainfundibulär gebildet wurden. Metagenomische Analysen ergaben, dass insgesamt Staphylococcus die am häufigsten vorkommende Gattung in läsionalen und nicht-läsionalen HFs von LPP- und FFA-Patientinnen war, während Lawsonella bei gesunden Personen und bei AA-Patientinnen dominierte. Wir fanden statistisch signifikante Unterschiede in der Ratio von Firmicutes

zu Actinobacteria zwischen gesunder Kopfhaut, läsionalen und nicht-läsionalen Proben von FFA- und LPP-Patientinnen, welche mir erhöhter Beta-Defensin-Produktion einherging.

Schlussfolgerung: Unsere Ergebnisse stellen den ersten Bericht einer Dysbiose bei LPP und FFA dar. Es bleibt weiterhin unklar, ob bakterielle Verschiebungen einen Zusammenhang mit dem entzündlichen Infiltrat entlang der HF haben. um die potenziellen Funktionen und metabolischen Aktivitäten des Mikrobioms innerhalb des HF zu identifizieren, sind größere Studien erforderlich, bei denen hochentwickelte metagenomische Technologien eingesetzt werden.

1 Introduction

1.1 Rationale for investigating the hair follicle microbiome

The skin microbiome has been thoroughly investigated throughout the years,(1, 2) and the role of skin-resident microorganisms in the establishment and preservation of cutaneous homeostasisis is well characterized. (3, 4) Additionally, the cutaneous microbiome has been shown to have a critical impact on the disposition and/or propagation of inflammation-mediated skin diseases, such as psoriasis, acne and atopic dermatitis.(5-8) There is proven evidence on the variability of the composition of cutaneous bacteria according to body site and physiological characteristics such as sebum, pH, temperature, or moisture. (8-10) Understanding this community variability across different skin sites can provide a valuable foundation for the understanding of disease predisposition of specific body sites. Due to their anatomical structure and unique microenvironment, skin folds and cutaneous invaginations, including sweat glands, sebaceous glands, and hair follicles (HFs) are associated with their own unique microbiota.(1)

Hair follicles serve as pivotal sites for intricate interactions among the skin barrier, microbiome, and associated immune cells.(4, 11) On the contrary, despite being riddled with terminal HFs, the human scalp remains inadequately explored concerning its microbial inhabitants. Given that chronic skin diseases are typically associated with barrier abnormalities, cytokine increase and immune cell infiltrates,(12) inflammatory triggers or stimulatory signals like bacteria are more likely to reach viable cells.(13) The role of the immune system, including the activation of regulatory cells in the regulation of the hair cycle and the status of stem cell niches, has been variously described on the basis of basic science.(14-17) However there is scarce information on how to implement this knowledge to understand disease processes. Understanding the correlation between the presence and diversity of microbial material within scalp HF and the immune or disease status, holds the potential to furnish crucial evidence regarding the clinical significance of these mechanisms in inflammatory scalp diseases.

The human skin, constituting the outermost layer of the body, serves as the interface with the environment. Consequently, it has traditionally been recognized as an anatomical barrier regulating the transit of compounds and pathogens between the internal and

external milieu. While this assertion holds true, particularly in the context of the uppermost skin layer, the stratum corneum (SC), which adequately prevents the penetration of various substances, it is noteworthy that HFs emerge as efficient pathways for the transcutaneous penetration of topically applied agents, particularly high-molecular-weight compounds, or particulate matter. (18) (Figure 1) Smaller nanoparticles exhibited better and deeper penetration into the HFs compared to larger particles, (19) as well as a prolonged storage in the follicular reservoir.(18, 20) We therefore hypothesized that HFs could similarly allow the penetration of microorganisms of a similar size (i.e. ~ 0.5 - 2 µm).

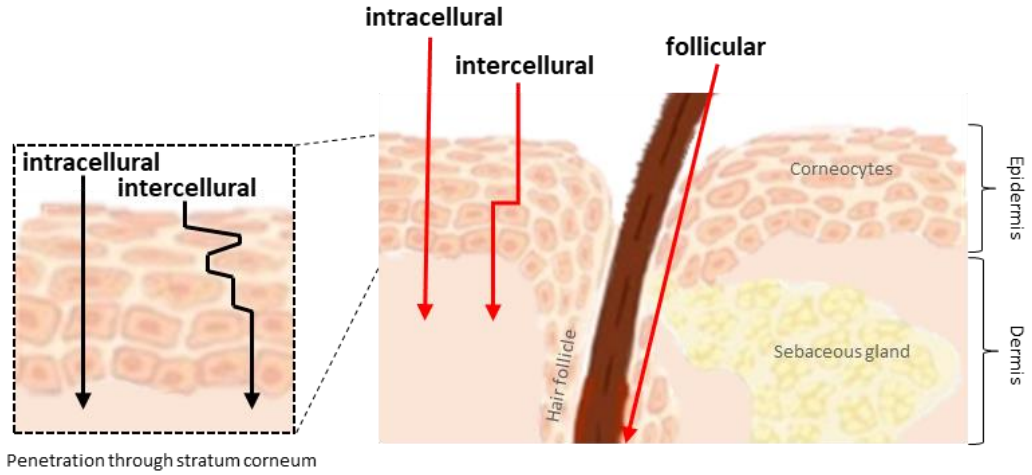

Figure 1: Illustration of skin penetration pathways: There are three different skin penetration pathways for topically applied particles; (1) through the intracellular route, (2) through the intercellular route, or (3) through the follicular route. Source: Own illustration.

Although earlier studies have addressed the role of hair follicles (HFs) as microbial reservoirs, the extent of microorganisms residing in the HFs compared to the overall cutaneous microbial population has not been investigated yet. (21) Despite the ever emerging research interest on the role of microbial presence and dysbiosis in human disease, the focus of these studies has mainly been the gut, where a substantial epithelial surface area facilitates extensive microbe-host interactions. (22) And despite the increasing attention toward skin microbiome research, our specific emphasis was directed toward the HF-unit. Similar to the gut, the epithelial surface of skin appendages warrants recognition as a significant interface for microbial communication, holding a great potential to influence the immune system. (22) (Figure 2)

Hair follicles are the most important appendages in terms of both surface area and skin depth, particularly due to the dense concentration of terminal HFs on the human scalp, extending into the deep dermis. (23) Hence a considerable amount of surface is provided

for follicular penetration processes, potentially hosting interactions between the microbiome and underlying immune processes. Hence, a precise understanding of the HF microbial composition in both healthy individuals and those with inflammatory hair diseases, could be of relevance for understanding the pathophysiology of skin inflammatory conditions.

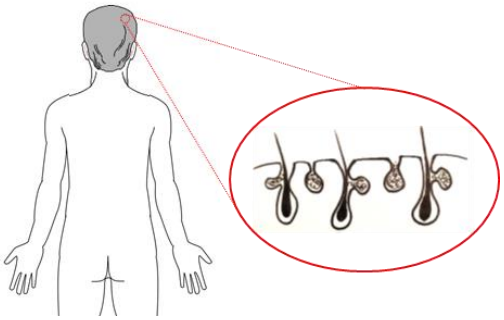

Figure 2: Estimated surface area of the skin: According to Gallo, since the human skin is not a flat surface, but is riddled with millions of appendages (HFs and glands), the total epithelial surface area that is accessible to microbes is now estimated to around 25m2; much greater than previously thought (~2 m2). (22) Source: Own illustration.

1.2 Skin and hair microbiome

1.2.1 Characterization of the microbiome

The cutaneous microbiome refers to the total genome of microorganisms residing on the skin.(24) Skin, being our exterior interface with the environment, hosts a plethora of commensal microbiota including bacteria, fungi, archaea, mites, and viruses, with whom a symbiotic relationship is established. Different sampling methods (e.g., swabs, skin stripping, biopsies) and evaluation techniques (i.e., cell cultures or next generation sequencing) are used to study the composition of the skin microbiome. Recent advances in sequencing technologies have allowed the extensive characterization of human microbiome in different sites, including the skin. Prior studies have found that the majority of skin bacteria belonged to the following phyla: Actinobacteria, Firmicutes, Proteobacteria and Bacteroides. The three prevailing genera identified encompass *Corynebacteria*, *Propionibacteria* and *Staphylococci*; however, their respective abundances exhibit variability contingent upon the anatomical site.(1, 2) Similarly, skin invaginations like HF openings and skin appendages (i.e. sweat and sebaceous glands) also constitute distinct habitats for microorganisms.(25) (Figure 3)

Human skin is first colonized at birth, and during the neonatal period an immune tolerance to commensal microbes is established.(26) By adulthood a rich and diverse microbiome is formed at different body sites.(27, 28) There is considerable variability in the microbial communities among individuals.(29, 30) The heterogeneity observed in the cutaneous microbiome among individuals, as well as alterations noted during an individual's life course, are subject to the influence of diverse factors, both exogenous and endogenous in nature.(31-34) These encompass variables such as sex, age, genetic makeup, climatic conditions, lifestyle choices, underlying medical conditions, medication usage, and anatomical body site. (1, 34, 35) For example, genomic analyses employing skin swabs across different anatomical regions, have shown that the physiological microenvironment (moisture, dryness and sebum) affects the local skin microbiome; The sebaceous sites, for instance, exhibit the lowest bacterial diversity.(2)

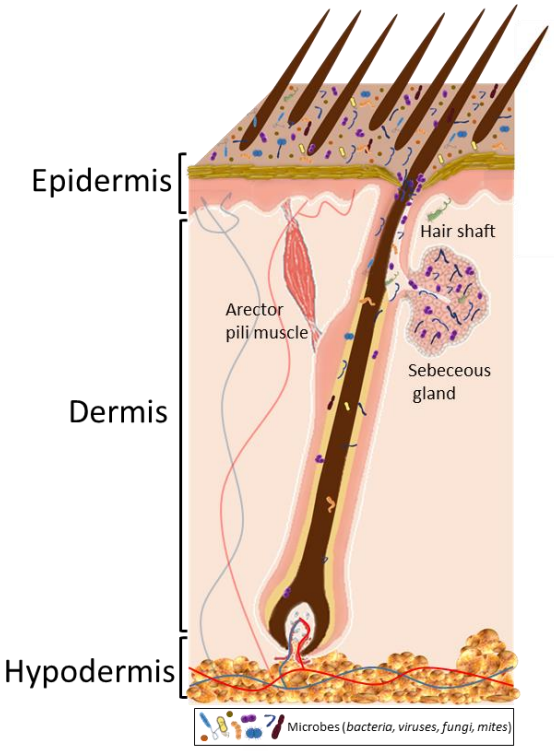

Figure 3: Schematic of a pilosebaceous unit of the skin with microorganisms: A pilosebaceous unit, comprised of the hair shaft, hair follicle, sebaceous gland, and arrector pili muscle. A variety of microorganisms (including Proteobacteria and Staphylococcus spp, Malassezia spp. and Demodex) reside on the surface of the skin and penetrate deep in the hair and glands. Source: Own illustration.

1.2.2 Microbiome – host crosstalk

Microbes inhabit many parts of the human body, and their contribution is well recognized, not only in diseases but rather in providing vital functions essential for the survival of their human hosts. Even though most of our knowledge on bacteria-dependent regulatory pathways derives from the gastrointestinal tract, the microbe-related pathways involved in the maintenance of homeostasis are possibly tissue specific (e.g. gut, skin). (36-38) The characterization of microbial components within the skin provides a fundamental basis for subsequent research, aimed at comprehensively elucidating both the interactions between microbes and host, and the interplay among different microbes (microbe-to-microbe interactions). It is widely recognized that microorganisms play a pivotal role, extending beyond the pathogenesis of diseases, in various physiological functions, such as lipid metabolism, tissue repair, colonization resistance, protection against infections and modulation of the local immune system. (4, 39)

The nexus between microorganisms confined in their niches and epithelial cells, lymphocytes, and antigen-presenting cells located in the epidermis and the dermis, merges innate and adaptive immunity in order to orchestrate well-organized immune responses. (25) Cutaneous bacteria promote the expression of innate immune factors, such as antimicrobial peptides (AMPs) (mostly cathelicidins and β-defensins) that directly eliminate a range of pathogens,(40) in addition to components of the complement system that opsonize pathogens and induce inflammation. (25) Skin resident microbiota also control local inflammatory milieus, through the interleukin-1 (IL-1) signaling pathway, a cytokine that regulates and initiates inflammatory responses. (25, 41, 42) (Figure 4)

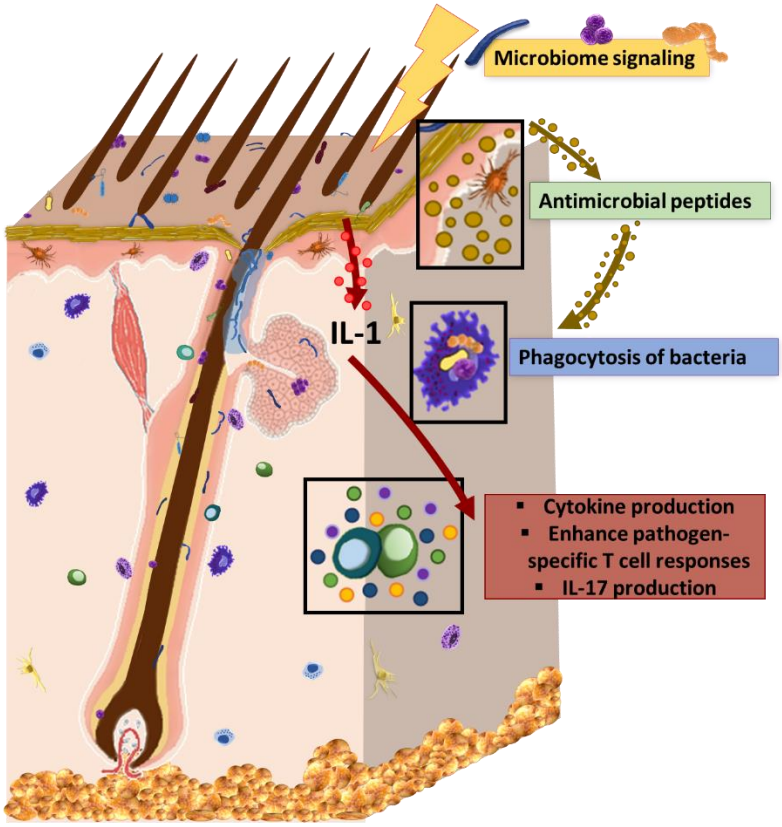

Figure 4: Microbiome and the cutaneous immune system crosstalk: A variety of microorganisms inhabit the surface of the skin and its appendages and are in constant contact with the cutaneous immune system and directly affect its responses. Microbiota signaling promotes the production of antimicrobial peptides and immune-mediators (e.g. complement, interleukin-1) by antigen-presenting cells and other immune cells. These molecules can further promote the secretion of cytokines by increasing skin's own antimicrobial functions, and by recruiting immune cells or modulating the activity of skin dendritic cells. Source: Own illustration.

Moreover, as demonstrated in a murine skin model of Candida albicans skin infection, specific subtypes of dermal dendritic cells (specialized antigen-presenting cells), are required for efficient cross-presentation and the elicitation of T helper-17 cell responses upon pathogen invasion.(43) Similarly, Langerhans cells have been shown to mediate tolerance or immunity through the induction of proliferation among pathogen-specific skin resident effector memory T cells and T regulatory cells, after exposure to pathogenic antigen.(44) Given that microbes occupying barrier sites, such as the gut or the skin, contribute to the regulatory pathways of local tissues, the breakdown of these pathways can lead to chronic inflammation and disease (e.g. inflammatory bowel disease and acne).(45) Shifts in the bacterial composition could therefore transform our microbial

allies into enemies and contribute to the development or the course of dysbiosis and disease. (42)

1.2.3 Skin and follicular microbiome in disease

1.2.3.1 Cutaneous diseases

Numerous studies have linked the skin microbiome to both the development and the severity of various inflammatory not-infectious skin diseases. Nevertheless, these associations differ across different diseases. For example, early clinical studies in patients with atopic dermatitis showed that a decrease in microbiome diversity correlates with disease severity and increased presence of pathogenic bacteria like *Staphylococcus aureus* (*S. aureus*), thus supporting the protective role of skin commensals.(7, 46-48) That said, the pathophysiology of atopic dermatitis is intricate and multifactorial, involving environmental triggers (like bacteria) alongside with genetic predisposition and immune dysfunction.(49) These findings underscore how intricate the interplay between host factors and bacteria-driven mechanisms.(47)

Psoriasis has been a subject of considerable scrutiny regarding its potential association with an altered skin microbiome. Numerous studies have reported differences between healthy or uninvolved skin and psoriatic lesions (50-54). However, the different methodologies, sample sizes, and body site variability make it difficult in formulating a definitive conclusion regarding the true implication of these changes. For example, a relative increase in *S. aureus* in both lesional and non-lesional psoriatic skin, and an underrepresentation in *Staphylococcus epidermidis* and *Propionibacterium acnes* in psoriatic lesions were reported by Chang et al. supporting the protective role of commensals. (8) Similarly the decrease of immunoregulatory microorganism like *Staphylococcus epidermidis* and *Propionibacterium* acnes may cause community instability and enable the overpopulation of pathogens like *S. aureus.*(8) Alekseyenko et al, found consistent decreases of taxonomic and species level diversity not only in the psoriatic lesions but also at the clinically unaffected skin of psoriasis patients, compared to healthy controls.(50) These observations indicate that psoriasis could affects the bacterial composition of the skin as a whole, and not specifically of the lesion sites.(50) Despite the findings of an altered cutaneous microbiome in psoriasis, the question still remains; does is play a role in the pathogenesis or propagation of psoriasis, or is this an epiphenomenon of the compromised skin barrier function?

1.2.3.2 HF-associated skin diseases

Acne, a prevalent dermatological condition, is characterized by a chronic inflammation affecting the pilosebaceous unit, consisting of the HF, sebaceous gland, and erector pili muscle. The pathogenesis of acne involves various factors such as an altered sebum production or lipids, inflammation, hormones, hyperkeratinization and bacteria.(55) While Cutibacterium acnes (formerly Propionibacterium acnes) typically inhabits healthy skin and HF, it is implicated in the inflammatory cascades of acne pathogenesis, despite not being inherently categorized as an infectious agent. Notably, research indicates that the the more pronounced differences observed in individuals with acne are not primarily in bacterial relative abundances but instead in the bacterial strain population and their metabolic activity. (6, 56, 57) This further highlights the complex nature of bacterial pathogenicity mechanisms.

Rosacea is another common chronic inflammatory skin disease exhibiting various clinical cutaneous manifestations (i.e. erythematotelangiectatic, papulopustular, phymatous or ocular), the exact etiology of which remains incompletely understood.(58) Some of the suggested pathophysiological mechanisms of rosacea include neurovascular dysregulation, immune system responses, and Demodex mites infestation.(59) Demodex, a genus of microscopic parasitic mites found inside or around HFs are typically found on the face, but can occasionally cause skin diseases.(60) A meta-analysis concluded that Demodex infestation is a crucial risk factor for rosacea and the severity of Demodex species infestation had a statistically significant association with the development of the disease.(61) The association between the Demodex infestation and rosacea was mainly based on histopathologic findings, in addition to a reduction in the density of demodex mites after treatment. (62, 63) However the role of Demodex in the pathomechanisms of rosacea is still controversial. There might be a genetic susceptibility in some individuals resulting in different immune reactions.(60)

Hidradenitis suppurativa (HS), is a complex, chronic inflammatory disorder, with currently an unclear pathogenesis, affecting HF in intertriginous regions that results in painful nodules and abscesses, fistulas, sinus tracts and eventually scarring.(64) HS pathogenesis encompasses genetic and environmental factors, lifestyle, hormonal status and microbiota, that result in immune activation around the terminal HF, hyperkeratosis

of the infundibulum and eventually follicular plugging and stasis.(65) While mild inflammation can happen at any stage, the breach in the cyst or lumen wall and extrusion of luminal cornified debris, act as a ‘booster’ for immune activation.(65, 66) Anaerobic bacteria, like *Prevotella* and *Porphyromonas*, coagulase-negative *Staphylococcus*, and *S. aureus* were regularly found in HS lesions.(67-70) Bacterial biofilm formation has also been reported in lesions of HS.(71)

1.2.3.3 Scalp diseases

Folliculitis decalvans (FD) represents a well-studied microbe-related inflammatory hair disorder. Classified as a rare form of neutrophilic primary cicatricial alopecia (PCA), FD's etiology remains partially unclear.(72) Nonetheless, *S. aureus* is suggested to be pivotal in its pathogenesis, as it has been consistently isolated from the majority of patients, and instances of remission post-antibiotic treatment have been documented.(73-75) Bacterial communities organized as biofilms formed exclusively of bacilli were observed in the infrainfundibular part of HFs in FD patients.(76) A recent retrospective cohort study identified a subset of patients with gram-negative infections, or no infections.(77) Similar to observations in acne and rosacea patients, a hypothesis posits that gram-negative folliculitis may arise as a complication of prolonged antibiotic therapy. This is attributed to the suppression of normal commensal flora and reduced levels of protective Propionibacterium acnes, which along with coagulase-negative staphylococci, also responds well to anti-staphylococcal antibiotics. (77, 78)

Immunohistochemistry of early FD lesions has revealed an infiltration of activated T-helper cells, with a mixed TH1/TH2 polarization.(79) Moreover IL-8 and ICAM-1 may chemoattract and recruit the neutrophils, while b-FGF and TGF-β may represent key mediators of late fibrosis observed in FD.(79) In addition, immune signals associated with inflammasome activation (e.g. NALP1, NALP3, and IL-1β) were significantly increased in HFs in FD, which could be a consequence of microbiota dysbiosis similar to psoriasis or HS.(80, 81) Asfour et al. showed significantly higher antibiotic resistance rates in FD patients, highlighting the need for non-antibiotic drug therapies (e.g., isotretinoin/dapsone/photodynamic therapy) to suppress inflammation and induce long-term remission, while preserving antibiotic treatments for disease exacerbations.(82)

Dandruff (seborrheic dermatitis on the scalp) is one of the most common scalp disorders in adults, and involves pruritus and desquamation without visible inflammation.(83) The

exact pathophysiology remains unclear, yet various inherent and environmental factors, such as sebaceous secretions, fungal colonization and individual susceptibility are discussed as contributing factors.(83) Excessive yeast presence/proliferation of the genus Malassezia on the scalp, has long been considered the main triggering factor, despite the lack of a proven solid causal relationship. This is unlikely to be the only cause, considering that Malassezia species are the dominant fungi found on healthy skin,(84) and therefore not considered to be de facto infectious.(85) On this account recent studies have shifted to a more global look into the microbiome aspect of seborrheic dermatitis.(86, 87) Park et al. found both bacterial and fungal communities to be different between disease and controls, and appeared to be associated with the scalp disorder.(86) Saxena et al. complimented their microbial analysis with a functional analysis, which revealed that the fungal microbiome was enriched in pathways greatly involved in cell-host adhesion in the dandruff scalp, while the bacterial microbiome showed a noticeable enrichment of pathways related to the synthesis and metabolism of elements vital for hair growth, like amino acids, biotin and other B-vitamins.(87) These findings highlight the importance of studying the microbial community of the scalp as a whole, rather than looking into specific bacteria or fungi.

1.2.3.4 Problems with the concept of dysbiosis

Dysbiosis is described as the alteration of the commensal community composition, usually towards a decrease in beneficial microbiota, resulting in impaired homeostasis. Over the past decade, investigations into the human microbiome have sought to delineate its characteristics and examine potential associations between humans and their commensals with respect to various diseases. The basis for the host–microbe equilibrium is fragile, balancing tolerance and inflammatory immune responses. Recent studies have begun to identify how microorganisms and their mechanisms are responsible for eliciting immune developments within the host.(88) However, the majority of studies on dysbiosis are mostly exploratory, and do not report the mechanisms in which microbiota influence host biology. Additionally, considering how variable the so-called “healthy” microbiome is, comparing patients against healthy controls in microbiome studies could also result in prejudices. (29, 89)

Current knowledge underscores that diseases are not exclusively associated with pathogens (known infectious agents); commensals can also adopt pathogenic traits under specific conditions. Afterall the pathogenetic mechanisms of microorganisms are

rather complex, stretching far beyond their abundance on/in the host. Crosstalk between the host and microbes, but also microbe-to-microbe interactions, in addition to the pathways of bacterial metabolism and metabolic interactions, might be able to shape the local microenvironment towards a disease or healthy status. Whereas a change in the bacterial presence/abundance of specific taxa, only provides us with a fraction of the knowledge needed to understand the pathophysiology of dysbiosis. The microbiome research is currently mainly descriptive, and there is a great deal of difficulties in translating our results into clinical therapeutic interventions, due to the large compositional complexity of the data that require sophisticated analyses.(90)

A disruption in the microbial communities colonizing the skin (skin dysbiosis), like an imbalance between the commensal and opportunistic microbiota due to factors such as drug intake, comorbidities, diet, or trauma, may potentially result in skin inflammatory processes. Having said that, we are only scratching the surface of comprehending the complexity and intimacy of host-commensal relationships. What remains to be elucidated is whether dysbiosis is pathogenic and a driver in the underlying immune processes of inflammatory skin disease, or a secondary outcome. We ought to be careful with the interpretation of such results, especially with respect to causal hypotheses, and avoid directly connecting findings of dysbiosis to a disease outcome.(91) Though taxonomic findings of dysbiosis are a starting point, they lack the capacity to differentiate causal connections between host microbes and disease, from secondary microbial changes that might accompany the disease course.(89, 92) To investigate that, the functional analysis of the microbiome is more relevant than a compositional analysis.(92)

1.3 NIH Human Microbiome Project as a model metagenomics approach

The outdated notions positing microbiota as inherently pathogenic or infectious entities for humans have been abandoned in recent years. Scientists now recognize that many microorganisms have a mutualistic relationship with their hosts, even providing vital functions essential for human survival. As ever-increasing studies have demonstrated that shifts in the microbial composition correlate with various disease states, the possibility that manipulation of these communities could be used to treat disease was suspected. This is how the Human Microbiome Project (HMP),(93) comprising a consortium of researchers, organized by the National Institutes of Health (NIH) was established. The mission of the HMP was to characterize the human microbiome, with

the aim of better understanding how microorganisms impact human health and disease. Initiated over a decade ago, their look on microbiome understanding was revolutionary; To comprehensively understand how microbes contribute to chronic disease, it is imperative to acquire data pertaining to the other integral component, the host.

The initial phase of the project (2008-2013), named HMP1, was an interdisciplinary effort to characterize the microbial communities from 300 healthy volunteers, across various body sites (i.e. nasal, oral, skin, gut, and urogenital tract), using 16S rRNA sequencing.(29) Using metagenomic whole genome shotgun sequencing they additionally investigated the functions and pathways present in the microbiome.(94) The second phase of the project, named the Integrative Human Microbiome Project (iHMP)(95) will study the microbiome to host interactions by analyzing microbiome and host activities in longitudinal studies of disease-specific, microbiome-associated cohorts (preterm birth, inflammatory bowel disease and type-2 diabetes) and by creating integrated data sets of microbiome and host functional properties.(96) In order to study the molecular microbial activity during dysbiosis, new computational tools are integrated. As a result of creating these metagenomic and metatranscriptomic data resources, the iHMP project has been a pioneer in paving the way for high quality analyses of human microbiome data. These data sets served as experimental test beds to evaluate new models, methods, and analyses, which have been invaluable and have laid the foundation for integrating such tools into countless future studies, ranging from basic to translational to clinical.(96, 97)

HMP generated massive open-source datasets for researchers to further investigate. The HMP website provides microbiome datasets and reporting standards, allowing researchers to query and retrieve metagenomic, metatranscriptomic, human genetic, microbial culture, and other data types from the project.(93) Granting researchers access to these complicated datasets and study protocols not only advocates for data transparency and reproducibility but also enables researchers to contribute or further enhance existing knowledge helps, maximizing data accuracy and consistency .

1.4 Cicatricial alopecia (LPP and FFA)

1.4.1 Epidemiology, clinical presentation, etiopathogenesis

Alopecia (i.e. hair loss) is classified as cicatricial (scarring), resulting in permanent hair loss, and non-cicatricial, when HF's regenerative capacity is preserved. Lichen planopilaris (LPP) and frontal fibrosing alopecia (FFA) represent primary cicatricial alopecia entities. FFA has been considered a variant of LPP due to their almost identical histopathology, despite their different clinical presentations.(98) Even though rare, FFA has globally emerged as the most frequent cicatricial alopecia diagnosis in hair clinics, followed by LPP. (99) The psychological impact of alopecia is devastating and can affects the patient's quality of life.

Lichen planopilaris is an inflammatory scalp disorder that usually presents with progressive hair loss patches (Figure 5Aa), and affects women more than men. Diagnosis is usually made based on clinical presentation, especially during the disease flare ups, however in the subacute, early or late disease stages a scalp biopsy might be needed.(100) Pruritus, pain and burning sensation are common symptoms during flare-ups. Dermoscopy reveals follicular hyperkeratosis, perifollicular erythema, obliterated follicular ostia (Figure 5Ab,c). Histology of active lesions shows a dense perifollicular lichenoid infiltrate composed mainly of lymphocytes, and progressive perifollicular fibrosis.

Although FFA was mostly reported in post-menopausal woman, it can also affect men and younger women. Clinically, FFA is characterized by progressive band-like recession of the hairline, often accompanied with thinning or total loss of eyebrows (Figure 5Ba,b).

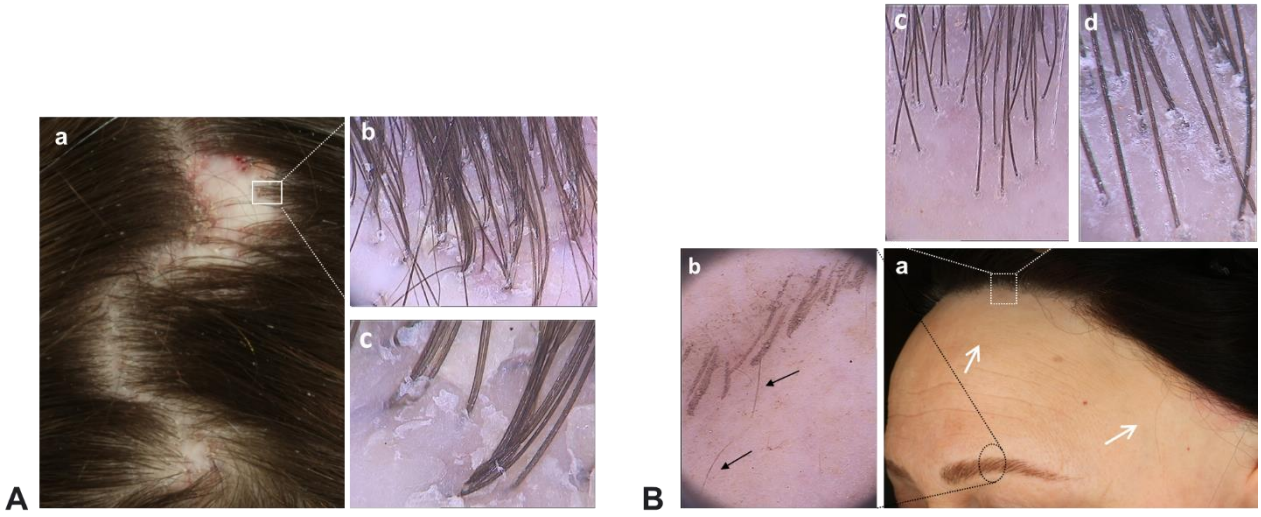

Figure 5: LPP and FFA clinical and dermoscopic photos: Clinical and dermoscopic pictures of the scalp of a LPP (A) and a FFA (B) patient. (A) (a) global hair photography shows active lesions with permanent hair loss patches, and perifollicular erythema on the edges. Dermoscopic scalp examination at (b) 20-fold and (c)70-fold magnification at the edge of the scalp lesions. (B) (a) global hair photography shows the frontotemporal recession of the hairline (white arrows). (b) Dermoscopy also revealed loss of eyebrow hairs. Though cosmetic camouflage techniques mimicking eyebrow hairs are often used by patients, dermoscopy shows that only scarce eyebrow hairs are still intact (black arrows). Dermoscopic scalp examinations at (c) 20-fold and (d)70-fold magnification on the edge of the hair loss region. Source: Own illustration.

The skin in the affected area is usually pale, due to lack of sun damage, and shiny, without visible HF openings (Figure 5Ba). Pruritus, pain, and increased scalp sweating were reported, though FFA can also be asymptomatic. Dermoscopy reveals perifollicular erythema, hyperkeratosis and obliterated follicular ostia (Figure 5Bc,d).

As accurately described by Harries et al. the etiopathogenesis of LPP and FFA mainly involves inflammation-associated, irreversible damage to epithelial HF stem cells (eHFSCs) of the HF in their immune privileged niche, located around the bugle area, causing irreversible hair loss and eventually scarring (Figure 6).(101) There are many proposed models for the pathogenesis of LPP and FFA, including the loss of CD200 ‘no danger signal’, PPPRγ deficiency, inflammatory and pro-inflammatory activation or altered lipid metabolism.(101) Many factors have been suggested to play a triggering role in the disease development including environmental, genetic or hormonal, especially due to the rising incidence of FFA cases, however the results are still conflicting.(101-103) Hence it remains to be proven if the immune privilege (IP) collapse occurs early in the disease process, or whether it is secondary to environmental factors, such as an infection, possibly affecting genetically predisposed patients.

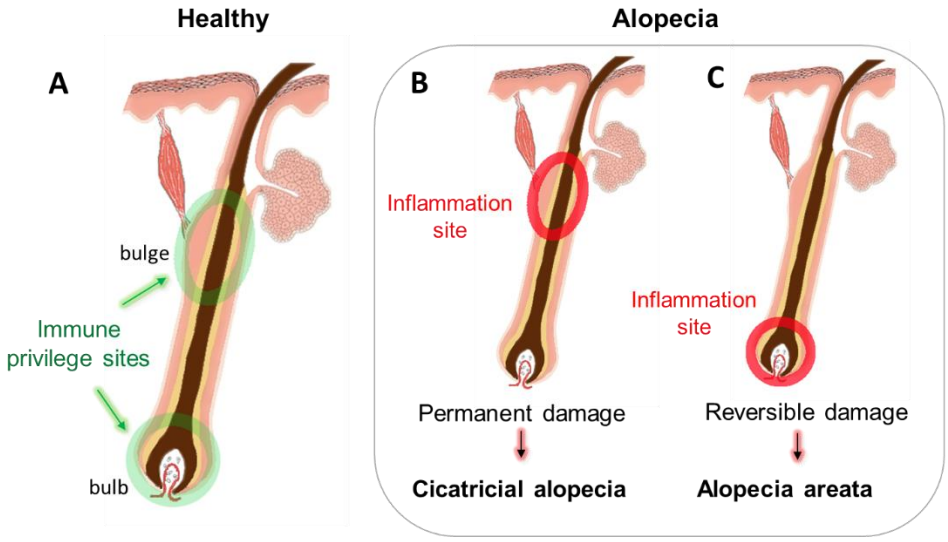

Figure 6: Immune privilege collapse in alopecia: (A) The bulge and bulb regions are recognized sites of immune privilege (IP). The bulge IP protects the eHFSC niche that is essential for HF regeneration. (B) An IP collapse and inflammation attack at the bulge area will cause the destruction of the eHFSCs, eventually resulting in permanent hair loss and scarring. This is considered to be a key event in the pathogenesis of PCA like LPP and FFA. (C) Whereas an IP collapse and inflammatory infiltrate around the hair bulb affect the hair cycle causing hair loss, while preserving the possibility of HF regeneration after remission. Source: Own illustration.

1.4.2 Therapeutic challenges

Despite the increase in the disease rates, LPP and FFA remain quite rare. Several therapeutic approaches have been proposed; nevertheless, the lack of randomized controlled trials has resulted in a paucity of efficacy data for these interventions. The therapeutic objective is to alleviate patient symptoms or discomfort and mitigate the underlying inflammation with the aim of stopping or delaying the progression of hair loss and scarring, thereby minimizing the formation of permanent bald patches.(100)

In general, first line treatment is potent topical corticosteroids and / or intracutaneous intralesional triamcinolone acetonide injections, whereas systemic therapies include antimicrobial, antibiotic or immunomodulating/immunosuppressive agents, both in combination with psychological support and camouflage/cosmetic techniques.(100) Even though there is promising evidence on the efficacy of different oral treatments, such as 5 alpha-reductase inhibitors (dutasteride and finasteride), topical calcineurin inhibitors (tacrolimus and pimecrolimus), hydroxychloroquine, peroxisome proliferator-activated receptor gamma agonists (pioglitazone and N-Acetyl-GED), and oral retinoid agents, more data are needed.(104)

1.5 Alopecia areata

Alopecia areata (AA) is a common autoimmune scalp disorder that results in unpredictable, non-scarring hair loss. Besides the scalp, any hair-bearing body area, or even the nails can also be affected. Oftentimes the clinical presentation and dermoscopy are sufficient for clinical diagnosis (Figure 7A, B). Contrary to LPP and FFA, the stem-cell niche is not affected in AA, and thus the eHFSCs, hair cycling and regeneration capabilities are preserved. However, the synthesis of hair shafts and pigmentation is compromised as the inflammatory attack specifically targets the bulb region, which houses the rapidly proliferating keratinocytes and pigment-producing melanocytes (Figure 6C, 7C).(105)

While a considerable number of patients may undergo spontaneous hair regrowth, the prevalence of relapses following remission, or the manifestation of a chronic course is common. This unpredictability in the course of AA poses a challenge in its management. There is still no curative treatment and often the treatment effectiveness and side effects must be weighted. Intralesional and topical corticosteroids are considered the first line treatment and may be used alone or in conjunction with each other or other treatments.(106) Other treatments include topical minoxidil, platelet-rich plasma, topical immunotherapy using diphenylcyclopropenone that causes an allergic contact dermatitis, methotrexate and janus kinase inhibitors.(106)

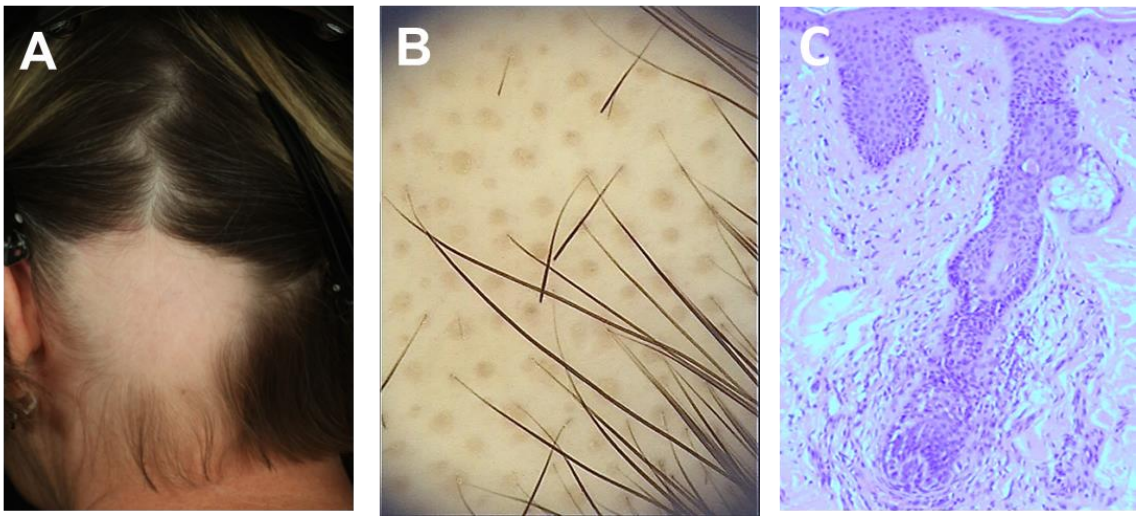

Figure 7: Alopecia areata: The clinical presentation of AA may vary, though the most common hair loss pattern is (A) circumscribed (patchy) AA. (B) Dermoscopy reveals hair loss patches with preserved empty follicular ostia and yellow dots. Exclamation mark hairs and a positive pull test

are signs of disease activity. (C) ‘Swarm of bees’ appearance of the lymphocytic infiltrate around the bulb of the HF. AA, alopecia areata. Source: Own illustration.

1.6 Project overview and objectives

1.6.1 Study plan and course

As outlined above, despite the continuous rise of FFA incidence, and the great emotional burden of patients with LPP and FFA, current treatment strategies face various challenges. In spite of the recognized role of the HF IP collapse in the pathomechanism of alopecia, we still do not know what triggers this collapse. Microbiome interventions have gained increasing attention in the treatment of inflammatory skin diseases, like atopic dermatitis and psoriasis. However, how the microbiome of human HFs affects the HF IP is yet mostly unexplored. Investigating possible triggering factors (e.g. microbiome shifts), could therefore help us identify what causes the IP collapse in the first place. Ultimately this would be of great value in shifting our therapeutic efforts towards targets that could protect or restore HF IP more effectively.

The presented work focuses on two different aspects of this complex topic:

Part one:

In the initial phase, we conducted an observational, cross-sectional, descriptive study with the primary objective of examining the increasing incidence of FFA, along with exploring the comorbidities and other relevant clinical data among FFA patients. This investigation was undertaken with the aim of identifying potential associations with disease severity, ultimately enabling us to elucidate correlations and triggering factors associated with FFA. In short, data of FFA patients treated in collaborating hair clinics in Paris and Germany were collected using standardized forms. The data included: demographic and clinical characteristics, comorbidities, laboratory test results (when available), regular use of cosmetics and FFA treatments used. We analyzed correlations between disease severity, clinical patterns, medications, and other characteristics.

Part two:

In the second phase, we postulated that dynamic alterations in the bacterial composition of the HF could serve as a trigger, eliciting an immunological response leading to the collapse of the IP and subsequent inflammation, ultimately resulting in alopecia.

To investigate a potential relationship between the inflammatory process of inflammatory hair diseases and microbes, we performed an investigator-initiated exploratory study to identify the microbiome composition of the scalp and HF, in healthy individuals and in patients suffering from inflammatory hair diseases (FFA, LPP and AA). Differences between healthy and patients, as well as between affected (lesional) and not affected (non-lesional) scalp areas in patients were compared. Our microbial investigation was complemented with clinical findings, in addition to the (semi-) quantification of the expression of inflammatory and immunological markers.

1.6.2 Study objectives

Primary outcomes:

- Describe demographic and clinical characteristics of FFA patients.
- Characterization of the scalp- and HF- microbiome in healthy individuals.
- Characterization of the scalp- and HF- microbiome in the different disease groups.

Secondary outcomes:

- Investigate possible correlations between the clinical data collected with the severity of FFA.
- Quantification of inflammatory and immunological parameters (cytokines, chemokines, antimicrobial peptides) from affected and not-affected scalp sites in the different groups.
- Investigate possible shifts in the bacterial composition between healthy and patients.
- Investigate possible shifts in the bacterial composition between affected and non-affected site in patients.

2 Methods

For publication 1: This descriptive, observational, cross-sectional study examined the characteristics, comorbidities, treatments, and cosmetic products used in FFA patients treated in the Department of Dermatology and Allergy of the Charité – Universitätsmedizin Berlin, Germany, and other collaborating clinics, in order to identify factors associated with the disease and disease severity. The data were collected in part via patient chart review, but for the most part via active patient interview. For the statistical analysis, bivariate associations were explored using a Spearman rho correlation matrix. More details are found in the original publication.(107)

For publication 2: For this investigator-initiated exploratory study, twelve healthy participants with no known skin or hair disease were included. After obtaining ethic approval from the Charité Ethics Committee (protocol code EA1/113/18) and informed consent, 12 healthy participants were included. Exclusion criteria were scalp diseases, known hormonal imbalances or viral infections, diabetes, and recent use or/and intake of antibiotics or immune treatments. The clinical data collected included subject demographics, skin physiological measurements, scalp imaging (global photography, trichoscopy and VISIA imaging) and scalp swabs for bacterial analysis. Additionally, about 50 hairs were plucked from two scalp sites (frontal and occipital). The HFs were further processed for 16s rRNA sequencing for bacterial analysis, fluorescence in situ hybridization (FISH) for bacterial visualization and ELISA for IL-17A and HβD2 quantification. For the protein extraction the HFs were cut at the lower isthmus level, and for the 16s rRNA sequencing they were further divided into infrainfundibular and bulbular part.

The histologic analysis included three scalp specimens from healthy individuals undergoing cosmetic surgery. To investigate deeper penetration of microbial material, gram staining and FISH were applied. Whereas the expression of IL-17A and HβD2 in deeper HF compartments was confirmed by immunohistochemistry in the scalp biopsies. The scalp specimens were further histologically analyzed using giemsa staining and immunohistological staining for CD3, CD4, CD8, FOXP3 and HβD1. More details about study procedures and exact names of materials used are found in the original publication.(108)

For publication 3: Following up on our exploratory investigations on healthy participants (see publication 2), hair loss patients suffering from AA, LPP and FFA were also recruited. Inclusion criteria were an established diagnosis confirmed by a dermatologist, and disease activity at the time of inclusion confirmed by a positive pull-test. Enrollment criteria, scalp photography, physiological measurements, samples collection and processing including next-generation sequencing, ELISA and the histology analysis were as described above (publication 2). The dermatology life quality index (DLQI) and disease severity scores were also collected. The sampling and clinical procedures were performed on two scalp regions, lesional (edges of active disease lesions) and non-lesional. For the histological assessment, biopsy samples collected from 19 alopecia patients for diagnostic purposes (n = 11 LPP, n = 8 FFA) were used. Lastly, the metagenomic analysis of the bacterial colonization was performed using QIIME2. More details about study procedures and exact names of materials used are found in the original publication.(109)

Publication 4 is a Review paper summarizing the recent literature reports on how HF microbiome alterations correlate with some inflammatory skin diseases, and how this might suggest a link between disease development or perpetuation and dysbiosis. In addition, the paper touches on how Metagenomics has revolutionized microbiology research and its potentials for clinical dermatology.

3 Results

Results of the present work have been already published in the following publications:

Publication 1: Varvara Kanti, **Andria Constantinou**, Pascal Reygagne, Annika Vogt, Jan Kottner, Ulrike Blume-Peytavi, Frontal fibrosing alopecia: demographic and clinical characteristics of 490 cases. J Eur Acad Dermatol Venereol. 2019.

Publication 2: 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 12.

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.

Publication 4: Andria Constantinou, Varvara Kanti, Katarzyna Polak-Witka, Ulrike Blume-Peytavi, George M. Spyrou, Annika Vogt. The Potential Relevance of the Microbiome to Hair Physiology and Regeneration: The Emerging Role of Metagenomics. Biomedicines. 2021 Feb.

Demographics and clinical characteristics of FFA

In our observational, cross-sectional study of FFA patients, data from 490 FFA patients were collected, including 23 male cases (5%). In regard to age of disease onset, the median age was 60 years, 16% of female patients were premenopausal, whereas the youngest female FFA patient was diagnosed at 15 years old. The diagnosis came on average 3 years after the first symptoms, however a delay in diagnosis of up to two decades was reported.

The hormonal status in female patients was mainly normal. Interestingly though, abnormal estrogen and testosterone values were associated with a lower disease activity. An increased prevalence of thyroid disease (38%) and arterial hypertension (18.4%) were reported in female patients, whereas the prevalence of hypercholesterolemia was high among all patients (43% in males, 47% in females). Apart from these, the correlation analysis did not support the correlation of other comorbidities. Dermatological and systemic autoimmune disorders were only reported in 7.3% of females. Similarly, despite the frequent use of leave-on products among patients (96% used facial moisturizers and 67% used sunscreen), the correlation analysis did not support their involvement in the pathogenesis of FFA.

Since no evidence-based guidelines exist, a variation of therapeutic interventions were documented. The most common were topical corticosteroids, followed by tetracyclines, intralesional corticosteroids, and less often hydroxychloroquine, finasteride, systemic corticosteroids, and methotrexate. While only in single cases topical 17a-estradiol, topical minoxidil, topical calcineurin inhibitors, systemic retinoids and platelet rich plasma injections were also reported.

Healthy scalp investigations

As a starting point for our exploratory investigator-initiated study the penetration depth and composition of bacterial microbiome on the scalp surface and along the scalp HFs of healthy individuals (n=12, 6 male and 6 female), and the expression of immunoregulatory molecules were examined. Herein, plucking hair as a minimally invasive method of collecting material for the analysis of microbiome and biomarkers is suggested (Figure 8).

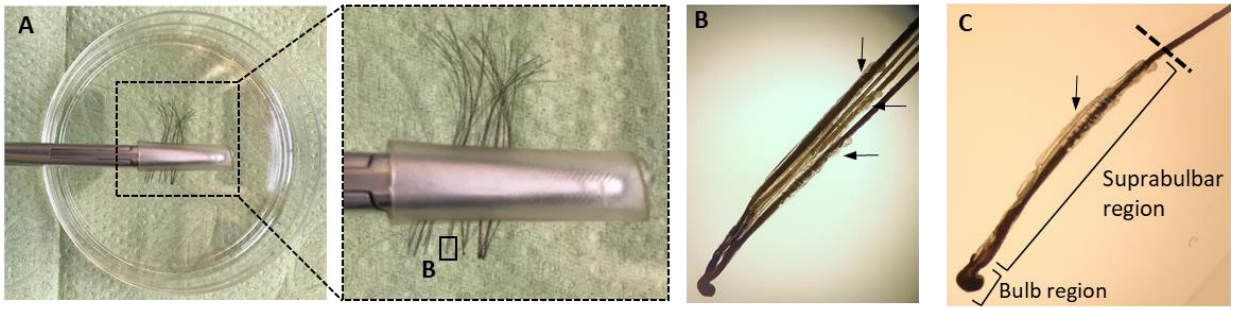

Figure 8: Plucked hair from human scalp: (A) Approximately 50 HFs were plucked from each scalp area, using standard trichogram procedures. The hairs were then transferred to a petri dish. (B) A naturally occurring bundle of hairs, part of a plucked follicular unit. The inner and outer root sheaths of the anagen HFs are fairly intact (arrow), while fragments of the dermal papilla and the HF epithelium can be left behind during plucking. (C) For further analysis, the hair roots were cut at the lower isthmus level (dashed line). For the microbiome analysis the hair preparation was performed under sterile conditions. Source: Own illustration.

The 16r RNA sequencing analysis showed that the genera Staphylococcus, Lawsonella and Cutibacterium dominated the swabs (scalp surface) of healthy participants. When moving deeper below the skin surface, the bacterial material extracted from two different depth levels of pulled HFs was examined, while Gram staining and FISH were applied to investigate deeper penetration of bacteria. The 16r RNA sequencing analysis of the HF bacterial colonization below the infundibulum level revealed that *Lawsonella clevelandensis* is by far the most abundant (>80%) bacteria, followed by *Staphylococcus* (Figure 9).

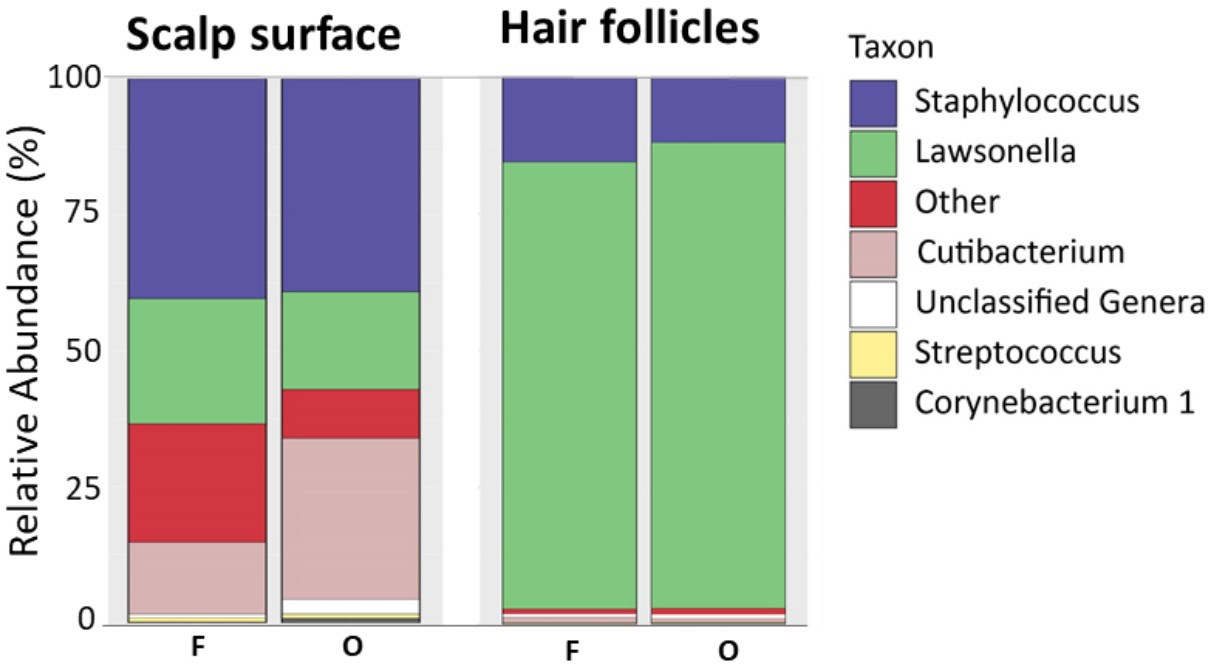

Figure 9: The bacterial composition on the scalp and hair follicles of healthy participants: Figure adjusted from Constantinou et al. Relative abundance (%) of the most abundant bacteria on the scalp surface collected from scalp swabs and plucked hair follicles of healthy subjects, at the Genus level. F, frontal; O, occipital. (n=12). Source: Own illustration.

The visualization of bacterial colonization in healthy skin was more pronounced around the HF openings and infundibulum, and gradually declined towards the bulb. Gram staining and FISH confirmed this bacterial colonization. More specifically, FISH of paraffin sections from healthy human scalp samples helped visualize DAPI (nucleic acid-specific stain 4',6-diamidino-2-phenylindole) positive microorganisms in the epidermis and the HF root sheath. Similarly in the Gram staining of healthy human scalp paraffin sections at different depths along the HF (infundibulum, isthmus, suprabulbar, proximal and bulb region) gram-positive bacteria structures were mainly found around the hair follicle openings (infundibulum) and only scattered below the infundibulum, in suprabulbar regions.

FISH and Gram staining of paraffin sections from healthy human scalp samples helped visualize DAPI (nucleic acid-specific stain 4',6-diamidino-2-phenylindole) positive microorganisms in the epidermis and the HF root sheath. Similarly in the Gram staining of healthy human scalp paraffin sections at different depths along the HF (infundibulum, isthmus, suprabulbar, proximal and bulb region) gram-positive bacteria structures were

mainly found around the hair follicle openings (infundibulum) and only scattered below the infundibulum, in suprabulbar regions.

When investigating healthy plucked HFs, FISH was able to visualize the formation of complex colonies/biofilms attached to the lining epithelium in deeper HF depths. (Figure 11).

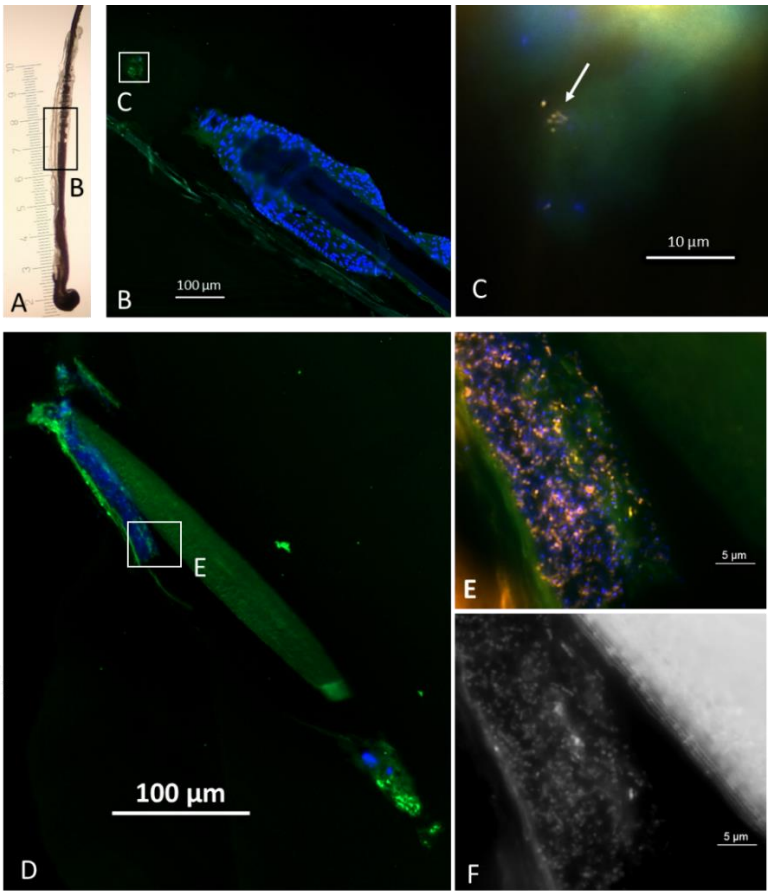

Figure 10: FISH of healthy plucked hair: (A) Plucked hair follicle, with parts of its root sheath intact. (B) FISH consecutive longitudinal sections of a plucked hair. Nucleic acid-specific stain 4',6-diamidino-2-phenylindole (DAPI, blue) visualizes cell nuclei of the outer root sheath. (C) Higher magnification of the marked area visualizes active microorganisms (based on ribosome content) by the pan-bacterial FISH probe (orange). Autofluorescence of the tissue background is shown in green. (D) Overview of a longitudinal section of a different plucked hair. The section was hybridized with the species-specific Cutibacterium acnes probe PRACCy3 (orange) and stained with DAPI (blue). Autofluorescence of the tissue background is shown in green. (E) Higher magnification of the marked area shows the overlay of the Cy3 and DAPI channels, visualizing FISH-positive Cutibacterium acnes biofilms. (F) Single fluorescence channels as a greyscale

image shows active Cutibacterium acnes bacteria in a biofilm. Source: Adapted from Polak-Witka et al. 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.

Marked presence of IL-17A and HβD2 in deeper HF compartments was confirmed by immunohistochemistry in scalp biopsies and ELISA analyses of protein extracts obtained from plucked HFs (Figure 12, 14 A).

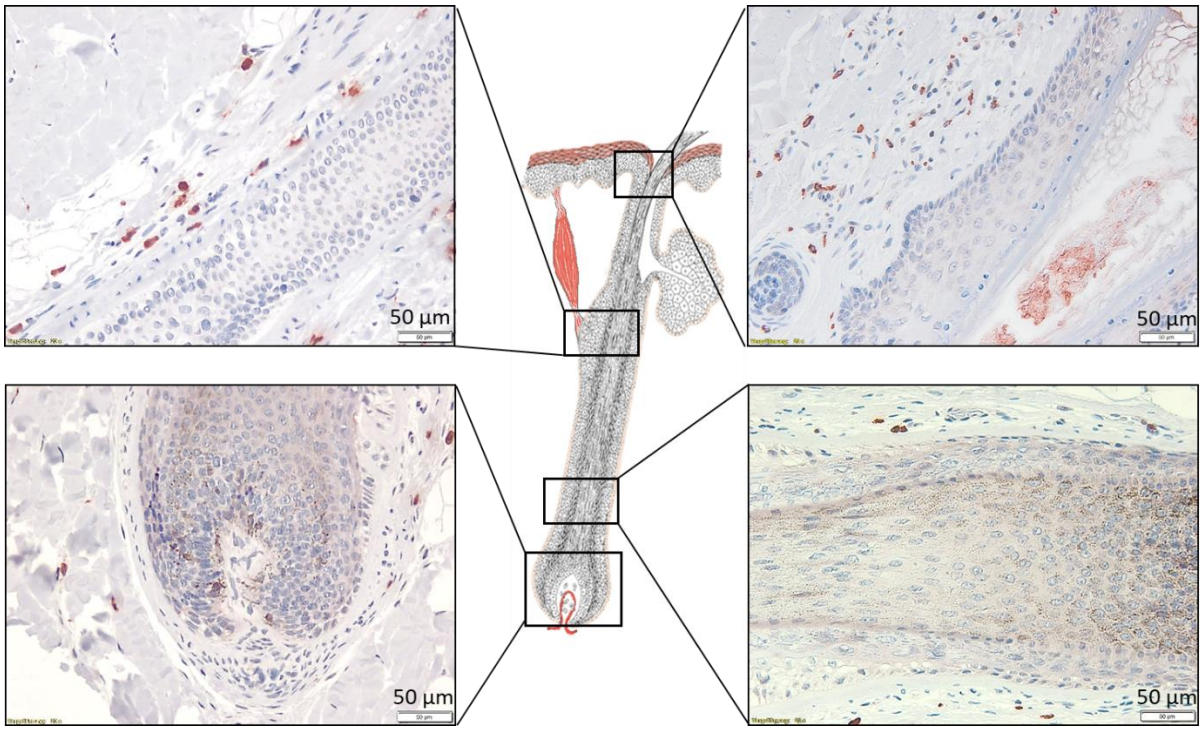

Figure 11: IL-17A expression in healthy scalp: Representative immunohistochemical staining of IL-17A in the scalp of a healthy control subject, in different levels along the terminal hair follicle (infundibulum, bulge region, proximal region and bulb). Scale bar 50 µm. Source: Own illustration.

Scalp investigations in alopecia patients

Herein, the distribution of bacteria on the scalp surface and in deeper HF compartments obtained from patients with LPP, FFA and AA and samples obtained from healthy individuals were compared. Additionally, lesional and non-lesional sites of patients were also compared. The analysis of the microbiome revealed profound differences in the bacterial compositions of patients' samples. Results are summarized in Figure 13. Briefly, Staphylococcus was the most abundant genus on the lesional scalp of all alopecia patients, but also in swabs collected from non-lesional scalp sites of LPP patients, and to a lesser degree from non-lesional AA sites. However, the most notable shift in the

bacterial composition was below the skin surface, in the bacterial analysis of plucked HFs. While Staphylococcus dominated both the lesional and non-lesional HFs of LLP and FFA, this was not the case for AA. In the HF samples of AA patients, similar to the samples of healthy individuals, there was a steep increase in the abundance of *Lawsonella*, compared to the skin surface.

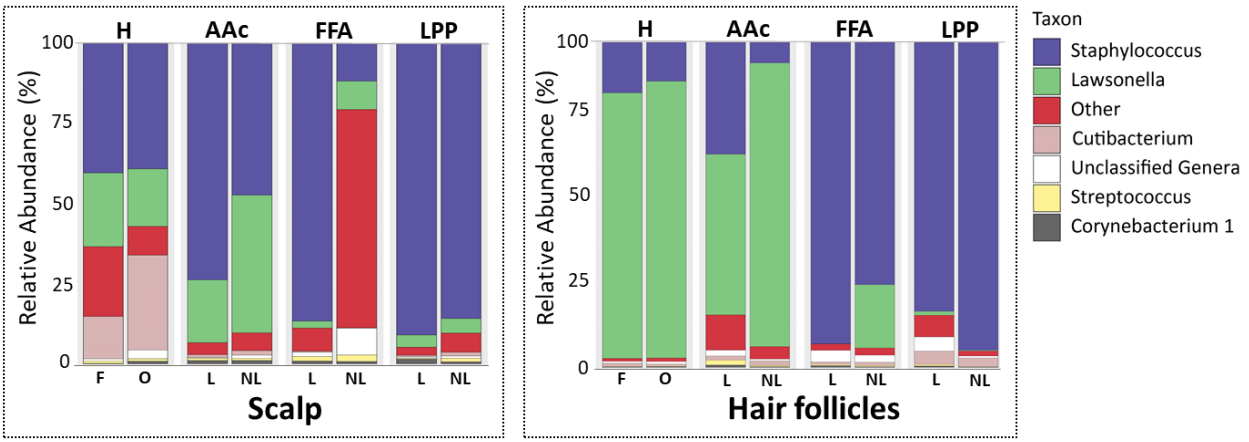

Figure 12: The bacterial composition of the scalp and hair follicles: Figure adjusted by Constantinou et al. Relative abundance (%) of the most abundant bacteria on the scalp surface collected from scalp swabs and plucked hair follicles of healthy subjects and alopecia patients, at the Genus level. F, frontal; O, occipital; L, lesional; NL, non-lesional; H, healthy; AAc, alopecia areata circumscripta; LPP, lichen planopilaris; FFA, frontal fibrosing alopecia. H (n=12), AAc (n = 6), LPP (n=6), FFA (n=6). Source: Adapted from Constantinou et al. Dysbiosis and Enhanced Beta-Defensin Production in Hair Follicles of Patients with Lichen Planopilaris and Frontal Fibrosing Alopecia. Biomedicines 2021, 9, 266.

The results obtained from this work demonstrated a pronounced expression of beta-defensins along the HFs affected by LPP and FFA, assessed by immunohistochemistry and in protein extracts from plucked HFs (Figure 14). These findings were complemented by the immunohistochemical characterization of the local inflammatory infiltrate in scalp biopsies from LPP and FFA patients, which showed the infundibulo–isthmic area as the main site of inflammation in LPP and FFA, with a shift towards $CD8^+$ T.

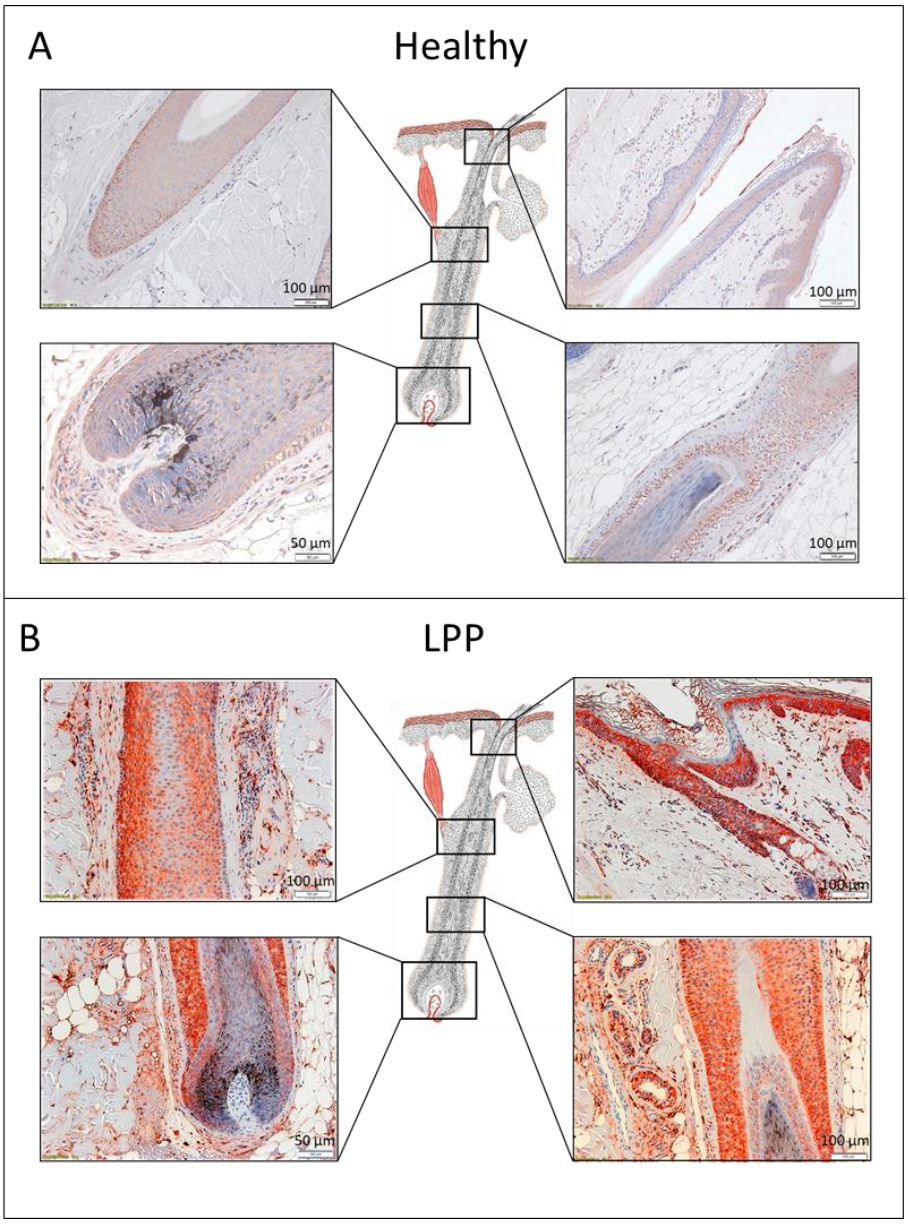

Figure 13: HβD2 expression along the hair follicle: Representative immunohistochemical staining of HβD2 in the scalp of a healthy control subject (A), and a skin biopsy obtained from a lesional site of a LPP patient (B) at different levels along the terminal hair follicle (infundibulum, bulge region, proximal region, and bulb). Scale bar 100 µm or 50 µm, as shown in each compartment. Source: Adapted from Constantinou et al. Dysbiosis and Enhanced Beta-Defensin Production in Hair Follicles of Patients with Lichen Planopilaris and Frontal Fibrosing Alopecia. Biomedicines 2021, 9, 266.

4 Discussion

4.1 Frontal fibrosing alopecia: rising incidence, clinical characteristics, and associations.

The incidence of FFA has been increasing in recent years. This type of alopecia mainly affects postmenopausal women, and despite being less common, reports of FFA in premenopausal women, and men are also reported. (107) The etiology of FFA is still unknown but various factors have been proposed to play a role (e.g. genes, allergens, cosmetic products, food) and different possible associations have been suggested (e.g. thyroid disease, rosacea, and hormonal alterations). (103) However, to date, there is no concrete evidence to verify these connections. Based on the predominance of FFA in postmenopausal women or the therapeutic use of anti-androgenic drugs, an underlying hormonal mechanism has been long postulated to play a role in the etiopathogenesis of FFA. (108) In conclusion, further studies are needed to improve the understanding of the controversially discussed role of environmental triggers in the pathophysiology of FFA.

4.2 Bacterial presence below the infundibulum

The fact that millions of microbes call human skin their home, is well established. As mentioned in the introduction the interaction and synergy between (skin) microbes and the human immune system is also recognized. Yet the HF microbiome has been mostly investigated in the context of HF-related diseases such as FD and HS. Several studies have addressed the composition of bacterial communities collected using non-invasive methods like swabbing. However, collecting samples from deeper strata of the skin, such as follicular microbiome, is more challenging. Only a few animal models are available for hair diseases and hair research largely relies on patient samples i.e. scalp biopsies. Cutaneous bacterial evidence has mainly been derived from cultures, and only recently next generation sequencing of skin swabs. These methods allow the characterization of the microorganisms, but their exact site of colonization within the local microenvironment cannot be determined.

Whereas methodologies enabling direct visualization of bacteria, such as FISH or Gram staining allow the localization of these communities. Hence, to test the robustness of our findings and support our data, a range of various methods were implemented, including

Gram staining, FISH, immunohistochemistry, and 16r RNA sequencing. Our hair plucking sample collection technique, similar to that of a trichogram, is a simple, semi-invasive, reproducible and inexpensive method that allowed us to reach below the skin surface and collect material, without the need of a skin biopsy or tape stripping (Figure 8). It also allowed the extraction of sufficient material for the next generation sequencing analysis of follicular microbiota, and protein extraction for the ELISA analyses.

In line with recent reports,(110, 111) our preliminary results suggested that the bacterial microbiome extends below the infundibulum, in proximity to structures of the IP status, essential for hair cycle and hair regeneration. Our bacterial analysis showed that *Lawsonella clevelandensis* was one of the most abundant bacteria on the scalp surface but especially along the HF of healthy participants. (Figure 9) *Lawsonella clevelandensis*, is a common commensal of the human skin, first described in 2016.(112) Even though it has low virulence, it is an emerging pathogen, that has been implicated in abdominal, breast, spinal and psoas abscesses in a limited number of cases. (113)

FISH revealed biofilm formations along healthy HFs (Figure 11).There were already reports on bacterial biofilm formations below the infundibulum on the HFs of FD patients, but also healthy controls.(76) Similarly, bacterial microcolonies/biofilms were found in the sebaceous follicles of acne lesions, but also healthy controls.(114, 115) In nature, microorganisms exist primarily by attaching to and growing upon living (e.g. human tissues such as tooth enamel, heart valves, or the lung and middle ear) and inanimate surfaces (e.g. aquatic systems or medical devices).(116) To do so, bacteria form a biofilm; bacterial biofilms are complex surface-attached communities of bacteria, held together by bacteria-produced polymeric substances.(117) It is a complex process, where bacteria irreversible attach to surfaces, and therefore biofilm formation can be detrimental in healthcare, drinking water distribution systems, food and marine industries, etc.(117) Hence the presence of biofilm formations along the HFs of healthy individuals, prompt the question as to whether these bacteria could play a role in the hair homeostasis and immune regulatory processes.

The findings of IL-17a and HβD expression, both within the infundibulum but also in the lower portion of the HF, might also support the presence of microbiota in these areas (Figure 12, 14A).(108) Resident cells of the epidermis not only form a passive mechanical barrier but also secret molecules to fight against exogenous pathogens.(118) Human β-

defensins are some of the most investigated human antibacterial peptides. In human epidermis HβD1 and HβD2 were induced by microbe-derived molecules through signaling involving either lymphocytes or monocyte-derived cells.(119) In addition to its antibacterial activity, HβD2 display proinflammatory properties, and activates several cells, such as dendritic cells, mast cells and neutrophils.(120) Early reports showed that HβD2 was highly effective in killing Gram-negative bacteria (E. coli, Pseudomonas aeruginosa), the yeast Candida albicans, and had a bacteriostatic effect on the Gram-positive *S. aureus.*(121) Numerous studies in mice indicated that IL-17 signaling is critical for protection against a variety of fungal and bacterial infections.(122, 123) Whereas in humans, the IL-17 receptor A deficiency was associated with a compromised mucocutaneous immunity to Candida and Staphylococcus.(124) IL-17A is involved in inflammation process and host defense against infection by inducing the expression of genes encoding proinflammatory cytokines, chemokines, antimicrobial peptides (including defensins), and matrix metalloproteinases.(125)

The preliminary data from healthy participants made us wonder what the impact of these findings could be on the onset or maintenance of inflammatory scalp diseases. Thus, our investigations were expanded in patients suffering from LPP, FFA and AA. A better understanding of the cytokine profile and an exploration of innate immunity in these diseases, with a possible correlation to the bacterial microbiome could finally help advance our understanding of inflammatory processes and provide the base for the exploration of novel disease-specific treatment strategies. What these diseases have in common is the IP collapse. As shown in Figure 6, lower compartments of the HF are characterized by an IP, protecting them from immune-mediated inflammation under healthy conditions. These sites include the bulge, where HF’s stem cell reservoir is located, and the bulb, where cells divide and grow to build the new hair shaft. However, HF IP is a relative, not an absolute state, implying an inherent danger of IP collapse. Indeed, both regions are sites of intense inflammatory infiltrate in some inflammatory hair diseases. The upper part of the HF around the bulge area is the target of destructive inflammatory process, which causes hair loss by replacing HFs with scar tissue, a condition that once established cannot be reversed by treatment. The question as to why some inflammatory processes involve the stem cells region (i.e. LPP and FFA) with subsequent destruction of the follicles, while others don’t (e.g. AA), is not clear yet. How

exactly these diseases begin and what causes the IP collapse is currently poorly understood, however, a possible role of microbial triggers is proposed.

Recent advances in the targeting of specific inflammatory mediators with biologicals, biosimilar or pathway-specific small molecules have revolutionized the treatment of chronic skin diseases. In contrast, topical and intralesional corticosteroids remain the primary treatment of choice for hair patients. Anti-inflammatory therapy is often not sufficient and must be combined with anti-microbial approaches. So, a better understanding of how shifts in the microbial colonization potentially contribute to the IP breakdown and the molecular details of the inflammatory process in different hair disease could open new therapeutic options.

4.3 Hair disease and dysbiosis

It is increasingly acknowledged that immunological interactions in and around the HF have profound effects on HF homeostasis and cycling. The sequence of highly immuno-active upper compartments and immune-privileged sites around the bulge and the bulb renders HFs particularly vulnerable for inflammatory insults. While minor disbalances may result in disturbed cycling and hair growth,(126) profound impairment of this finely tuned equilibrium may cause inflammatory destruction of key compartments, as observed in inflammatory hair diseases. Current research largely focuses on immunological aberrations within the tissue. However, the contribution of external stimuli to the development and course of inflammatory hair diseases is less understood.

Among different contributing factors, bacteria are of special interest as their extensive and continuous presence in follicles is well established. Findings of follicular microbiome alterations in other HF-related inflammatory cutaneous diseases like acne vulgaris, HS and FD suggest a link between their pathophysiology and dysbiosis. Even though scalp/hair disorders are not widely researched in regard to their microbiome involvement, this concept was recently extended to androgenetic alopecia and AA. In androgenetic alopecia patients, alterations in the follicular microbiota were shown, i.e. an increased abundance *of C. acnes* in the middle and lower compartments of miniaturized HFs.(127) Whereas a decrease of *Staphylococcus epidermidis*, accompanied by a significant increase of *C. acnes*, were found on the scalp of AA patients; similar to acne vulgaris reports.(6, 128)

The aim of this study was to investigate if the microbiome or the penetration depth of bacterial material into the HF could be related to HF homeostasis and inflammatory processes along the HF. We thus compared the scalp- and HF- microbiome of healthy individuals with scarring and non-scaring alopecia patients, as well as lesional and non-lesional scalp sites in patients. With the help of collaboration partners experienced in bioinformatics and metagenomics, the analysis of the microbiome revealed profound differences in the bacterial compositions of patients' samples; first evidence for dysbiosis. The results also demonstrated a pronounced expression of beta-defensins along HFs affected by LPP and FFA, assessed by immunohistochemistry and in protein extracts from plucked HFs. (Figure 13)

Lawsonella dominated in healthy and AA patients, whereas *Staphylococcus* was the most abundant in lesional and surprisingly also non-lesional HFs of LPP and FFA patients; these findings are quite remarkable. Notably, samples from LPP and FFA patients show a clear shift towards a Staphylococcus dominance, not only on a superficial level but also well below the skin surface. The depth of the dysbiosis does not reflect the more superficial perifollicular lymphocytic cell infiltrate, found mainly at the infundibulum and isthmus level, and even the upper dermis. In addition, considering that LPP can potentially affect any area of the scalp, it is worth noticing that lesional and non-lesional samples showed similar results. And even though below the skin surface non-lesional FFA samples were similar to LPP samples, (i.e. dominated by Staphylococcus) this was not the case for the scalp surface (scalp swabs). It is still early to say whether this could be explained by the fact that the clinical presentation of FFA has a more predictable localization, meaning our non-lesional samples were collected from sites that are not typically affected from the disease.

On the contrary, the superficial colonization of AA patients, especially in the lesional sites, was notably dominated by Staphylococcus. Whereas the colonization of the deeper compartments along the HF, especially the non-affected areas, was more similar to healthy. This was quite unexpected, considering that the inflammatory infiltrate observed in AA, is located in the lower peribulbar region of the HFs. Additionally, since the clinical patterns and severity of hair loss in AA vary, it is hard to evaluate if the "non-lesional sites" investigated could potentially be pre-lesional sites. Especially considering the small number of patients included and the fact that no follow up was performed after the sample collection.

The novelty of these findings is that the investigated disease entities have not been previously considered to be infectious, nor has the immune response implicated in the diseases' pathogenesis been considered bacteria-mediated. Still, it is not clear whether this reported dysbiosis is a secondary phenomenon of the inflammatory processes of the disease, or if under certain conditions (e.g. impaired skin barrier) this marked shift in the relative abundance of Staphylococcus can constitute a starting point, by triggering an imbalance in the finely tuned homeostasis of the HF. Nevertheless, it would be interesting to see whether the restoration of the microbiome could help facilitate the regulation of the cutaneous immune system.

Considering that the outer surface of the HF epithelium is lined with bacteria that extend all along the infundibulum and beyond,(4) the question arises as to how shifts of its microbial colonization and their metabolic activity may affect the underlying tissue. Hair follicle stem cell activity is subject to autonomous regulation, which allows the eHFSC-niche the ability to begin a regenerative scheme or remain quiescent by responding to its environment.(129) In adaption to varying physiological conditions and the ever-changing external environment, the stem cell niche has evolved to detect these changes and to communicate with remote cells/tissues to tailor their activity for organismal needs.(129)

For example, the inflammation observed in LPP and FFA is typically located around the infundibulum, suggesting that the destruction of the bulge region may be the result of collateral damage. Yet, recent investigations point towards a more subtle crosstalk that link HF immunology with altered cycling, even in the absence of prominent inflammation. Therefore, we hypothesize that changes in the composition of bacterial communities in the scalp might trigger an immunological activation and eventually the collapse of the IP, resulting in hair diseases. (Figure 15) Even though our results cannot verify this hypothesis, they encourage further research.

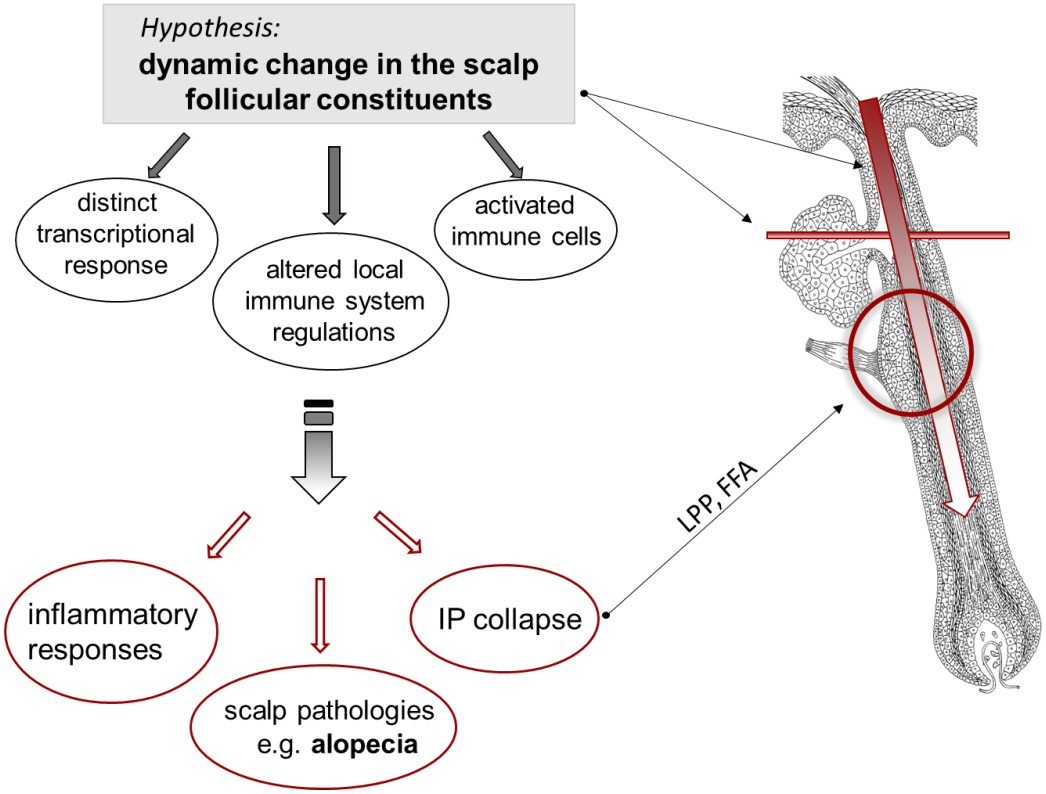

Figure 14: Our hypothesis: We hypothesize that alterations in the micro-environment of the HF might alter the local immune regulation, eventually resulting in hair disorders. Source: Own illustration.

4.4 Could environmental factors act as a triggering factor for HF immune activation?

For the last decades, the skin penetration of topically applied molecules and nanoparticles for drug delivery have been the focus of our research group and other colleagues at the Dermatology Department of the Charité.(109) The skin barrier is robust and thus particle penetration is complex. There are different penetration routes through the stratum corneum: the intercellular, intracellular and follicular pathway (Figure 1). While the intercellular penetration is often difficult, the HF has been proven to be a relevant penetration pathway for particles, but also a long-term particle- or even bacterial-reservoir. (21, 110) The healthy skin retains strong protective properties, yet microorganisms can penetrate when the skin barrier is injured or disturbed, or through the hair and sebaceous follicles.

The hair follicle is a typical stress-responding, sensory miniature organ associated with distinct immune responses. The anatomy of the HF, and its unique topography reaching

deep into the dermis while maintaining constant contact with the outside world, make HF a unique interface with the environment. As a barrier site, HFs not only provides penetration pathways for macromolecules but also facilitate their interactions with the underlying tissue. Considering the perpetual exposure of HF openings, the translocation of bacteria along the HF epithelium and their subsequent immune recognition (e.g. from Langerhans cells), may be able to disturb the finely tuned homeostasis in the area and initiate downstream immune responses that could potentially result in inflammatory infiltrate around the HF, often with devastating sequences for the mini-organ.

4.5 Limitations

Our microbiome analysis study involved a small sample size; hence the results cannot be accurately interpreted for a generalized population. And even though our project has enabled us to explore our scientific questions, no concrete conclusion can be derived from it yet. Our small sample size reduces the statistical power of the study, making it challenging to detect subtle but meaningful differences in microbial composition or diversity. Additionally, there is a recognized sampling bias since the small sample becomes more challenging to control for confounding variables or add robust subgroup analyses. In summary, while microbiome studies such as this can offer preliminary insights, their limitations in statistical power, generalizability, and ability to identify meaningful patterns underscore the importance of validating findings through larger-scale research efforts.

Considering the small number of participants in our groups, the herein presented findings cannot be conclusive on the role of dysbiosis in the development or progression of inflammatory hair diseases. Homeostasis reflects the stability of a system, however there is a great degree of complexity in its preservation. In smaller, pilot, exploratory studies, like ours, one should avoid prejudicing high bacterial abundance as a disease causative. And even though no causality between the dysbiosis and the local immune dysregulation could be defined based on our findings at this point, it has enabled us to set a strong foundation for exploring our primary questions.

To compensate for the small sample size, various approaches were implemented, to ensure that our results were consistent and robust. Nevertheless, the results presented herein are preliminary data of a pilot, investigator-initiated, exploratory study on this topic,

to provide evidence for the feasibility of our research project and help design a larger-scale study. Hence this work is a starting point for bigger scale studies as follow-up projects are needed in order to better investigate the meaning of these findings.

A further weakness of this study is the unmatched sex composition between the patients and the control group. All patient samples and n=6 out of 12 healthy controls were collected from female donors. Considering the relatively small number of patients pro disease entity included, only female patients were included to ensure the patient-population was more homogenized, in order to limit any other possible factors (e.g. sex, hormonal differences) that could potentially affect their bacterial composition. To address the unmatched sex composition a preliminary analysis of our data excluding the male subjects from our healthy control (found in the supplementary material of our original publication) had not given statistically significant differences. Thus, we decided to keep all controls, including males, in the analyses.

Furthermore, there was a big mean age difference between our control group and patients group. However, a review of the current literature did not justify the bacterial shifts observed in our patients. More specifically, previous studies comparing the cutaneous microbiome composition of younger and older women, did not reveal a shift towards Firmicutes (/Staphylococcus), similar to the findings of this study (references are addressed in more detail in the original publication). Therefore, since no study has shown a shift in bacterial colonization of older females towards Staphylococcus yet, or even towards the broader phylum of Firmicutes, we believe that our findings cannot be simply explained by the age difference between the groups. Still to really address these limitations, we plan to expand our investigations into bigger patients' groups, where age and sex stratification will be possible.

4.6 Current challenges and future potentials

4.6.1 Current challenges and perspectives

For years the focus of the pathogenetic mechanisms of LPP and FFA was mainly the immune system itself. Our novel findings represent starting points that might help shift the focus and add external factors (such as bacterial colonization) into the equation. Having said that, future studies could take various points into consideration. For example, to better define what constitutes the "normal" microbiome, and which microorganisms are

potentially “pathogenic”, bigger sample sizes are needed. Furthermore, other factors that can potentially influence the microbiome could be included, like adding before- and post-treatment groups amongst patients. Then we could see how or if the microbiome changes, even under a standard anti-inflammatory or immunomodulating therapy. Or if a distinctive microbiome profile could be differentiated in patients with better therapeutic outcomes. If so, maybe it would be possible to explore new therapeutic approaches by mimicking these “responders” profiles, using microbiome-altering interventions, in patients with poorer therapeutic outcomes.

Even though the skin and its appendages are our interface with the environment, microbiological hair research is still in the early stages. As skin microbiology enters the metagenomics era, interdisciplinary collaborations can introduce novel computational methodologies and technologies, enabling a new multi-dimensional level of investigations on the effects of microorganisms and metabolism on host tissue, and thus help focus new therapeutic approaches towards the immune system or the HF unit.(42)

The field of microbiome research has grown expeditiously in the last decades and has become a topic of great interest in science and medicine. As a result of this rapid growth, we are currently lacking a clear commonly agreed definition of different terms or consensus of methodology.(130) Current challenges, common in such projects, are environmental contaminants, bioburden, lack of a standardized mean to collect skin microbiome samples, overcoming low biomass, but also the fact that skin swabbing remains the most common method for sample collection, only allowing the collection of surface material. While the optimization of sample collection and processing workflows remains a priority, the heterogeneity of data analysis methods and approaches adds further challenges in the interpretation and comparison of these results.

Nevertheless, the true meaning of microbiome research findings in the physiology of the human body or the pathophysiology of diseases is not fully understood yet, and therefore impulsive causative assumptions should be avoided. Further evaluation is needed using bigger studies, in order to better understand the cytokine profile and innate immunity of these diseases, and explore a possible correlation to the bacterial microbiome. Additionally, most studies only sequence bacteria, whereas we know that viruses, fungi and archaea also inhabit the skin. However, this does not lessen the importance of current microbiome discoveries, such as bacterial alterations or imbalances. These constitutes

the first step in enabling the scientists to identify new therapeutic targets, and could finally help advance our understanding of inflammatory processes and provide the base for the exploration of novel disease-specific treatment strategies. Despite the advances in microbiome research and the hype of such discoveries, it is becoming increasingly clear that translational challenges remain. The time from bench to bed is long, as the clinical translation of new interventions or drugs requires robust testing, in the form of randomized placebo-controlled clinical trials in large cohorts, as only then the efficacy and safety issues can be assessed.

Additionally, the contribution of microbiome data in skin disease might also end up not being treatment-related. An evaluation of possible inflammation-contributing factors (e.g. external stimuli like bacteria) could help us identify features amongst individuals, in order to optimize non-invasive diagnostic techniques to ensure early selection of an appropriate therapy. Or identify prognostic factors able to aggravate a disease phenotype for disease screening purposes. Moreover, recent studies have shown drug-microbiome interactions, that could explain the contrasting responses of hair patients to treatment. Hair disease treatment remains a challenging task. Most of these entities are characterized by chronic, frequently progressive inflammatory processes, which often require long-term anti-inflammatory therapy. Yet, remission is not always achieved, and some patients are faced with total or sometimes permanent hair loss, with massive psychosocial stress. The ability to predict such drug/host-microbiome interactions will help physicians practice precision medicine. However, to do so, microbiology needs to be combined with more modern meta-analysis tools like metagenomics and artificial intelligence. Artificial intelligence can significantly contribute to advancing microbiome research in several ways.

4.6.2 Future potentials: the multi-omics approach

Preliminary data like ours help build bigger, more sophisticated projects. And even though the scope of this work is the microbiome of the HF, the concepts shared here can be applied to any skin microbiome study, or even microbiome research in regard to diseases in general. The future holds many exciting microbiome-related innovations with significant contributions to human and animal health, chronic diseases, antibiotic resistance, environmental sustainability, and even food production. However, to reach this level we need to study how bacteria interact with the environment, the host and with

each other. But for that we need a combined approach of methods, as each method has its advantages and limitations.

There is no one-size-fits-all method, so the better approach to investigate the microbiome will always be the multi-omics approach, as we need to utilize different approaches to address different questions. (Figure 16) Current dermatological research is mainly focused on bacterial abundance in patients. However, in this new era of metagenomics, the key is not to just focus on the microorganisms present within our samples, but rather to question what can they do and what are they actually doing.(130) Considering how complex homeostasis is and what is needed to preserve it, prejudicing the high bacterial abundance of a specie as the disease causative, might easily results in efforts for its eradication. However, if this is not indeed the case, we risk causing further perturbations in the system, complicating the imbalance even more. Until we fully understand how microbiota interact with the environment, the host and with each other, we will not be able to fully translate the compositional findings into clinical findings or interventions.

Not only has microbiome research radically changed our understanding of human microbiome functioning, but it has also opened new possibilities for the treatment of inflammatory diseases. It is believed that in the future, microbiome modulations will become promising and efficient therapy targets for various immune diseases (Figure 16). Lasting or temporal modifications of the HF microbiome, achieved by topical or systematic interventions (e.g. antibiotics, probiotics, prebiotics, anti-inflammatory or immunomodulating drugs) may cause transient microbiome shifts, stabilization or increase of the microbial diversity, and restoration of the dysbiosis, by utilizing targeted shifts toward beneficial members of the skin flora, or suppression of potential pathogens. For example, current studies are working on efforts to impede quorum sensing in harmful bacteria and promote quorum sensing in beneficial bacteria.(131) Still, as already mentioned, even after the collection of quality and robust data sets, the translation of these data to the clinical setting remains challenging.

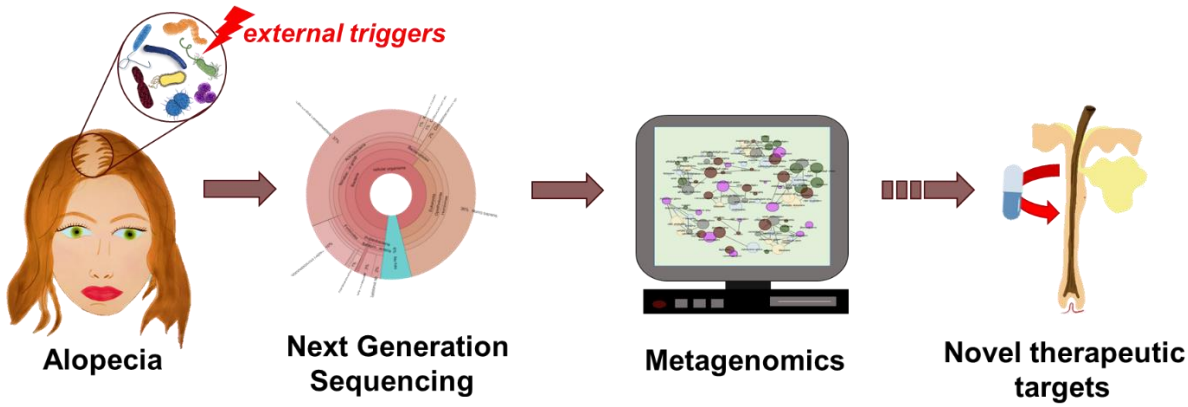

Figure 15: Microbiome alteration therapies: The important role of microbial alterations in the pathogenesis of inflammatory diseases is emerging. Interventions able to reverse these alterations, might therefore be able to restore symbiosis and subsequently reduce inflammation. Identifying the external triggers and their effect on immunological pathways of the host, could help realize the development of novel targeted treatments. Source: Own illustration.

Modern, complex microbiome research can cover many aspects of the microbiome and its interaction with its host. In that context, the focus moves from the microbial potential (learning about available microbiota in the given habitat) over to the metabolic potential (deciphering available genetic material) towards microbial functioning (e.g. the discovery of the active metabolic pathways).(130) Integration of data gives a completer and more accurate picture of the system, similar to different puzzle pieces coming together to form a picture. (Figure 17) Besides the characterization of bacterial abundances, the understanding of the effects of skin microbiome requires the integrated use of data from several omics.

Most microbiome studies employ various “meta-omics” approaches, including 16S rRNA gene sequencing, metagenomics, metatranscriptomics, metaproteomics, and metabolomics, which directly examine the phylogenetic markers, genes, transcripts, proteins, or metabolites from the samples that are associated with the development and treatment of human diseases. (130) (132) For example, computational tools have allowed the investigation of the functional activity, the functional potential of microbes within their complex communities and the host, as well as the metabolic pathways involved. Furthermore, the metabolic pathways of microbiota can be predicted in relation to pathways linked to certain conditions. Interestingly specific bacterial strains or bacteria-derived metabolites can cause host epigenetic aberrations (e.g. affect the host’s own metabolism) which can result in the initiation or progression of diseases.(133)

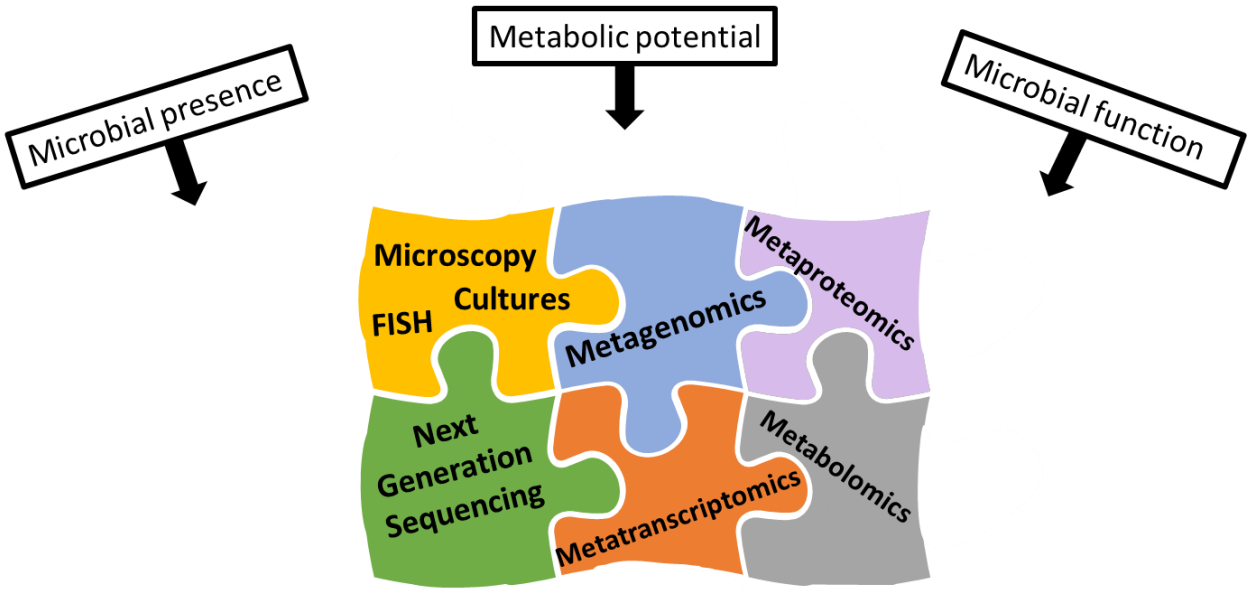

Figure 16: Multi-omics approach in microbiome research: Our prospect for skin microbiome research, especially in regard to inflammatory skin diseases, is to move beyond the analysis of skin inhabitants and instead try to paint the full picture of the cutaneous ecosystem, and the interactions between its flora and immune system using the multi-omics approach. The best method for investigating the effects of the microbiome is the combination of different omics approaches. Source: Own illustration.

Furthermore, the integration of artificial intelligence into microbiome research holds great potential for accelerating discoveries, that would have otherwise needed an enormous human effort costing time and money. It can advance microbiome research by efficiently analyzing complex datasets, identifying microbial patterns, predicting community dynamics, enhancing metagenomic analysis, and advancing our understanding of the complex interactions within microbial communities. Integrating artificial intelligence into microbiome studies enhances analytical capabilities and provides valuable insights into the relationships between microbial communities and various health conditions. This, in turn, may lead to new insights into health and disease and the development of innovative therapeutic strategies.

5 Conclusion and outlook

5.1 Conclusion

The data presented in this thesis have made important contributions in the field of inflammatory hair diseases. The characterization and the penetration depth of the bacterial colonization of the HF in healthy individuals but also in patients with inflammatory hair diseases (AA, LPP and FFA) has been studied in detailed. For decades the traditional diagnostic or experimental gold standard for inflammatory scalp diseases remains skin biopsy. Non-invasive sampling of the epidermis for the collection of stratum corneum samples includes repeated tape stripping, however there are limitations for hair-bearing areas like the scalp. Here hair plucking is demonstrated as a robust, easy and cost-effecting minimally invasive collection technique to collect samples, able to yield sufficient bacterial material for DNA sequencing but also for protein (e.g. cytokines) extraction and quantification.

This study provided evidence for the colonization of the HF with bacteria and biofilm formations, not only at the level of the HF openings (infundibulum) but also deeper, in close proximity to the sensitive bulge area, where the HF stem cells are located. Under a healthy state, this region, similar to the bulb region, enjoys a relative immune privilege status, protecting the hair unit from immune-mediated inflammation. However, both regions are sites of intense inflammatory infiltrate in some inflammatory hair diseases resulting in permanent or reversible hair loss. Therefore, when investigating the bacterial penetration of the HF, the penetration depth was crucial.

For the first time a marked dysbiosis towards a *Staphylococcus* dominance was demonstrated in patients with LPP and FFA. Remarkably, this dysbiosis was evident not only superficially, in proximity to the observed inflammatory infiltrate in these conditions, but also at a deeper level. Moreover, contrary to the FFA samples, the LPP samples were predominantly dominated by Staphylococcus in both the lesional and non-lesion scalp areas, on the surface level, as well as in plucked hairs. Considering how FFA has long been considered a LPP variant with identical histological features but distinct clinical presentations, the differences in the microbial profile of these diseases might be of relevance for the pathophysiology of the disease. Additionally, the results demonstrated a marked expression of AMP (HβD1 and HβD2) on the lesional sites of LPP and FFA

patients. The AA results were also quite interesting as they demonstrated a closer resemblance to healthy samples (with the dominance of *Staphylococcus* and *Lawsonella*), especially in the lower follicular departments, despite the inflammatory infiltrate in AA being concentrated around the bulb area.

In conclusion, the scalp bacterial microbiome in healthy individuals has been characterized, while new findings for the follicular micro-environment in patients with LPP and FFA were discovered.

5.2 Outlook

Over the last decade, particularly since the initiation of the Human Microbiome Project of the NIH, the human microbiome has emerged as one of the most active areas of research in the field of microbiology and bioinformatics. Simultaneously, there is a growing interest in the cutaneous microbiome with fundamental discoveries of dysbiosis involving inflammatory skin diseases. Despite the remarkable advances, the current state is still primitive, compared to what is needed for a robust and thorough evaluation of the role of microbiome in the pathogenesis of such diseases. One of the long-standing issues in achieving clear and conclusive results is the single-level analysis of the skin microbiome, solely based on the bacterial abundances found on lesional sites compared to healthy. Indeed, an important drawback of the majority of studies is the lack of functional analysis of the microbiome, in order to identify possible pathways involved in the pathogenesis of the diseases.

Regarding hair loss diseases, an initial step towards such findings was made with our proof-of-concept project, whereby an interdisciplinary collaboration was created. The highly interdisciplinary character of our project encompasses a broad spectrum of fields in medicine, microbiology as well as in computer science and bioinformatics. Importantly, our recent results are precursors for creating a more sophisticated analysis via the functional characterization of the bacteria found in alopecia patients. In line with our broader research scope towards a more holistic microbiome research approach, basic, initial findings such as ours provide an attractive ground to expand our investigation on the role of microbiota in inflammatory hair loss. The herein demonstrated results are of great interest for enhancing our understanding of the pathogenesis of LPP and FFA, and of potential therapeutic importance.

Massive technical complexities are required, in order to translate even the fundamental microbiome information with adequate accuracy for identifying novel therapeutic targets, as illustrated in Fig. 16. Still, the microbiome-related results obtained in this study bring a very pragmatic opportunity for the field of dermatology to transit from the current one-dimensional approach to interdisciplinary collaborations in order to achieve a complicated multi-omics approach. The current prospect for skin microbiome research, especially in regard to inflammatory skin diseases, is to move beyond the analysis of skin inhabitants and instead try to paint the full picture of the cutaneous ecosystem, and the interactions between its flora and immune system. Creating a consensus of theoretical and experimental tools to analyze the human microbiome, would help us study the role of microbiota in the propagation or preservation of diseases, diagnostic screening or even therapeutic outcomes.

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Statutory Declaration

“I, Andria Constantinou, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic Hair follicle bacterial dysbiosis: a distinct finding in patients with inflammatory hair diseases / Bakterielle Dysbiose der Haarfollikel: ein deutlicher Befund bei Patienten mit entzündlichen Haarerkrankungen, 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 own contribution to the publications

Andria Constantinou contributed the following to the below listed publications:

Original articles

Publication 1: Varvara Kanti, **Andria Constantinou**, Pascal Reygagne, Annika Vogt, Jan Kottner, Ulrike Blume-Peytavi, Frontal fibrosing alopecia: demographic and clinical characteristics of 490 cases. J Eur Acad Dermatol Venereol. 2019.
Contribution: Literature review, writing – original draft preparation and tables were created by me.

Publication 2: 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 12.
***equal contribution*
Contribution: AC's contributions were mainly focused, but not limited, to the superficial part of the hair follicle and hair openings, whereas K.P-W' s contributions were mainly focused on the lower compartments of the hair follicle. Specifically, AC's contributions: development and design of methodology (part), creation of study protocols and SOPs, subjects' recruiting (as contact person), clinical data collection and analysis (including skin physiological measurements, global photography and trichoscopy, VISIA, questionnaires, scalp swabs), hair samples collection and preparation (DNA extraction and protein extraction), ELISA (HβD2), microbiome analyses of the superficial part of the hair follicle (scalp swabs), graphs were created by me using MATLAB, figures and figure legends were created by me, writing, commentary and revision of the original draft.
KPW's contributions: Topic conceptualization, development, and design of methodology (part), ELISA (IL-17A), microbiome analyses of the deeper compartments the hair follicle (plucked hair follicles), IMHC staining of human tissues, analysis of IMHC staining and creation of the corresponding graphs, literature review, writing – original draft preparation

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.
Contribution: development and design of methodology (part), creation of study protocols and SOPs, subjects' recruiting (as contact person), clinical data curation and analysis (including skin physiological measurements, global photography and trichoscopy, VISIA, questionnaires, scalp swabs), hair samples collection and preparation (DNA extraction and protein extraction), ELISA, immunohistochemistry staining and analysis, figures 1, 2, 4 b,c, 5 b,c, 6 (including the statistical analysis) and 7 and all figure legends were

created by me, writing of the original draft.

Review articles

Publication 4: **Andria Constantinou**, Varvara Kanti, Katarzyna Polak-Witk, Ulrike Blume-Peytavi, George M. Spyrou, Annika Vogt. The Potential Relevance of the Microbiome to Hair Physiology and Regeneration: The Emerging Role of Metagenomics. Biomedicines. 2021 Feb.
Contribution: Topic conceptualization, literature review, writing – original draft preparation and figures were created by me.

_____

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

_____

Signature of doctoral candidate

Excerpts from Journal Summary List

For publication 1

Journal Data Filtered By: **Selected JCR Year: 2017** Selected Editions: SCIE,SSCI
Selected Categories: **"DERMATOLOGY"** Selected Category Scheme: WoS
Gesamtanzahl: 63 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	JAMA Dermatology	3,787	8.107	0.016170
2	JOURNAL OF THE AMERICAN ACADEMY OF DERMATOLOGY	26,450	6.898	0.035000
3	JOURNAL OF INVESTIGATIVE DERMATOLOGY	28,457	6.448	0.038800
4	BRITISH JOURNAL OF DERMATOLOGY	26,280	6.129	0.034830
5	Pigment Cell & Melanoma Research	4,430	6.115	0.007840
6	Advances in Wound Care	1,543	5.200	0.005240
7	JOURNAL OF THE EUROPEAN ACADEMY OF DERMATOLOGY AND VENEREOLOGY	9,711	4.287	0.019840
8	CONTACT DERMATITIS	5,484	4.275	0.003800
9	JOURNAL OF DERMATOLOGICAL SCIENCE	4,421	3.675	0.007260
10	DERMATOLOGIC CLINICS	1,988	3.214	0.002940
11	MELANOMA RESEARCH	2,356	3.135	0.004620
12	ACTA DERMATOVENEREOLOGICA	5,818	3.127	0.009260
13	AMERICAN JOURNAL OF CLINICAL DERMATOLOGY	2,160	3.018	0.003200
14	WOUND REPAIR AND REGENERATION	5,625	2.952	0.006310
15	MYCOSES	3,378	2.793	0.004990
16	JOURNAL OF DERMATOLOGY	4,252	2.788	0.007490

For publication 2

Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCI Selected Categories: **"DERMATOLOGY"** Selected Category Scheme: WoS
Gesamtanzahl: 66 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	JAMA Dermatology	4,578	7.995	0.021050
2	JOURNAL OF THE AMERICAN ACADEMY OF DERMATOLOGY	27,929	7.102	0.036590
3	BRITISH JOURNAL OF DERMATOLOGY	27,173	6.714	0.031730
4	JOURNAL OF INVESTIGATIVE DERMATOLOGY	30,038	6.290	0.036990
5	CONTACT DERMATITIS	5,478	5.504	0.003510
6	JOURNAL OF THE EUROPEAN ACADEMY OF DERMATOLOGY AND VENEREOLOGY	11,252	5.113	0.020730
7	Pigment Cell & Melanoma Research	4,249	4.172	0.006340
8	JOURNAL OF DERMATOLOGICAL SCIENCE	5,066	3.986	0.007150
9	JOURNAL DER DEUTSCHEN DERMATOLOGISCHEN GESELLSCHAFT	2,750	3.924	0.003550
10	AMERICAN JOURNAL OF CLINICAL DERMATOLOGY	2,574	3.840	0.004390
11	Advances in Wound Care	2,002	3.714	0.005730
12	Dermatology and Therapy	567	3.615	0.001900
				. . .
15	LASERS IN SURGERY AND MEDICINE	5,244	3.262	0.003720
16	EUROPEAN JOURNAL OF DERMATOLOGY	2,816	3.094	0.003600
17	MYCOSES	3,919	3.065	0.005380

Selected JCR Year: 2018; Selected Categories: “DERMATOLOGY”

For publications 3 and 4

Journal Data Filtered By: **Selected JCR Year: 2019** Selected Editions: SCIE,SSCI
Selected Categories: **“MEDICINE, RESEARCH and EXPERIMENTAL”**
Selected Category Scheme: WoS
Gesamtanzahl: **138 Journale**

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE MEDICINE	85,220	36.130	0.168730
2	Science Translational Medicine	34,479	16.304	0.116030
3	JOURNAL OF CLINICAL INVESTIGATION	109,020	11.864	0.125830
4	JOURNAL OF EXPERIMENTAL MEDICINE	63,562	11.743	0.067350
5	TRENDS IN MOLECULAR MEDICINE 10,618 11.099 0.018720	10,618	11.099	0.018720
6	Annual Review of Medicine	6,267	9.716	0.009390
7	MOLECULAR ASPECTS OF MEDICINE	6,207	9.577	0.005750
8	MOLECULAR THERAPY	17,977	8.986	0.030980
9	EMBO Molecular Medicine	8,366	8.821	0.022770
10	Theranostics	12,995	8.579	0.029740
11	Clinical and Translational Medicine	1,349	7.919	0.003280
12	Wiley Interdisciplinary Reviews-Nanomedicine and Nanobiotechnology	2,819	7.689	0.004240
13	Molecular Therapy-Nucleic Acids	5,024	7.032	0.013550
14	JCI Insight	7,697	6.205	0.034400
15	Cold Spring Harbor Perspectives in Medicine	7,647	6.000	0.016800
16	ALTEX-Alternatives to Animal Experimentation	1,413	5.787	0.002210
		. . .		
31	Biomarker Research	768	4.866	0.002100
30	Biomedicines	1,156	4.717	0.002850
33	mAbs	4,906	4.634	0.011110

Selected JCR Year: 2019; Selected Categories: “MEDICINE, RESEARCH and EXPERIMENTAL”

Printing copy of the publications

5.3 Publication 1

Varvara Kanti, Andria Constantinou, Pascal Reygagne, Annika Vogt, Jan Kottner, Ulrike Blume-Peytavi, Frontal fibrosing alopecia: demographic and clinical characteristics of 490 cases. J Eur Acad Dermatol Venereol. 2019 Oct;33(10):1976-1983. https://doi.org/10.1111/jdv.15735

5.4 Publication 2

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. https://doi.org/10.1684/ejd.2020.3948.

*equal contribution

5.5 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 Mar 7;9(3):266. 9, 266. https://doi.org/10.3390/biomedicines9030266

5.6 Publication 4

Andria Constantinou, Varvara Kanti, Katarzyna Polak-Witk, Ulrike Blume-Peytavi, George M. Spyrou, Annika Vogt. The Potential Relevance of the Microbiome to Hair Physiology and Regeneration: The Emerging Role of Metagenomics. Biomedicines. 2021 Feb 26;9(3):236. https://doi.org/10.3390/biomedicines9030236

Curriculum Vitae

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

Publication list

Work related to the thesis

Publication 1: Varvara Kanti, **Andria Constantinou**, Pascal Reygagne, Annika Vogt, Jan Kottner, Ulrike Blume-Peytavi, Frontal fibrosing alopecia: demographic and clinical characteristics of 490 cases. J Eur Acad Dermatol Venereol. 2019 Oct;33(10):1976-1983. doi: 10.1111/jdv.15735. IF: 4.287

Publication 2: Katarzyna Polak-Witka***, Andria Constantinou***, Rolf Schwarzer, Johannes Helmuth, Al-exandra 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. IF: 3.094 **equal contribution*

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 Mar 7;9(3):266. doi: 10.3390/biomedicines9030266. IF: 4.714

Publication 4: **Andria Constantinou**, Varvara Kanti, Katarzyna Polak-Witk, Ulrike Blume-Peytavi, George M. Spyrou, Annika Vogt. The Potential Relevance of the Microbiome to Hair Physiology and Regenera-tion: The Emerging Role of Metagenomics. Biomedicines. 2021 Feb 26;9(3):236. doi: 10.3390/biomedicines9030236. IF: 4.714

Other publications

U. Blume-Peytavi, K. Hillmann, **A. Constantinou**, A. Vogt. [Frontal fibrosing alopecia-update]. Hautarzt. 2022 May;73(5):344-352. doi: 10.1007/s00105-022-04983-w. Epub 2022 Apr 8.

D. A. Lintzeri*, **A. Constantinou*,** K. Hillmann, K. Ghoreschi, A. Vogt, U. Blume-Peytavi. CME Article. Alopecia areata – Current understanding and management. Journal der

Deutschen Dermatologischen Gesellschaft. 2022 Jan;20(1):59-90. doi: 10.1111/ddg.14689. **equal contribution*

P. Sidiropoulou, K. Tsaoutou, **A. Constantinou**, L. Marinos, D. Voudouri, T. Iliakis, G. Kanellis, E. Pouliou, A. Stratigos, V. Nikolaou. New insights into granulomatous mycosis fungoides (GMF): a single-center experience. European Journal of Cancer. 2021 Oct.

A. Vogt, **A. Constantinou**, F. Rancan, K. Ghoreschi, U. Blume-Peytavi, B. Combadiere. A niche in the spotlight: Could external factors critically disturb hair follicle homeostasis and contribute to inflammatory hair follicle diseases? Experimental Dermatology. 2020 Oct 8. doi: 10.1111/exd.14212.

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