SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of the immune status in healthy and inflamed skin

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The Examination is held online and starts at 11:00 on 10th December, 2021.

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Publicity is provided online. If you wish to participate, please indicate it in a message sent to the e-mail address jenei.adrienn@gmail.com by 2 p.m. on 9 December, 2021 latest. After the deadline it is no longer possible to connect to the PhD defense due to technical reasons.

Introduction

The skin is the first line of defense in the human body, it protects against physical, chemical and microbiological harm coming from the environment. Based on anatomical and physicochemical properties, there are 3 topographically different skin regions: ‘sebaceous gland rich’, fatty (SGR), ‘apocrine gland rich’, moist (AGR) and ‘gland poor’, dry (GP) skin regions.

Previous research has shown that topographical different regions of healthy skin have a different microbiome composition. According to our data, the immunological features of SGR skin regions are also different compared to GP skin regions. The immune milieu of AGR skin areas have not been investigated yet.

Hidradenitis suppurativa is an inflammatory skin disease localized to AGR skin areas. In previous studies, the immunological status of HS was compared to the immunological status of GP skin region, which led to inaccurate results and hindered our understanding of the development of inflammation. Furthermore, changes in the immune milieu of AGR skin during HS development has not been examined yet.

Establishing the antimicrobial peptide (AMP) profile of the skin has both diagnostic and predictive value. AMPs as markers can inform us about the development, status and severity of skin diseases. Our research group could identify significant differences in gene and protein expression of certain AMPs when comparing GP, AGR and SGR skin regions.

The number of suitable methods available for a quick, minimally invasive and repeatable sampling are limited. Current approaches for the identification of AMPs typically rely on antibody-based epitope recognition by ELISA and immunohistochemical (IHC) assays using skin biopsies. However, the process of sampling is associated with pain and scarring and cannot be repeated in the same skin area. Only minimally invasive sampling coupled with a quick, reliable analytic method may be suitable to monitor the change of skin immune status in healthy subjects or cases of subclinical and manifest inflammation. A sensitive and accurate detection method for AMPs will enable prophylactic interventions and may help to monitor the efficacy of therapies.

In my PhD work, I studied the immunological features of healthy AGR skin and HS lesions and developed a new, minimally invasive method to detect the diagnostic and predictive markers for the immune status of stratum corneum.

Objectives

Aims of this doctoral dissertation:

I/1. To explore the differences between immune and barrier features of healthy apocrine and sebaceous glands poor (GP) and apocrine glands rich (AGR) skin regions.

I/2. To investigate the changes in the features of apocrine glands-rich skin regions during the development of hidradenitis suppurativa (HS).

II/1. To optimize a minimal invasive SC sampling method based on a tape stripping technique.

II/2. To optimize a high specificity PRM-based mass spectrometry method to SC sample.

II/3. To develop a new, hexane - free protein elution method and compare to the hexan-based methods.

II/4. To determine the composition of AMP content in stratum corneum of the different skin regions.

Materials and methods

All volunteers and patients involved in this study were recruited from the outpatient clinic of the Departments of Dermatology and Surgery, Faculty of Medicine, University of Debrecen. All participations signed the informed consent form. Sample collection was performed according to the guidelines of Helsinki Declaration and this study was approved by the local ethics committee of the University of Debrecen, Hungary (ethical approvals nr.: 4492-2015 – DE RKEB/IKEB, 5064 – 2018 - DE RKEB/IKEB, 17/09/2018). We used samples from healthy volunteers and patients. Healthy volunteers did not have a history or any manifestations of inflammatory skin diseases. Patients suffering from inflammatory skin diseases had moderate to severe skin symptoms. No medical treatment occurred during the investigation in the patients enrolled.

1. Sample collection

1.1. Skin biopsies collection

Skin biopsies were collected from normal skin of healthy individuals (8 samples from the axillary region representing AGR, 8 from shin or arm regions representing AGP and from axillary lesions from 8 patients with HS. Samples were classified according to the number of apocrine glands and were defined as AGR (apocrine gland-rich) skin when containing $n \geq 2$ apocrine glands in the field of view on 100x magnification in the microscope.

HS patients were enrolled according to the following criteria: individuals (over 18 years of age) with clinically diagnosed moderate-to-severe HS with at least 6 months disease duration. All patients were biological therapy naive before skin biopsy and any previous conventional systemic therapies were washed out 4 weeks and topical treatments 4 days prior to skin biopsy.

One part of the biopsies was stored in RNAlater at -70°C until RNA isolation for RT-PCR, another part of the biopsies was formalin-fixed and paraffin-embedded and used for IHC.

1.2. Stratum corneum samples collection

A total of 60 samples from 23 adult volunteers (19 healthy controls and 4 patients, Table S1) were collected and divided into optimization and test groups. The optimization group contained three subgroups used for (1) optimization of sample collection (samples from 2 healthy volunteers: 1 female, age: 41 years; 1 male, age: 34 years), (2) design and optimization of PRM-based mass spectrometry method (samples from 1 female patient with severe HS, age: 44 years; 1 male patient with moderate Ps, age: 30 years; 1 female patient with severe PPR, age: 40 years; 1 male patient with moderate AD, age: 44 years; 1 healthy male donor, age: 34 years) and (3) optimization of protein elution (samples from forehead region of 1 healthy male donor, age: 34 years), according to the study workflow.

The test group included samples from 15 healthy volunteers: 10 female, median age: 28 ± 9.7 years; and 5 male donors, median age: 35 ± 10.8 years.

2. RNA Isolation, Reverse Transcription and Real-Time Quantitative PCR

All samples were homogenized in TriReagent solution with Tissue Lyser using previously autoclaved metal beads. Total RNA was isolated from the human skin. The concentrations and purities of the RNA samples were measured by means of a NanoDrop spectrophotometer), and its quality was checked using an Agilent 2100 Bioanalyser (Agilent RNA were reverse transcribed into complementary DNA (cDNA) using the High Capacity cDNA Archive Kit according to the manufacturer's instructions and the indicated thermal protocol. Previously, samples were treated with DNase I. qRT-PCR measurements were carried out in triplicate using pre-designed FAM-MGB assays as well as TaqMan® Gene Expression Master Mix ordered from Applied Biosystems. All reactions were performed with a LightCycler® 480 System. Relative mRNA levels were calculated using either the comparative Ct method or based on a standard curve, and normalized to the expression of PPIA mRNA.

3. Routine and immunohistochemistry staining of skin segments

In the first step we classified skin segments according to the number of apocrine glands. Determination of tissue details was carried out with May-Grünwald-Giemsa staining. The pathological severity of skin samples from HS lesion was determined with hematoxylin-eosin

(H&E) staining. Pathological examination of skin segments was carried out by pathologist of Institution of Dermatology, at the University of Debrecen. After classification of the skin samples, amount of target proteins was determined by IHC staining.

3.1. Immunohistochemistry Staining

For IHC analyses freshly prepared paraffin-embedded sections from HS patients and healthy control skins were used. After deparaffinizing and rehydrating the samples, endogenous peroxidase activity was eliminated with 3% H_2O_2 for 15 minutes. Subsequently, heat-induced antigen retrieval was performed. After blocking in 1% bovine serum albumin (BSA) solution, sections were incubated with primary antibodies overnight at 4°C. Subsequently, anti-mouse/rabbit HRP-conjugated secondary antibody was employed. Before and after incubating with antibodies, samples were washed with TBST for 5 minutes, 3 times in each step. Signals were detected with the Vector® VIP and ImmPACT™ NovaRED™ Kit. Background staining was performed with methylene green.

The detection of each protein was carried out on all sections in parallel at the same time to enable us to evaluate comparable protein levels. Positive, Ig, and isotype controls were also used to normalize staining against all proteins [mouse and rabbit IgG, rabbit immunoglobulin fraction].

4. Stratum corneum sample collection

SC samples were collected from the skin surface with tape stripping technique using D-Squame discs. In the course of each examination, D-Squame discs were always used by the same person wearing powder-free latex gloves. All volunteers were asked not to wash the area of sampling with soap, not to use any cosmetics 24 hours prior to sample collection. No disinfectant was used to clean the skin surface before sampling. D-Squame discs were pressed to the skin surface for 10 seconds with medium force before being peeled off. All D-Squame discs were stored in a wet chamber with samples facing upwards at room temperature until processing. Sample processing was carried out on the sampling day.

5. Protein elution of stratum corneum from D-squame discs

Proteins from the collected tape stripping discs have been eluted with hexane as previously described. Briefly, the D-Squame discs removed from the skin surface were glued to silane-coated slides and soaked in hexane at room temperature, overnight. The proteins were denatured on the discs with 8 M urea for 30 minutes at room temperature in a wet chamber by evenly spreading urea solution across the disc surface. The solution of denatured proteins was collected to Eppendorf tubes for digestion.

To improve the recovery of skin-derived AMPs from the SC, a hexane-free elution method was applied. The hexane-based elution method was modified as follows: after removal from the skin the D-Squame discs were placed in a wet chamber with samples facing upwards and 8 M urea solution was added immediately and the denatured proteins were collected to Eppendorf tubes.

6. Measurement of protein concentration from stratum corneum

Discs containing SC samples were placed in 10 ml of 0.1% SDS and were sonicated for 2 minutes. Then, samples were centrifuged for 10 minutes at 4 °C, and 5500 rpm. Next, samples were dried and re-dissolved in 2 ml ultra-pure water.

Protein concentration was determined using the Pierce BCA Protein Assay Kit according to the manufacturer's instruction in 96 well plastic plates. 0.1% SDS was used as negative control. The protein measurement was performed at 595 nm wavelength on a Labsystems Multiskan MS spectrophotometer.

7. Protein digestion of stratum corneum

The proteins eluted from the discs were digested with trypsin. Reduction was carried out using 10 mM dithiothreitol for one hour at 37 °C, followed by alkylation with 20 mM iodoacetamide for 45 minutes at room temperature in the dark. Before digestion, urea was diluted to 1 M with 25 mM ammonium bicarbonate. Finally, trypsin digestion was carried out overnight at 37 °C using stabilized MS grade TPCK-treated bovine trypsin applying a 1:25 trypsin: protein ratio. The digested peptides were dried in speed-vac and then, re-dissolved in 100 µl 1% formic acid and desalted with C18 Pierce Tip.

The eluates were dried and kept at -20 °C until mass spectrometry analysis. Prior to the injection to the mass spectrometer the peptides were re-dissolved in 10 µl of 1% formic acid.

8. PRM assay design

The 18 amino acid sequences of the thirteen selected AMPs characteristic for the skin surface were retrieved from UniProt database (www.uniprot.org). Based on literature and our previous data, the selected proteins were appropriate for characterization of healthy and inflamed skin.

In silico trypsin digestion of the protein sequences were carried out using the ExPaSy PeptideCutter (https://web.expasy.org/peptide_cutter/) tool accessible from the UniProt. In order to collect unique, protein-specific sequences, the peptides with 100% cleavage probability and 5-14 amino acid length were subjected to BLASTp search (https://blast.ncbi.nlm.nih.gov) using default settings, the NCBI non-redundant database and "Homo sapiens" as the query species. The protein-specific unique peptide sequences were used for PRM analyses and their stable isotope labeled (SIL) counterparts were ordered from JPT Peptide Technologies GmbH, Berlin, Germany.

9. Mass spectrometry experiment

Digested samples were randomized and analyzed in duplicates under identical conditions. Before the mass spectrometry analyses the same amount of SIL synthetic peptide mixture was added to each sample.

PRM analyses were carried out on an Easy nLC1200 nanoUPLC coupled to Orbitrap Fusion mass spectrometer.

Peptides were separated using a 60-minute water-acetonitrile gradient at a flow rate of 300 nl/min on an Acclaim PepMap RSLC (150 mm x 50 µm, 2 µm particle size, 100 Å pore diameter, Thermo Scientific, Waltham, Massachusetts) column after desalting on Aclaim PepMap C18 (20 mm x 75 µm, 3 µm particle size, 100 Å pore diameter, Thermo Scientific, Waltham, Massachusetts) column. The buffer A was LC grade water with 0.1% formic acid and buffer B was LC grade acetonitrile with 0.1% formic acid. During the chromatography, buffer B ratio was increased from 5% to 20% over 5 minutes followed by an increase to 45% over 40 minutes. Next, the buffer B ratio was increased to 85% over a 3 minutes period and

kept at 85% for 5 minutes. Then, buffer B ratio was decreased to 5% in 1 minute, and held constant for 6 minutes.

PRM analyses were performed with targeted MS2 approach. The peptide masses were set as precursor ions. After precursor ion selection in the quadrupole analyzer the peptides were fragmented with 30% normalized CID energy, then, fragments were analyzed in the 114-1000 m/z range in the Orbitrap analyzer (50000 resolutions, AGC target: 1.0e4, centroid mode) in positive polarity mode.

10. Data evaluation and statistical analysis

IHC and RT-qPCR examination of biopsies were carried out at least in double repetition and biological triplicate. To determine the statistical significance between the groups, One-way analysis of variance (ANOVA) test and Newman-Keuls post hoc test were used (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Graphs demonstrate the mean and the corresponding 95% upper and lower confidence intervals (boxes) as well as maximum and minimum values of protein levels measured by Panoramic Viewer. Statistical analyses were performed using GraphPad Prism software version 6.

For SC protein quantification, the Skyline software was used. Shapiro-Wilk test was used for normality testing. As far as the data did not follow normal distribution the three groups were compared by Kruskal-Wallis test followed by Dunn's post hoc analysis. Statistical analyses were carried out by using GraphPad Prism 8.0.1. The data were presented as mean ± SEM. The results were considered to be statistically significant when the p-value was less than 0.05 (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Results

1. Immune milieu of healthy AGR skin and HS

In previous studies we showed that there are various features in the immune and permeability barrier of the sebaceous glands rich skin (SGR) compared to glands poor skin (GP) regions. Therefore, we examined the immunological characteristics of the apocrine glands rich, moist skin.

1.1 Examining immune cells in AGR skin and HS

First, we compared AGR skin to the GP skin region. IHC revealed significantly elevated numbers of $CD11c^+$ DCs and $CD4^+$ T cells in AGR region compared to GP without prominent DC activation, while no significant differences in the number of LCs ($CD1a^+$) and macrophages ($CD163^+$) could be detected between the two healthy skin regions. The gene expression and protein levels of the activation and maturation markers (CD80, CD83, CD86) of DCs was investigated without any prominent differences between two healthy skin regions. Quantitative real-time PCR analyses showed no significant differences between the two regions in the expression of T-cell-related factors.

Next healthy AGR and HS biopsies were examined. In HS, the numbers of DCs and T cells further and significantly increased, DCs became activated, and $CD163^+$ macrophages appeared in a significantly enhanced number compared to AGR (IHC). Activated status of DCs was confirmed by RT-qPCR measurements.

1.2 Th subtypes related factors

Expression of different T cell subtypes related cytokines and transcription factors was examined. Although quantitative real-time PCR analyses showed no significant differences between the two regions in the expression of T-helper subtype related factors, by IHC both $IL\text{-}17^+$ and $IL\text{-}10^+$ cells were detected in significantly elevated numbers in AGR and were nearly

absent from GP. Moreover, a few IFN-g^+ cells were found in AGR only, and were totally absent from AGP.

In HS significantly higher expression of T-helper 17-related (IL-1B, IL-17A, IL-23A, transforming growth factor-β1) and even T-helper 1-related molecules (IL-12B, TBX21, tumor necrosis factor-α) were detected at the mRNA level. Furthermore, by IHC, the number of IL-17^+ and IL-10^+ cells further and significantly increased, and a robust presence of tumor necrosis factor-α and IFN-γ^+ cells became detectable.

In case of Th2-, Th22- and T_{reg}-related molecules we did not detect any differences between the two healthy skin regions.

1.3 Th17-related factors

Next, we investigated important chemokines, AMPs, barrier and proinflammatory molecules of the skin immune system. Although, when comparing AGR to GP skin, no significant difference could be detected at the mRNA level, except for the higher expression of KRT79 in AGR, protein levels of IL-17-related chemokines (CCL2, CCL20), AMPs (TSLP, LCN2), and one of the barrier molecules (FLG) were significantly altered in AGR. When comparing HS and AGR samples, quantitative real-time PCR revealed significantly increased expression of chemokines (CCL3, CCL19, CCL20, CCL23) and AMPs (DEFB4B, S100A7, S100A8, S100A9, LCN2), as well as altered mRNA level of KRT17 barrier molecule in HS. The significantly and highly elevated level of T-helper 17erelated chemokines (CCL2, CCL20) and AMPs (S100A8, LCN2) were also confirmed by IHC, together with the altered expression of barrier molecules (KRT17, LOR), which also reflected an IL-17 effect. Expression of pro-inflammatory molecules (Toll-like receptor 2, Toll-like receptor 4, NLRP3, IL-1b, tumor necrosis factor-α) was low in both GP and AGR, while in HS, a robust and significant increase was observed in their mRNA and protein levels, with the exception of NLRP3.

2. Stratum corneum of healthy skin regions examination with mass spectrometry method

2.1. Optimizing the method for the examination of AMPs

The aim of our second research project is the identification of AMPs in the SC of the different skin regions and the comparison of their expression levels. In healthy and inflamed skin we optimized our sampling method of protein elution and PRM-based mass spectrometry, in order to determine AMP content in SC. Proteins were collected by specific D-Squame discs from healthy, untreated and uncleaned skin surface. SC samples were collected from the forehead and cheek of healthy volunteers.

2.1.1. Optimization of sample collection

The total protein amount that can be obtained from SC by D-Squame discs is limited. In order to extract the highest protein amount, we tested several sampling methods. Samples were collected from SC of healthy study subjects from the forehead and cheek. 5 or 10 individual discs were pressed onto the surface of the forehead and cheek, respectively, to the same area. Proteins removed with 5 or 10 discs, respectively, were pooled and the total protein concentration was measured in each collected sample.

The SC total protein concentration was rather characteristic to the volunteer and not to the number of collected discs or place of collection.

Taking into account that stripping the same area three or more times caused significant discomfort to volunteers, for all subsequent samplings only one disk was used for each area.

2.1.2. Design and optimization of PRM-based mass spectrometry method

Our previous studies at the mRNA and protein levels demonstrated significant differences in the expression levels of the S100A7, S100A8, S100A9, hBD2, LCN2 and TSLP among the different healthy skin regions. In addition to those, in our current study we investigated further AMPs (hBD1, 3, 4, LL-37, LACRT, LYZ and DCD), which are known to be present in the epidermis under both steady-state and diseased conditions.

The protein sequences of selected AMPs were subjected to bioinformatics analyses and the unique protein-specific tryptic peptide sequences were identified. The selected unique peptides were used for PRM analyses.

The SIL synthetic counterparts of the selected peptides were ordered and used for quantification. The PRM data were analyzed with the Skyline software and all the recorded data were uploaded to the Panorama (https://tinyurl.com/mbbuwrw). A peptide was considered to be present in the sample when the peaks characteristic for the endogenous peptides showed coelution with the peaks characteristic for their SIL synthetic counterparts. Peaks showing no coelution with their SIL counterparts were excluded from the analyses. The integration of the spectra was performed by the software and the ratios of the endogenous and SIL peptides were determined. The calculated ratios were used for the comparative analysis of the selected AMPs in the studied groups.

In order to detect both constitutive and inducible AMPs, SC samples were collected from 4 patients suffering from various inflammatory skin diseases: HS, Ps, PPR, AD, as well as from a healthy volunteer. HS samples were collected from armpit, Ps from the limbs, PPR from the cheek and AD from thigh, while healthy control samples were originated from the forehead region. SC samples were pooled to have a cocktail of AMPs for testing.

According to the applied criteria, ALNSIIDVYHK and GADVWFK peptides from S100A8, VPLQQNFQDNQFQG peptide from LCN2, ENAGEDPGLAR peptide from DCD, SILLTEQALAK peptide from LACRT and WESGYNTR peptide from LYZ could be observed both in healthy and inflamed skin sample, while DLYNFLK peptide from S100A9, LLGDFFR peptide from LL-37 and TFVPGCQPGEFTLGNIK peptide from LCN2 were only detected in the inflamed skin sample. The SIIGMIDMFHK peptide from S100A7, IQGTCYR peptide from hBD1, GIGDPVTCLK peptide from hBD2, GIINTLQK peptide from hBD3, ICGYGTAR peptide from hBD4, SYNVTSVLFR peptide from LCN2, CLEQVSQLQGLWR peptide from TSLP, QELNPLK peptide from LACRT and GISLANWMCLAK peptide from LYZ could not be detected in any of the samples.

2.1.3. Optimization of protein elution

From the 18 examined peptides 9 (DLYNFLK peptide from S100A9 protein, LLGDFFR peptide from LL-37 protein, TFVPGCQPGEFTLGNIK peptide from LCN2 protein, ALNSIIDVYHK and GADVWFK peptides from S100A8 protein, VPLQQNFQDNQFQGK

peptide from LCN2 protein, ENAGEDPGLAR peptide from DCD protein, SILLTEQALAK peptide from LACRT protein, WESGYNTR peptide form LYZ protein) could be detected in either healthy or inflamed skin samples. Another 9 peptides could not be detected in the samples examined. In order to elucidate whether the failure to detect 9 peptides was due to the low protein yield obtained with the hexane-based elution the effect of elution on protein recovery was tested. Ten SC samples from the forehead of a healthy volunteer (male, age 30) were collected and discs were divided equally into 2 groups: one group was used for hexane-free and another one for hexane-based elution method.

The proteins eluted from the discs either with the hexane-based or hexane-free method were digested and examined with mass spectrometry. For comparison five peptides from four proteins previously detected in both sample types were used. The light-to-heavy ratio often used for relative quantification was calculated in case of both elution types. The ratio refers to the peptide amount in the sample normalized to the amount of synthetic SIL peptide; higher ratios indicate higher peptide amounts in the sample.

According to our observations the extracted peptide amounts were markedly higher in hexane-free elution compared to the hexane-based elution in SC samples. Therefore, the hexane-free method was applied for the examination of the AMPs in all further experiments.

2.2. Comparative analysis of AMP levels of different healthy skin regions

Following the optimization experiments, the comparative analyses of skin-derived AMPs in GP, SGR and AGR healthy skin regions were performed. SC samples were collected from GP, SGR and AGR skin regions of 15 healthy volunteers (10 women, 5 men). SC samples were collected from forearm, forehead and armpit, representing GP, SGR, and AGR skin regions, respectively. The collected samples were examined by the optimized sample elution, digestion and targeted proteomics analysis workflow. The light to heavy ratios for peptides of the 18 skin-derived AMPs were determined in the samples collected from GP, SGR and AGR skin regions.

Six peptides from five proteins were suitable for quantification. ALNSIIDVYHK peptide of S100A8 and VPLQQNFQDNQFQGK peptide of LCN2 could not be detected, but GADVWFK peptide of S100A8 was detectable in low amounts in GP skin, while in AGR skin, these peptides were present in higher amounts. These peptides could be detected in significantly elevated levels in SGR skin area compared to AGR and GP skin regions. The SILLTEQALAK peptide of LACRT and the WESGYNTR peptide of the LYZ could not be detected in AGR

skin, however SILLTEQALAK peptide of LACRT was present in GP skin in low amount, and the WESGYNTR peptide of the LYZ was undetectable. These two peptides were detected in relatively high amounts in SGR skin region and the difference between SGR and GP and SGR and AGR, respectively, was statistically significant. ENAGEDPGLAR peptide from DCD could be quantified in samples originating from all skin regions; however, the amounts did not show any significant topographical differences among the skin regions.

Discussion

1. Differences in healthy and inflamed skin

In our present study, we could unambiguously show that moist/AGR skin possesses distinct immune and barrier milieu compared to dry/GP areas and between AGR skin region and HS inflamed skin localized on AGR skin. In AGR skin, this is a non-inflammatory IL-17/IL-10^{+} containing environment with significantly higher T cell and DC attendance, without any signs of activation or inflammation, accompanied by IL-17-related chemokines, AMPs, and barrier characteristics.

Previous data have shown a subclinical inflammatory state preceding the manifestation of HS, which is started with an aberrant keratinocyte response to commensal follicular bacteria. Healthy AGR skin disposes region-specific microbiota influenced by the chemical milieu, moisture content, and temperature of this area, but in accordance with our present results, this specific commensal population can live in a homeostatic state with the region-specific immune and barrier milieu of AGR skin.

We hypothesize that, during the development of HS, this "AGR-specific" homeostatic symbiosis between microbiota and skin immune system can be modified (or not fully developed), and this alteration of the non-inflammatory IL-17/IL-10 milieu leads through a gradual subclinical progression to a severe IL-17/IFN-γ type apparent inflammation in HS.

The mentioned pathomechanism of HS highly resembles that of Crohn's disease, as proposed by current literature, which can be an additional link between the two diseases.

The data of our study also suggest that the non-inflammatory IL-17 milieu of AGR skin may prone this area to develop specifically an IL-17 type inflammatory disease, as all the characteristic adaptive and innate immune or barrier features of AGR skin were also present in HS in robust and widespread forms, accompanied by activation and inflammation markers, ultimately resulting in a full-blown inflammatory IL-17 environment. This inflammatory IL-17 milieu was reflected by significant chemokine, DCs, AMP, and barrier alterations. High IFN-γ and TNF-α presence in HS was also confirmed by this study, although previous literature data were inconsistent regarding their levels.

In conclusion, our previous and present data suggest that not only the microbiota and chemical content of human skin show three main topographical areas (dry, moist, oily/ sebaceous), but probably in correlation to this, the immune and barrier characteristics of these

topographical regions are also distinct, which can make these skin regions become prone to the development of “region specific” inflammatory skin diseases, like HS on AGR and acne and rosacea on sebaceous gland rich areas.

2. Differences of AMP composition of healthy skin SC in GP, AGR and SGR skin regions

Although AMPs participate in the maintenance of skin homeostasis in several ways, AMPs stand out as the most relevant players in suppressing microbial invasion in the skin. Presence or absence, the elevated or suppressed levels of AMPs may be considered as markers reflecting the immune status of the skin.

As the cohesive forces gradually increase towards the deeper layers in SC, the amount of proteins that can be extracted is exponentially reduced. The greatest amount of protein can be removed from the outermost layers of SC with D-Squame discs. The outermost surface of the skin represents the first line of defence upon contact with microbiota, making the surface and the top layer of the skin the most valuable area for retrieving diagnostic material.

In addition, the change in the composition of AMPs can have a predictive value and under pathological conditions they may indicate the severity of the inflammation.

Both in the examination of the topographically different healthy skin areas and the pathomechanism of inflammatory skin diseases, the analysis of SC have relevance. The most commonly used methods for the molecular analysis of the skin rely on invasive sampling, such as biopsies, and are associated with scarring. There is an increasing demand for a novel, less invasive sampling methods without pain and scarring. In clinical practice, this method can complement previous protocols and can help form accurate diagnoses and select appropriate therapy.

In previous articles, the tape stripping sampling method, which we also used, was applied for proteomic analysis of SC, but in this case the protein content was collected from the skin surface with more discs. Then various AMPs (hBD, LL-37, RNase 7, S100A7, DCD) were detected by antibody-based (ELISA, IHC) molecular biology methods.

We developed a novel, hexane free elution method against the hexane-based elution method. D-Squame discs were used to sampling. Because the amount of protein that can be removed with D-Squame disc is limited, we aimed to increase the amount of extractable protein. Comparing the hexane-based elution method to hexane-free method, we extracted significantly higher protein amount from surface of discs with the hexane-free method.

Previously, AMPs that were removed with tape stripping technique were investigated by ELISA, RT-qPCR and IHC methods. We selected a Parallel Reaction Monitoring (PRM) based mass spectrometry method for the analysis of samples. The high specificity PRM MS is suitable for the analysis of low protein content samples.

In previous studies, SC was removed by 6 or more discs from the same place and protein content of the first disc was excluded from the investigation. These studies concluded, that the removable protein content decreased exponentially towards deeper layers of SC due to increasing cohesive force between cells. During sampling, more discs applied to the same place caused increased discomfort. Because the skin, as a barrier first encounters pathogens at the outermost layer of the SC, we used the protein content of the first disc for our investigation and removed only one disc from one site.

We examined 6 AMPs (S100A7, S100A8, S100A9, hBD2, LCN2, TSLP) in SC samples and we determined their expression levels in skin segment during the first part of workflow. We selected another 7 AMPs (hBD1, 3, 4, DCD, LACRT, LYZ, LL37) in the second part of the workflow, which are the characteristic markers of healthy or inflamed skin. 6 peptides of 5 proteins (ALNSIIDVYHK and GADVWFK peptides from S100A8 protein, VPLQQNFQDNQFQGK peptide from LCN2 protein, ENAGEDPGLAR peptide from DCD protein, SILLTEQALAK peptide from LACRT protein and WESGYNTR peptide from LYZ protein) from the examined 13 AMPs could be detected in different healthy skin regions. 4 proteins from 5 (S100A8, LCN2, LACRT and LYZ) were detected in significantly higher amounts in SGR skin region compared GP and AGR skin regions. We also found that ALNSIIDVYHK and GADVWFK peptides of S100A8 and VPLQQNFQDNQFQGK peptide of LCN2 were present in notably higher amounts in AGR skin compared to GP skin and could be detected in significantly increased amounts in SGR skin compared to GP and AGR skin regions. These data are consistent with our results from the first part of our studies.

Moreover, we could identify two previously unexamined AMP molecules (SILLTEQALAK peptide from LACRT and WESGYNTR peptide from LYZ), that were detected in significantly higher amounts in SGR skin compared to GP and AGR skin areas. The absence of LCN2, LYZ and the low level of S100A8 and LACRT suggest that GP skin might be associated with the less pronounced steady-state immune activity.

The amount of DCD produced by eccrine glands was detected to be comparable in the three healthy regions, which is consistent with previous findings. Since DCD is present in the sweat of the eccrine gland in healthy skin, but its amount is significantly reduced or completely

disappears during inflammation, its altered level can indicate inflammatory skin diseases in their initiation phase.

AMPs play an important role in the development and pathogenesis of certain skin diseases and have significant impact on their severity. PRM- based mass spectrometry analysis of SC protein extracted with tape stripping technique can provide an opportunity for the detection and quantitation of AMPs. Detection of AMPs can help research the pathomechanisms of various skin diseases, as well as aid the clinical follow-up of skin diseases. Monitoring therapeutic efficacy can provides an opportunity to determine the AMP composition of the skin surface before and after therapy.

Summary

Based on our results, the three main topographically different skin regions (gland-poor [GP], sebaceous gland-rich [SGR], apocrine gland-rich [AGR]) are equipped with a significantly different immune and barrier supply, similarly to the previously reported topographical distinctions of their microbiota and chemical milieu. The variances in immune and permeability barrier characteristics among the skin regions may explain the fact that different region-specific skin diseases localize to different skin areas. For instance, AD is typical on GP, HS is common in AGR and acne or PPR occur on SGR skin regions. First, we investigated the immune status of healthy AGR skin regions and that of HS, an inflammatory skin disease specially localized to AGR areas. Our results revealed a non-inflammatory IL-17/IL-10 cytokine environment on AGR skin compared with GP skin, with increased $CD4^+$ Th and non-activated $CD11c^+$ DC counts. The expression of IL-17 related chemokines, AMPs and barrier molecules were increased significantly in AGR skin, compared to GP skin. In HS, the AGR specific non-inflammatory IL-17/IL-10 status turns to an inflammatory environment characterized by IL-17/IFN-γ cytokine milieu supplemented with further inflammatory markers. The amounts of cellular components associated with enhanced immune response and their activation markers, Th1/Th17 related factors, chemokines, AMPs and barrier molecules were increased significantly in HS compared with healthy AGR skin.

Next, we developed and optimized a PRM-based mass spectrometry method to detect the AMP content of SC in healthy and inflamed skin using a minimally invasive sample collection technique. We observed that the abundance of AMPs in the SC collected by D-Squame discs was specific to the individual, but were independent from the skin regions. Significantly higher AMP amounts could be extracted from the discs using our novel hexane-free elution technique, compared with hexane-based methods. Comparing the AMP content of healthy SGR skin with that of GP and AGR regions, we could detect significant differences in the case of 5 peptides of 4 proteins (two peptides from S100A8, one from LCN2, LACRT and LYZ). No significant differences could be detected in the case of the peptide of DCD protein in the SC of different skin regions. Applying this method for the investigation of inflammatory skin diseases may help us to better understand their pathomechanisms and may provide information on disease severity (based on AMP quantitation) in the clinical practice.

Publication list

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Doctoral School: Gyula Petrányi Doctoral School of Allergy and Clinical Immunology
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List of publications related to the dissertation

1. **Jenei, A.**, Kalló, G., Dajnoki, Z., Gáspár, K., Szegedi, A., Kapitány, A., Csősz, É.: Detection of Antimicrobial Peptides in Stratum Corneum by Mass Spectrometry.
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4. Bukva, M., Dobra, G., Gomez-Perez, J., Koós, K., Harmati, M., Gyukity-Sebestyén, E., Bíró, T., **Jenei, A.**, Kormondi, S., Horváth, P., Kónya, Z., Klekner, Á., Buzás, K.: Raman Spectral Signatures of Serum-Derived Extracellular Vesicle-Enriched Isolates May Support the Diagnosis of CNS Tumors.
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