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

Analysis of antimicrobial proteins of tears and sweat in health and pathological conditions

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The Examination took place at Research Center for Molecular Medicine, Faculty of Medicine, University of Debrecen, at 11:00 a.m. on 30th of June, 2016

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The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 13:00, 29th of November 2016.

1. Introduction

1.1 Antimicrobial and immunomodulatory proteins in the formation of chemical barriers

At those sites where the human body may come in contact with potential pathogens, well-defined chemical barriers exist. These chemical barriers provide passive protection against infections by diluting the pathogens and by the secreted antimicrobial and immunomodulatory proteins/peptides (AMPs) actively inhibit bacterial growth. The human body contains several contact sites: the eye, the oral cavity, the nose, the skin, the intestinal surface and the urogenital tract. Each site is protected by a chemical barrier maintained by different body fluids such as tears, sweat, saliva, nasal secretion, urine and intestinal mucus.

These chemical barriers are made up of secretions of various glands and epithelial cells and the characteristic composition of the chemical barrier makes the secreted AMP cocktail specific for each body fluid. Regarding the protein composition of the body fluids providing chemical barriers it was observed that the highly abundant proteins characteristic for body fluids are part of the immune system and have protective roles. While some proteins such as defensins and LL-37 cathelicidin were first isolated due to their antimicrobial properties (so called prototypic AMPs), other highly abundant proteins were initially recognized for their other functions and later they or their peptides were found to have antimicrobial activity. Besides the prototypic AMPs, there are several proteins with much higher concentration compared to prototypic AMPs. These proteins e.g. lactotransferrin, lipocalins, lysozyme-C, extracellular glycoprotein lacritin, prolactin-inducible protein etc. are the highly abundant body fluid proteins with defense functions.

1.2 AMPs as potential biomarkers for disease detection

According to the National Institutes of Health Biomarkers Definitions Working Group, biomarker is “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”. In some conditions, proteins are considered biomarkers when identified as having a central role in normal or pathological functions, and their presence or absence causes the malfunction leading to the disease. Consequently, these biomarkers are at the same time targets for drug design and therapy. In other cases, biomarkers are not suitable targets for therapeutic intervention; their presence, absence or differential expression is the consequence and not the

cause of the disease, hence, they cannot be used as target for therapies. In the recent years, studies aiming to identify biomarkers specific for different pathological conditions emerged and hundreds of potential biomarkers were identified.

One of the bottlenecks of biomarker studies is the availability of samples. In many cases, samples originating from the tissue or the biological material in which the alteration happens are not available for biomarker studies because of the highly invasive collection methods. Advances in proteomics and metabolomics techniques had led to improved sensitivity, and provided the possibility to detect protein or metabolite changes in body fluids which are not necessarily in proximity to the affected area and could be collected by non-invasive methods. The emerging omics technologies provide new possibilities for identification of biomarkers from the continuously available body fluids that can be collected by non-invasive means, as tears, saliva, sweat, nasal secretion or urine.

As far as different concentrations of the various host defense proteins can be observed in the chemical barrier, practically an AMP cocktail is present. Most probably the composition of the chemical barrier is characteristic to the stimulus to which the organism has to adapt or to the pathological condition causing the alteration of the chemical barrier leading to the characteristic symptoms. In this way, the changes of the composition of the host defense proteins in different body fluids as a response to pathological conditions may provide a feasible source for biomarker studies.

1.3 Tear fluid, the chemical barrier of the eye

Tear fluid is a complex mixture of proteins, lipids, salts and other organic molecules produced by the lacrimal glands, meibomian glands and conjunctival goblet cells. The functions of the tear film are the lubrication of the eye, delivery of nutrients and the maintenance of the refractivity of the cornea. Besides these roles, tear creates an effective chemical barrier on the surface of the eye *via* secreted AMPs which provide protection against pathogens. Major tear proteins; such as lysozyme-C, prolactin-inducible protein, lactotransferrin, lacritin etc., are involved in the defense against pathogens. While many of the tear proteins are produced by the lacrimal glands, some of them originate from epithelial cells; such as dermcidin, defensins, etc., and there are also proteins filtered from the blood such as albumin.

Tear fluid is one of the non-invasively obtainable body fluids that is relatively easy to collect, and the examination of tear components may help understand the pathogenesis of ocular and some systemic diseases; thus, tear is a possible source of potential biomarkers. Analysis of tear protein profile can provide useful information on understanding the molecular mechanisms of ocular diseases; such as dry eye syndrome, blepharitis, diabetic retinopathy, keratoconus etc., and can also reflect systemic diseases such as multiple sclerosis. The tear fluid was also used to study the neuroinflammation in Parkinson's disease and increased TNF-α levels were found in the tears of these patients compared to controls, therefore tear is a possible source for biomarkers specific to neurological disorders as well.

1.4 Sweat, the chemical barrier of the skin

The skin acts as an effective barrier against pathogens at the first line of host defense. Besides providing a physical barrier, the skin also creates a chemical barrier *via* AMPs secreted by epithelial cells, sebocytes and keratinocytes. Similarly to tear fluid, the abundant sweat proteins are part of the innate immune system; its protein content provides an effective defense against pathogens, and is involved in tissue regeneration after injury. Some AMPs were shown to be expressed constitutively while others were found to be inducible upon pathogenic stimuli. Besides these prototypic AMPs, the presence of lysozyme-C and lactotransferrin in the sweat has been reported as well.

The non-invasive collection and the continuous availability of sweat make it an excellent source for biomarker studies. Sweat samples from patients with skin disorders such as ectodermal dysplasia, cystic fibrosis and atopic dermatitis were analyzed using quantitative proteomics methods and reduced level of proteins involved in the host defense and tissue regeneration were demonstrated. The sweat proteome has been analyzed in order to identify biomarkers for systemic diseases as well.

1.5 Intestinal surface, the physical and chemical barrier of the digestive tract

The large mucosal surface of the intestinal epithelium acts as an important physical and chemical barrier and confers a first line of defense against pathogenic bacteria, viruses and other microorganisms. In the secreted AMP cocktail, the ubiquitous lysozyme-C and lactotransferrin are present, but the major constituents of this chemical barrier are prototypic AMPs, such as β-defensins (hBDs) and LL-37 cathelicidin. In the small intestine hBD1 was described as a

constitutively expressed AMP, its expression was not regulated by proinflammatory cytokines. The level of hBD4 can be upregulated as a result of bacterial infection but not by classical proinflammatory signals such as IL-1β. On the contrary, hBD2 and hBD3 were described as inducible AMPs, which respond to stimulation caused by proinflammatory cytokines like IL-1β. Inflammatory bowel diseases such as ulcerative colitis and Chron's disease are associated with increased levels of inducible β-defensins leading to epithelial cell proliferation, immune cell migration and enhanced production of proinflammatory cytokines.

1.6 Targeted proteomics with Selected Reaction Monitoring

Selected Reaction Monitoring (SRM) is one of the most commonly used targeted mass spectrometry approach in proteomics and in biomedical analyses, being a specific scan mode of the triple quadrupole-containing mass spectrometers. The first quadrupole transmits only a well-defined precursor ion which will be fragmented in the second quadrupole functioning as a collision cell. The third quadrupole transmits only one specified fragment resulting in a signal when the precursor ion and its selected fragment are present at the same time. These SRM transitions provide high specificity and sensitivity. The area under the curve (AUC) of the specific signal corresponds to the amount of the compound entering the mass spectrometer therefore SRM analyses provide quantitative data as well.

When functioning in a semi-quantitative setup, SRM experiments allow the relative quantification of specified analytes but absolute quantification is also possible. The monitoring of at least two SRM transitions for each peptide is mandatory for reliable analysis, and for more accurate absolute quantification, 3 to 5 SRM transitions per peptide is required. Depending on the size of protein multiple peptides per protein are required for SRM analyses. The "gold standard" of SRM is the application of the stable isotope-labeled (SIL) synthetic peptides. These SIL peptides are introduced into the samples in order to serve as internal controls during the experiments. During SRM scan, the mass spectrometer can analyze hundreds of transitions; therefore, multiple proteins can be analyzed in one sample. The sensitivity of SRM is outperformed by antibody-based experiments utilizing signal multiplication systems, although the dynamic range of SRM is usually wider than of antibody-based methods. The sensitivity of SRM can be increased by the administration of specific enrichment techniques. The high specificity, sensitivity, the large dynamic range and the multiplex feature of SRM is highly relevant to

biological applications where the amount of the samples is usually limited. SRM technique requires *a priori* information about the proteins to be studied and the limitation of this approach could be the restricted availability of unique, protein-specific peptides.

1.7 Label-free quantification mass spectrometry

Label-free quantification is a mass spectrometry technique where either the signal intensity of the precursor ion or the number of MS/MS events is used to gain information about the relative amounts of peptides entering into the mass spectrometer. In the first case, the AUC values of well-defined precursor ions are compared between the analyses and used for relative quantification. The second method is based on the correlation between the protein quantity and the number of recorded MS/MS spectra. The high scan rate and accuracy provided by the high resolution mass spectrometers, preferably Orbitrap- or FTICR-containing instruments, make relative quantification accessible, even in case of large sample number. This technique combines protein identification and protein quantification in the simplest way, thus label-free quantification is one of the most commonly used shotgun methods.

Label-free quantification has the benefits of versatility; and the fact that any kind of sample could be analyzed and compared without previous information about the proteins makes this mass spectrometry technique important in medical sciences. One of the drawbacks of label-free quantification is the need for a very stable and carefully controlled system to avoid possible errors caused by inappropriate chromatographic separation.

2. Aims of the study

- Design and development of an SRM-based targeted proteomics method for the detection and quantification of human β-defensins (hBDs) and their analysis in cell culture samples and tears
- Analysis of the components of the chemical barrier in the tears of patients with Alzheimer's disease compared to matched controls in order to identify potential tear biomarkers for Alzheimer's disease
- Analysis of the components of the chemical barrier in the sweat of healthy volunteers in order to identify the highly abundant human sweat proteins

3. Materials and methods

3.1 Cell cultures

For cell culture studies, we have used human HT-29, SW-1116 and Caco2 colonic epithelial cell lines derived from various types of human colorectal adenocarcinomas. HT-29 and SW-1116 cells were grown in RPMI medium supplemented with 10% fetal calf serum (FCS) while Caco2 cells were grown in 20% FCS containing RPMI medium. Mediums were supplemented with 100 U/mL penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1% non-essential amino acids at 5% and cells were grown at 5% CO2 and 37 °C. Resting cells (5×10^5/ml) were stimulated with 10 ng/ml IL-1β for 1 hour followed by the removal of the supernatant which was replaced by fresh medium and the cells were incubated for 5 hour before harvesting. The cell culture supernatants were collected without disturbing the cellular monolayer and trypsin-EDTA (Sigma) was added to collect the cells. The detached cells were washed twice with PBS in order to avoid contamination by the cell culture supernatant, and lysis buffer was added to collect the cell lysates. The protein concentration of the samples was determined with the Bradford method.

3.2 Tear collection

In total, 26 donors were recruited, 14 patients with Alzheimer's disease (AD) and 9 age and sex matched controls and three young healthy volunteers. Sample collection complied with the guidelines of the Helsinki Declaration and ethical approval was obtained from the University of Debrecen Ethics Committee (DEOEC RKEB/IKEB 2980–2009) and the subjects gave written informed consent.

In the case of patients with AD sample was collected in the presence of a caregiver. The diagnosis of AD was based on the NINCDS-ADRDA and the DSM-IV-TR criteria. Besides psychiatric and neurological assessment, patients underwent basic laboratory testing and CT or MR imaging of the brain in order to rule out other causes of dementia.

The clinical evaluation of the age matched control subjects consisted of a structured interview, demographic information, medical history, current medication, history of alcohol consumption and a subjective assessment of memory problems using Mini-Mental State Examination. Only controls without any signs of cognitive impairment were included in the

study. Patients with systemic inflammation, autoimmune disorders or ophthalmological disorders were excluded.

After the assessment of the anterior ocular status of each patient, tear was collected from the inferior meniscus of both eyes. The non-traumatic tear collection was carried using standard capillary collection technique; samples were centrifuged and the protein concentration of each tear sample was measured from the supernatant with the Bradford method. The samples were frozen and stored at -70^{o}C until analysis.

3.3 Sweat sample collection

Ten male and ten female healthy young adults were recruited into the study. Heat-induced sweat was collected in an 80°C electric Finnish-type sauna. Volunteers were asked not to use any cosmetics that day, and before entering the sauna, they were required to take a shower using only water and dry their skin. Volunteers were instructed to stay in the sauna for 20 minutes, thereafter, take a shower, dry their skin and rest for 10 minutes, followed by another 20 minutes sauna time. All sample collection complied with the guidelines of the Helsinki Declaration, approved by the Regional Ethical Committee of the University of Debrecen (DEOEC RKEB/IKEB 4078/2013 and 2885/2008) and the subjects gave written informed consent.

The collected samples were transported to the laboratory on ice in less than 30 minutes from sample collection. Samples were centrifuged and the clear sweat was concentrated, redissolved in 0.1 M ammonium bicarbonate buffer (pH 7.8) and pooled. The protein concentration of the pooled sweat was determined by Bradford method, and the concentrated sample was stored at -70°C until further processing. The pooled sample was precipitated twice, using six volumes of ice-cold acetone and the pellet was solubilized in 0.1 M ammonium bicarbonate buffer (pH 7.8) and micro bicinchoninic acid protein assay was performed according to the manufacturer's instructions to determine the total protein content.

3.4 ELISA

Determination of hBD2 protein level in tears was performed by sandwich ELISA in three biological replicates using the EK-072-37 kit (Phoenix Pharmaceuticals Inc.) according to the protocol provided by the manufacturer. Analyzes were performed in a volume corresponding to 5 μg total tear protein.

3.5 SDS-PAGE analysis

20 μg tear protein from three randomly selected AD patients and two controls were subjected to SDS-PAGE analysis on a 10% SDS polyacrylamide gel. The electrophoresis was done in a Bio-Rad mini tetra cell (Bio-Rad) on 100 V constant current for one hour. The protein bands were visualized using Coomassie PageBlue (Fermentas) stain solution and scanned with a Pharos FX Plus laser scanner (Bio-Rad). The image analysis was carried out using the QuantityOne software (Bio-Rad) and the band intensities in each case were determined and statistically analyzed performing Mann–Whitney U test using the SigmaPlot 12.0 software.

3.6 LC-MS/MS-based protein identification of tear proteins

The bands with significantly different intensities between AD and control samples were excised and in-gel digested with trypsin. First a reduction was performed using 20 mM dithiothreitol (Bio-Rad) followed by alkylation with 55 mM iodoacetamide (Bio-Rad). The overnight trypsin digestion was carried out using stabilized MS grade TPCK-treated bovine trypsin (ABSciex) at 37^{o}C and the digested peptides were extracted and lyophilized. The peptides were re-dissolved in 10 μl 1% formic acid and used for LC-MS/MS analysis.

Prior to mass spectrometry analysis, the peptides were separated using a 90 minutes water/acetonitrile gradient with an increase in acetonitrile concentration from 0% to 100% during 60 minutes on an EasynLC II system (Bruker). Desalting was performed on a Zorbax 300SB-C18 column (5 × 0.3 mm, 5 μm pore size; Agilent) followed by separation on a Zorbax 300SB-C18 analytical column (150 mm × 75 μm 3.5 μm pore size; Agilent). Solvent A was 0.1% formic acid in LC water, solvent B was acetonitrile containing 0.1% formic acid. The flow rate was 300 nl/min.

Positive mode LC-MS/MS scans were performed on a 4000 QTRAP (ABSciex) mass spectrometer using a NanoSpray II MicroIon source and was controlled by the Analyst 1.4.2 software (ABSciex). Collision induced dissociation (CID) spectra were acquired in Enhanced Product Ion mode and rolling collision energy was applied. Information Dependent Acquisition method was utilized; after the first mass scan, an enhanced resolution scan was carried out to establish the charge state of the precursor ions followed by the acquisition of MS/MS spectra of the two most intensive ions.

The acquired LC-MS/MS data were subjected to protein identification with the help of ProteinPilot 4.0 (ABSciex) search engine searching the SwissProt database (release: 2015.07, 548872 sequence entries) using the biological modification table included in the ProteinPilot 4.0 software. Minimum of two peptide sequences with ≥95% confidence were used for protein identification.

3.7 Label-free quantification (LC-MS^E) and LC-MS/MS analysis of sweat proteins

Label-free quantification and LC-MS/MS analyses were performed at the Center for Toxicology, University of Arizona. 4.2 µg pooled sweat protein samples were first reduced and alkylated, followed by tryptic digestion overnight at 37°C. For LCMS^E, 600 ng digested sample containing 50 ng of a T33V Rhodobacter cytochrome c digest as an internal standard, were injected onto a NanoAcquity UPLC (Waters) coupled to Waters QTOF Premier mass spectrometer equipped with a nanoESI source. LC separation of peptides was performed in triplicate using a Symmetry C18 pre-column (20-mm x 180 µm, 5 µm pore size; Waters) and a BEH130 C18 analytical reversed phase column (100-mm x 100 µm, 1.7 µm pore size; Waters). Solvent A was water with 0.1% formic acid and solvent B was acetonitrile with 0.1% formic acid. The peptides were separated with a gradient of 2-35% solvent B over 150 min followed by a rise to 95% of solvent B over 2 min and a 5-min rinse with 95% of solvent B, after which the system returned to 2% solvent B in 2 min. The flow rate was 750 nl/min and the column temperature was 35°C. Accurate mass LC-MS data were collected in an alternating low energy and elevated energy mode of acquisition.

The digested sweat protein sample was also analyzed by LC-MS/MS using an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) equipped with an Advion nanomate ESI source (Advion), following ZipTip C18 sample clean-up according to the manufacturer's instructions (Millipore). 240 ng tryptic digest was eluted from a C18 pre-column (2 cm x 100 µm, 5 µm pore size; Thermo Fisher Scientific) onto an C18 analytical column (10 cm x 75 µm, 3 µm pore size;Thermo Fisher Scientific) using 5% solvent B for 5 min, 5-10% gradient of solvent B over 5 min, 10-35% gradient of solvent B over 35 min, 35-50% gradient of solvent B over 20 min, 50-95% gradient of solvent B over 5 min, and finally 95% solvent B for 4.6 min. The flow rate was set to 400 nl/min. Data-dependent acquisition was performed by the Xcalibur v 2.1.0 software (Thermo Fisher Scientific) using a survey mass scan in the Orbitrap analyzer, followed by CID spectra of the 14 most intense ions were acquired in the linear ion trap.

3.8 Analysis of LCMS^E and LC-MS/MS data

LC-MS/MS spectra were searched against human proteins downloaded from the Uniprot database on August, 2013 (88323 entry) using Thermo Proteome Discoverer 1.3 (Thermo Fisher Scientific) considering tryptic peptides with up to two missed cleavages. Iodoacetamide derivatives of cysteines, and oxidation of methionines were specified as variable modifications. Proteins were identified at 99% confidence with XCorr score cut-offs. The protein and peptide identification results from the LC-MS/MS experiment were analyzed and visualized with Scaffold v 4.0.5 (Proteome Software Inc.) software as well. Those proteins were accepted that passed the criteria of a minimum of two peptides identified at 0.1% FDR at peptide level and 1% FDR at the protein level.

For LCMS^E data analysis, all LCMS^E data were processed and searched using ProteinLynx GlobalServer version 2.4 (Waters). Protein identifications were obtained by IdentityE algorithm and searching a human database downloaded from Uniprot to which the primary sequence of the T33V cytochrome c from *Rhodobacter capsulatus* was appended. A minimum of three fragment ions matched were required per peptide, and a minimum of one peptide was required per protein for identification.

3.9 Sample preparation for SRM analysis

Proteins were denatured with 6 M (Bio-Rad) urea and were reduced with 10 mM dithiothreitol. The reduced samples were alkylated with 20 mM iodoacetamide and diluted with 25 mM ammonium bicarbonate to decrease the urea concentration to 1 M. Trypsin digestion was performed at 37°C overnight by adding MS-grade modified trypsin in 1:25 enzyme-to-protein ratio. The digested peptides were lyophilized and dissolved in 1% formic acid. The samples were desalted with C18 ZipTip (Millipore), lyophilized and re-dissolved in 1% formic acid.

3.10 Development of SRM-based targeted proteomics method

Amino acid sequences of hBD1–4, lipocalin-1, lactotransferrin, extracellular glycoprotein lacritin, lysozyme-C, lipophilin A, Ig λ-chain C region, prolactin-inducible protein, Zn-α-2-glycoprotein, galectin 3 binding protein and dermcidin were utilized from the UniProt database and were subjected to *in silico* trypsin digestion by the PeptideCutter software. Tryptic fragments with 100% cleavage probability were selected for BLASTp analysis and the NCBI non-redundant

protein sequence database was searched to determine the unique, protein-specific tryptic peptide sequences. Design of the SRM transitions was performed by the Skyline software. SIL crude peptides were obtained from JPT Peptide Technologies GmbH, Germany, while purified hBD2 SIL peptide was obtained from PepscanPresto, The Netherlands. The SRM spectra of all singly charged "y" ions were recorded on a 4000 QTRAP mass spectrometer and the transitions with the highest intensity were used for further analyses. The optimization of collision energy (CE) and declustering potential (DP) was performed with the Skyline software using the SIL peptides. The best transitions along with the optimized CE and DP values were included into an SRM method file and tested on tears and sweat samples from healthy volunteers while the method for hBDs were tested on cell culture supernatants.

3.11 SRM analysis

In case of cell culture and tear samples, sample blocking was carried out. In case of the cell culture supernatants a randomly selected control and a randomly selected IL-1β-treated sample was paired and digested together and analyzed one after the other using the same conditions. The same procedure was applied for cell lysates. In case of tear samples a randomly selected AD sample was paired with a randomly selected control sample and analyzed one after the other using the same conditions. SIL peptides were added to the samples immediately before the analyses. All measurements were carried out in triplicates.

Chromatographic separation was performed with an EasynLC II system (Bruker). Desalting was performed on a Zorbax 300SB-C18 column (5 × 0.3 mm, 5 μm pore size; Agilent) followed by separation on a Zorbax 300SB-C18 analytical column (150 mm × 75 μm 3.5 μm pore size; Agilent). Solvent A was 0.1% formic acid in LC water, solvent B was acetonitrile containing 0.1% formic acid. The flow rate was 300 nl/min, and 30 minutes water/acetonitrile gradient with a continuous increase of solvent B from 0% to 100% during 15 minutes was applied.

SRM-based analyses using the designed and optimized SRM transitions were carried out on the 4000 QTRAP (ABSciex) mass spectrometer using a NanoSpray II MicroIon source controlled by the Analyst 1.4.2 software (ABSciex). The spray voltage was 2800 V, the ion source gas was 50 psi, the curtain gas was 10 psi and the source temperature was 70 °C. The cycle time was 1.15 sec in case of hBDs, 2.5 sec in case of the analysis of tears from patient with AD and matched controls and 1.5 sec for sweat analysis. These configurations provide nearly 30

data points/peak for hBDs, 16 data points/peak for tear proteins and approximately 20 data points/peak for the analysis of sweat proteins.

SRM data were analyzed by using the Skyline software, the AUC values for endogenous and synthetic SIL peptides and the endogenous:SIL ratios were calculated by the software. The lowest detected amount of peptide was determined based on the signal registered during the analyses of different concentrations of SIL peptides added to tear or cell culture samples. The mass spectrometry analyses were carried out in triplicates. For the calculation of the linear dynamic ranges, mean log AUC values were plotted against the log concentration values and logistic regression was applied for curve fitting. The linear dynamic range and the limit of quantification (LOQ) were defined based on these curves.

3.12 Statistical analysis

Statistical analysis was performed with the SigmaPlot 12.0 software using Student's t-test, the level of significance was set to $p \leq 0.05$.

In case of the analysis of tears from patients with AD and matched controls, the calculated AUC data were transformed into MSStats R-package format using an in-house developed software. After the normalization with the SIL peptides and log2 transformation of data, the differences between the groups were examined by a mixed-effect variance analysis. The groups were modeled as fixed effect while the subject level variances were modeled as random effects. After the analysis the raw p-values were adjusted by the Benjamini and Hochberg type false discovery rate method for multiple testing purposes. Besides the adjusted p-values, the log2 fold change, the t-values and the standard error were examined as well. Receiver-operating characteristic (ROC) analyses were also carried out, the accuracy and the 95% Confidence Interval (CI) values were calculated.

4. Results

4.1 Analysis of hBDs in cell culture and tear samples

Classical antibody-based methods are widely used for the quantitative or semi-quantitative analyses of proteins and peptides but the dynamic range in the quantitative settings can be a potential limiting factor and the determination of more than one analyte as well as lack of suitable antibodies may restrict the utilization of these methods. The SRM-based methods offer multiplexing; several proteins could be analyzed in one sample and are more cost-effective than the classical antibody-based techniques. In order to establish a cost-effective and flexible multiplex method for the analysis of the most common hBDs and to better understand the role of hBDs in innate defense mechanisms, we have developed an SRM-based method that could be applied for the determination of hBD1-4 levels in different biological samples.

Sequences of hBD1-4 were retrieved from the Uniprot database and were subjected to *in silico* trypsin digestion using the PeptideCutter software. The peptides with 100% cleavage probability were further subjected to BLASTp analysis in order to identify the unique, protein-specific peptides. BLASTp searches demonstrated the presence of peptides specific for the four studied hBD, thus IQGTCYR was specific for hBD1, GIGDPVTCLK was specific for hBD2 and GIINTLQK was specific for hBD3. We could identify four specific sequences for hBD4 and from these using the ICGYGTAR sequence was selected for the SRM assay using the CONSeQuence prediction algorithm. All transitions were designed using Skyline and were analyzed on 4000 QTRAP (ABSciex) mass spectrometer. The DP and CE were optimized with the help of the Skyline software using the SIL peptides. The best transitions were selected for further analyses.

4.1.1 Linear dynamic range of hBD peptides

Determination of the range where the AUC is proportional to the amount of peptide is critical in quantitative proteomics. The linear dynamic range was determined by using increasing amounts of SIL hBD peptides in the arbitrary selected dilution range of 1000 fold- to 5-fold. SIL peptides were added to cell culture supernatant and tear fluid samples. Using the developed SRM the 1000-fold dilution (approximately 75 fmol) of each hBD peptide could be detected while the limit of quantification (LOQ) and the linear dynamic range was different in case of each peptide.

The LOQ was typically 250-fold dilution in cell culture supernatant and 500-fold dilution in tears.

4.1.2 Analysis of hBD levels in cell lysate and cell culture supernatant samples

Considering that β-defensins are produced in the cytosol but exert their biological activities outside the cell the levels of hBD1, hBD2, hBD3 and hBD4 were analyzed both in cell lysates and cell culture supernatants of three different colonic epithelial cell lines. In order to examine the induction of hBDs during inflammation, IL-1β treatment was applied. The level of hBD1 did not change significantly in SW-1116 and HT-29 cells upon IL-1β stimulation; however, significant decrease was detected in Caco2 cell lysates while significant decrease of secreted hBD1 levels was observed in stimulated HT-29 cells. Changes in the endogenous:SIL peptide ratio showed significantly elevated intracellular and secreted hBD3 level in all stimulated cells compared to their unstimulated counterparts. As a result of IL-1β stimulation, the level of intracellular hBD4 did not change significantly and similar tendency has been observed as shown for hBD1. The level of secreted hBD4 decreased significantly only in the HT-29 cell culture supernatants, while in SW-1116 and Caco2 cells, hBD4 levels followed the pattern of hBD1. In our experiments, we could detect hBD2 only in CaCo2 cell lines; in the HT-29 and SW-1116 cells the level of hBD2 was under the detection limit of the mass spectrometer. We could demonstrate that CaCo2 cells responded to the IL-1β stimulation with increased hBD2 levels both in the cytosol and in the cell culture supernatant. Our data demonstrate the feasibility of the SRM-based method in the comparative analysis of hBD levels in biological samples and provide further evidence for the inducible feature of hBD2 and hBD3 in inflammatory environment.

4.2 Analysis of tear proteins of patients with AD

AD is one of the most common age-related dementia and several studies showed that these patients have a variety of visual problems and amyloid plaques were found in the eye of patients with AD; therefore we hypothesized that the disease may alter the levels of several tear protein as well. In order to assess whether the newly developed SRM method could be also utilized for the analysis of biological fluids, we collected tear samples from healthy volunteers and patients with AD and analyzed them. In case of the tear samples from patients with AD the level of hBDs was under the detection limit of the mass spectrometer, but from the samples

obtained from healthy volunteers the quantification of hBD1, hBD2, hBD3 and hBD4 peptides was successfully performed by the SRM method. The level of hBD2 was the lowest and the level of hBD3 was the highest, while the levels of hBD1 and hBD4 were almost the same in tears. The hBD levels in tear samples were found to be lower than those detected in the supernatants of colonic epithelial cells indicating tissue-specific expression of hBDs. In a next step we compared the efficacy of the ELISA and the newly developed SRM method for the analysis of hBD2 levels derived from the same samples and both methods gave comparable results. The data demonstrate that the developed SRM method is suitable for the relative quantification of hBDs also in body fluids typically of low sample volume such as tears.

As far as the levels of hBD1-4 in tears from patients with AD were under the detection limit of the mass spectrometer, the protein contents of tears of patients with AD have been analyzed and targeted proteomics approach was used to identify possible tear biomarkers for AD.

4.2.1 Tear protein profile changes in AD

By monitoring the production rate and protein concentration of tears, significant differences were observed. The protein concentration observed in controls (4.4±1.4 μg/μl) was significantly lower than that in patients with AD (8.8±2.9 μg/μl) along with a significant increase in tear flow rate from 6±2 μl/min in controls to 12±2 μl/min in patients with AD.

In order to examine the tear protein profile, equal amounts of tear proteins originating from three patients with AD and two controls were analyzed by SDS-PAGE. After visualization of the bands 13 bands have been observed and their densitometric evaluation indicated significant decrease in band intensity in AD samples in case of 11 bands. The bands were excised and digested with trypsin followed by LC-MS/MS based protein identification.

Based on the LC-MS/MS data 17 proteins have been identified from the 13 excised bands. These proteins are well characterized tear proteins originating from the lacrimal glands. By examining the function of the differentially expressed proteins, we observed that they are involved in the host defense mechanisms and are components of the chemical barrier of the eye. These data suggest that AD can alter the composition of the chemical barrier and is in accordance with previous experiments showing alterations of the chemical barrier by different stimuli and pathological conditions.

4.2.2 SRM-based quantitative proteomic method development for tear proteins

In order to validate the changes in chemical barrier present in the tears of the patients with AD studied, an SRM-based targeted proteomics approach was developed. The major tear proteins showing reduced level in AD samples, such as lipocalin-1, lactotransferrin, lysozyme-C, extracellular glycoprotein lacritin, Ig λ chain, Zn α2 glycoprotein, prolactin-inducible protein and lipophilin A were chosen for further analyses. It is well known that the major proteins of sweat and tears have a role in host defense against potential pathogens thus based on these data and on our previous experiments galectin-3 binding protein and dermcidin, as proteins having important role in host defense and immunomodulation were included in the panel of analyzed tear proteins. SRM-based quantitative proteomic experiments were designed for the selected proteins with the help of the Skyline software and optimized with the help of SIL peptides.

The sensitivity of the SRM method was assessed by analyzing increasing amounts of SIL peptides added to tears in the range of 10000 fold - 5 fold dilutions. Most of the peptides were detectable in 10000-fold dilution (approximately 7.5 fmol) and the SRM signal intensity was proportional with the amount of the peptides introduced to the mass spectrometer in a broad range of dilutions. Based on the results, it appears that the developed SRM method is sensitive enough and has broad dynamic range to be a useful tool to monitor the changes in the amount of the studied tear proteins.

4.2.3 Changes in the tear chemical barrier composition upon AD

In order to study the changes in the tear chemical barrier upon AD, the level of the selected antimicrobial and immunomodulatory proteins was analyzed using the developed SRM methods in 37 tear samples of 14 patients having AD and 9 controls. In line with the results of electrophoresis, the levels of lipocalin-1, lactotransferrin, extracellular glycoprotein lacritin, lysozyme-C, and prolactin-inducible protein were significantly decreased, while the level of dermcidin was significantly elevated in AD tears, as compared to those of the controls. The down-regulated proteins are expressed by the lacrimal glands indicating lacrimal gland dysfunction associated with AD.

4.2.4 Identification of possible AD-specific tear biomarkers

Proteins differentially expressed in the tears of patients with AD were subjected to further analyses in order to examine their potential as future predictive biomarkers for AD. ROC analysis was carried out for each significantly expressed protein alone and multivariate ROC curves were constructed to test the different combinations of the potential biomarkers. The best sensitivity was given by the combination of lysozyme-C and extracellular glycoprotein lacritin with 91% sensitivity, but the most balanced performance was achieved when the lipocalin-1, dermcidin, lysozyme-C and extracellular glycoprotein lacritin were combined. In this case, the AUC was 0.80, the sensitivity was 81% and the specificity was 77% indicating a well-performing biomarker combination.

4.3 Analysis of sweat proteins

The skin acts as an effective physical barrier against pathogens and by secreting sweat as an effective chemical barrier as well. Sweat as a continuously available and easy to collect sample is a good source for biomarker studies. Beside the fact that sweat has been used for the identification of possible biomarkers specific to skin disorders or systemic diseases, the normal sweat proteome has not been examined in details. In order to identify disease-specific changes, we ought to first understand the role and composition of the protein mixture present on the surface of the intact, healthy skin under physiological conditions.

4.3.1 Sweat protein identification and label-free quantification

A sweat pool collected during sauna bathing from healthy adult volunteers was examined it in order to gain more insights into the protein composition of sweat. Using consecutive mass spectrometry analyzes, 95 proteins were identified by high resolution LC-MS/MS, 20 of them being novel proteins that were not reported in sweat previously. The levels of proteins identified from human sweat were determined based on the spiked 850 fmol T33V *Rhodobacter capsulatus* cytochrome c internal standard. Dermcidin was the most abundant protein in the sweat having 1.14 pmol/μg concentration. Besides dermcidin, the levels of clusterin and apolipoprotein D were shown to be considerably high as well. Dermicidin, clusterin, apolipoprotein D and prolactin-inducible protein (PIP) accounted for 46%, 17%, 15% and 8% of the secreted sweat proteins, respectively, while the serum albumin accounted only for 6%. To the best of our knowledge, this is the first study to present a broad overview of the sweat protein profile, providing comparative

analysis of individual protein levels. Based on our quantification, the five most abundant sweat proteins; dermcidin, clusterin, apolipoprotein D, PIP and serum albumin constituted 91% of secreted sweat proteins.

4.3.2 SRM-based quantification of dermcidin and prolactin-inducible protein

In order to validate our results obtained by label-free quantification, we have designed SRM experiments to analyze dermcidin and PIP levels in human sweat. After the *in silico* determination of tryptic sequences BLAST searches were performed to identify sequences specific for dermcidin and PIP and the peptide ENAGEDPGLAR for dermcidin and peptides YTACLCDDNPK and TVQIAAVVDVIR for PIP were used to design SRM transitions. SRM transitions were designed with the help of the Skyline software and optimized for all three peptides using the SIL counterparts.

SIL peptides were administrated right before the analysis and the relative amount of dermcidin and PIP in the sweat pool was determined in two parallel experiments Both the SRM-based targeted proteomics experiment and the label-free quantification support the evidence that dermcidin is the most characteristic sweat protein.

4.3.3 Role of highly abundant proteins in sweat

In order to gain information about the role of the identified sweat proteins the functions of these proteins have been revealed by GeneOnthology (GO) annotation. Based on the GO annotation, the number of identified sweat proteins involved in immune homeostasis and defense against microorganisms is high. To better understand the function of highly abundant sweat proteins, the scientific literature was reviewed. The detailed analysis of these highly abundant sweat proteins revealed their possible implication in protecting the skin from invading pathogens as part of a chemical barrier whose alterations may lead to an increased rate of skin infections.

5. Discussion

Body fluids are easily accessible, continuously produced sources for biomarker studies. Numerous studies have shown that changes of the protein composition of different body fluids may reflect both local and systemic pathological conditions. Considering that body fluids contain AMPs in relatively high quantity and the fact that the composition of AMP cocktail changes constantly to cope with environmental or internal challenges, AMPs are potential targets for biomarker studies. This study focuses on mass spectrometry method development and the examination of quantitative differences of AMPs in colonic epithelial cells, tears and sweat.

During method development for optimization of various parameters usually high amount of sample is required. In order to have adequate amount of biological material for consecutive optimization experiments a two-step procedure was applied: first for method development a cell culture model system was chosen followed by analysis of body fluids using the optimized method.

Colonic epithelial cells are well known models for the study of inflammatory bowel diseases. hBDs secreted by colonic epithelial cells are essential components of the chemical barrier of the gut and play an important role in the pathophysiology of inflammatory bowel diseases, making these cells an excellent sources to study hBD levels in various conditions. In the UniProt database six human β-defensin types are listed, but when our studies were carried out, protein data regarding β-defensins 5 and 6 had not been published yet. Our goal was to develop and optimize SRM-based targeted proteomics methods for the detection and relative quantification of intracellular and secreted hBD 1-4. We have identified unique, protein-specific sequences for all studied hBDs and we have designed SRM transitions. Using SIL peptides, we have examined the sensitivity and the dynamic range of the developed method. Based on data obtained by SRM analyzes the developed method was sensitive and specific for each defensin. Compared to classical antibody-based experiments such as ELISA or Western-blot, the SRM method had a lower sensitivity due to the lack of signal amplification system characteristic for the advanced antibody-based methods, but the dynamic range was wider in the studied matrices. The advantage of the SRM method relies on its multiplex feature that allows for a cost-effective simultaneous analysis of multiple analytes derived from the same sample, which is indeed crucial to biological and medical applications, where the amount of sample to be studied is limited.

With the developed SRM method, we analyzed the levels of hBD 1-4 in control and IL-1β-stimulated HT-29, SW-1116 and Caco2 cell lysates and cell culture supernatants. We could identify and quantify hBD1, hBD3 and hBD4 in all analyzed cells, while hBD2 was only detectable in Caco2 cells. Members of the hBD family can be induced upon pathogenic or inflammatory stimuli or can be constitutively expressed. It has been demonstrated that proinflammatory cytokines, such as IL-1β can enhance the expression of hBD2 and hBD3 therefore, they are considered as inducible AMPs. Data suggest that hBD1 is constitutively expressed and cannot be induced by inflammatory stimulus. Our results demonstrated that the level of the intracellular and secreted hBD1 was not elevated after IL-1β challenge therefore we confirmed the non-inducible feature of this AMP, while the inducible feature of hBD2 and hBD3 was confirmed in the studied cell lines proving that the quantity of these AMPs change upon inflammatory stimuli. In contrast to hBD1-3, hBD4 has been less explored; few publications reported its inducible feature and functions. Literature data suggest that the level of hBD4 can be induced by pathogens albeit not by the classical proinflammatory cytokines. In accordance with literature data, our results have shown that the expression of hBD4 was not induced by IL-1β and interestingly the level of hBD4 followed the same tendency observed in case of hBD1. García *et al.* have shown that hBD4 collaborates with hBD3, resulting in the enhancement of antimicrobial activity, yet this functional interplay is unlikely to require elevated hBD4 expression. Our data suggest that hBD1 and hBD4 exhibited similar expression profile upon inflammatory stimulus; however, we could not find information in the scientific literature about the relationship between hBD1 and hBD4.

Our data demonstrate the feasibility of the developed SRM-based method for the comparative analysis of hBD 1-4 levels in cell culture samples and provide further evidence of the inducible feature of hBD2 and hBD3 in inflammatory environment.

The tear fluid is an excellent candidate for biomarker studies, due to the ease of its collection by non-invasive means, and the information it provides on local ocular conditions such as dry-eye syndrome, diabetic retinopathy, in addition to systemic pathophysiological processes. Tear samples collected from healthy volunteers were analyzed with the developed SRM method in order to determine whether or not the method may also be utilized for the analysis of hBDs in biological fluids with very low volume. With the aid of the new SRM method, the relative quantification of hBD1, hBD2, hBD3 and hBD4 was successfully performed in tear samples. The

level of hBD2 was the lowest and the level of hBD3 was the highest in the tear samples, while the levels of hBD1 and hBD4 were almost the same. Levels of hBD1-4 were found to be lower in tear samples than in the supernatants of colonic epithelial cells, suggesting distinct tissue distribution of hBDs. SRM analyses of hBD2 in tears were also confirmed by ELISA and both methods provided comparable results. The data demonstrate that the newly developed SRM method is suitable for the relative quantification of hBDs in body fluids as well, this is indeed relevant to medical sciences. The SRM-based method may be used as an alternative to the antibody-based quantification methods, and can be especially useful in those cases where the determination of more than one hBD is needed and the sample volume does not permit the use of consecutive ELISA analyses.

Equipped with a well-performing test, our aim was to examine the amount of antimicrobial and immunomodulatory proteins in biological fluids originating from patients. For our studies, Alzheimer's disease; one of the most common age-related dementia affecting millions people worldwide, was chosen.

The etiology of AD is still unknown. Evidence suggests that abnormal production and accumulation of β-amyloid peptides, the microtubule-associated protein tau and α-synuclein, are involved in the pathogenesis of AD. The pathological hallmarks of AD are the appearance of senile plaques and neurofibrillary tangles in the brain, but based on literature data, AD affects the entire visual system as well. AD-related changes have been observed in the eye, the visual pathway, as well as the visual cortex. The presence of amyloid plaques was demonstrated in the retina and lens of patients with AD, and based on the results obtained from animal models; there is a correlation between amyloid depositions in the retina and brain. Changes in the retinal morphology and retinal vasculature were shown in the eyes of patients with AD, explaining the reduced visual performance observed in patients with AD. We hypothesized that changes in the retinal morphology and blood flow related to AD can alter the microenvironment of the eye and this alteration can be reflected at the level of secreted tear proteins as well.

Our aim was to analyze the tear proteins of patients with AD using proteomics approaches. The level of hBD 1-4 was examined in the tears of patients with AD and controls but the level of hBD 1-4 was under the detection limit in the tears of patients. In order to gain more insights into the eye-related protein changes of AD, a more detailed analysis was performed.

A significant increase in the flow rate and protein concentration along with a significant decrease in the amount of the ten examined tear proteins was observed in AD suggesting

extensive ocular alterations related to the disease, which is in accordance with previously presented literature data. The decreased levels of tear proteins in AD observed with the electrophoretic analysis of 5 samples were validated by SRM-based targeted proteomics analyses on 37 samples. Based on SRM analyses, the tear levels of lactotransferrin, lipocalin-1, lysozyme-C, extracellular glycoprotein lacritin, prolactin-inducible protein was significantly decreased in patients with AD compared to those in the control group. The only increase in tear proteins characteristic to AD has been observed in case of dermcidin which is produced by epithelial cells and is the main sweat AMP with a broad range of antimicrobial activity. The down-regulated proteins are produced by the lacrimal glands being involved in the first line defense of the eye. The altered composition of the chemical barrier may imply an increased risk of ocular infections, yet there is no reported increase in the occurrence of ocular infections in patients with AD. However, reduced corneal sensitivity and abnormal tear functions were reported in patients with AD and other neurodegenerative diseases compared to controls. While expression of major AMPs originating from lacrimal glands decreased, dermcidin with its broad antimicrobial spectrum appears to be a good candidate to limit bacterial growth, and hence, possible infections. In order to test which proteins with a significant difference between the AD and control groups can be used as potential biomarkers, ROC analyses were performed for each potential biomarker, and for different combinations of the proteins. While the best sensitivity (91%) was given by the combination of lysozyme-C and extracellular glycoprotein lacritin, the most balanced performance was achieved when lipocalin-1, dermcidin, lysozyme-C and extracellular glycoprotein lacritin was combined. In this case, the AUC of the ROC curve was 0.80, and the sensitivity and specificity were 81% and 77%, respectively, indicating a well-performing combination.

Taking advantage of the easy, non-invasive collection of the tears, an easily accessible bedside test may be developed. Those patients who have increased tear flow rate along with increased tear protein concentration, and are positive for the combination of extracellular glycoprotein lacritin, lipocalin-1, lysozyme-C and dermcidin biomarkers, can be subjected to further imaging, neuropsychological testing and cerebrospinal fluid analyses. Considering the small sample size analyzed in our pilot study, more analyses carried out on large populations are required in the future, in order to evaluate the applicability of the proposed biomarkers.

Besides tear and gut, the human skin also possesses an effective antimicrobial defense system, formed by a complex physical and chemical barrier that is in cooperation with other cellular components of the innate immune system and normal microbial flora of the skin. Little is known about the causes and consequences of qualitative and quantitative changes of the human sweat proteins, moreover, the composition of the sweat proteome itself has not been fully explored.

In our study, we have analyzed sweat pools of healthy volunteers collected during sauna bathing. Using the state of the art mass spectrometry techniques, we have identified 95 proteins in the pooled sweat samples, 20 of which had never been reported previously in human sweat. Data obtained by label-free quantification mass spectrometry revealed that 91% of the sweat proteome is made up of six highly abundant proteins. The most prominent protein was dermcidin, accounting for 46% of the sweat proteins, followed by clusterin (17%), apolipoprotein D (15%), PIP (8%) and serum albumin (6%). In order to validate these findings, SRM-based targeted mass spectrometry method was developed for the relative quantification of dermcidin and PIP. SRM analyses confirmed the results obtained by label-free quantification regarding dermcidin, which is the most characteristic protein of the human sweat.

The identified sweat proteins were subjected to GeneOnthology analysis, which revealed that many of them are involved in immune system processes and defense against bacteria and fungi. In order to better understand the function of the highly abundant dermcidin, clusterin, apolipoprotein D, PIP and serum albumin, the scientific literature was reviewed. The detailed analyses of these sweat proteins revealed their role as part of the chemical barrier of the skin.

Taking into account our findings and the information available in the scientific literature about the highly abundant sweat proteins, it seems evident that these proteins are essential to the formation of the chemical barrier of the skin. Dermcidin and PIP most probably play an important role in antimicrobial defense, while clusterin functions as a chaperone and as an inhibitor of matrix metalloproteinase activity. It seems obvious that the primary role of apolipoprotein D in the sweat is the inhibition of lipid peroxidation, while the presence of albumin might provide either a scavenger function; by binding different molecules preventing their enrichment on the skin surface, or an antioxidant effect provided by its N-terminal tetrapeptide.

The intense research effort aiming for the identification of new biomarkers from easily accessible and non-invasively collected samples, and the recent advances in analytical techniques

enable the study of not only body fluids with relatively high protein concentration but also facilitate the analysis of samples with low protein amount or volume, such as human sweat and tears. The emerging proteomics techniques and the continuous development of more and more sensitive instrumentation allow for the in-depth analysis of body fluids with low quantity thereby, new sources for potential biomarker are revealed. Studies have demonstrated that changes in the level of the AMPs can reflect either local or systemic disorders; therefore, AMPs are good candidates for biomarker studies.

During our work, we have successfully developed and validated an SRM-based targeted proteomic approach for the relative quantification of hBD1-4, lipocalin-1, lactotransferrin, extracellular glycoprotein lacritin, lysozyme-C, lipophilin A, Ig λ-chain C region, PIP, Zn-α-2-glycoprotein, galectin 3 binding protein and dermcidin, in complex biological samples, as an alternative approach to the classical antibody-based detection and quantification systems.

Tear fluid and the sweat are good candidates for biomarker studies due to their continuous availability; however, considering the secretion rate, the amount of the collected samples is usually low. Considering that the normal sweat proteome has not been discussed in details, we have examined the protein content of the human sweat by quantitative proteomics approaches, in order to provide insights into the constituents of the skin's chemical barrier. Our findings may serve as a starting point for further biomarker studies, and may aid in the diagnosis of skin-specific or systemic disorders.

With the help of the multiplex SRM-based targeted proteomics analyses, we have demonstrated the utility of tears in biomarker studies, and have identified a panel of potential biomarkers which may aid in the diagnosis of AD. If the suggested biomarkers are to be validated, tear analysis can be used in population screening by the general practitioners, and patients with positive test results can be sent to clinical centers for further examinations in order to establish the diagnosis of the disease. If the diagnosis and treatment is provided as early as possible, the quality of life can be improved for patients with AD and their caregivers (families, relatives etc.) decreasing the socio-economic burden.

Our data demonstrate the feasibility of the SRM-based approaches to utilize the non-invasively collectable body fluids in the biomarker studies, and also provide an accessible method to be utilized in standardized biomarker verification and validation workflows.

6. Summary

The thesis focuses on the examination of quantitative changes of AMPs in colonic epithelial cells, tears and the sweat using targeted proteomics approaches. SRM-based targeted proteomics approach was developed and optimized for the semi-quantitative analysis of β-defensin 1-4, lipocalin-1, lactotransferrin, extracellular glycoprotein lacritin, lysozyme-C, lipophilin A, Ig λ-chain C region, prolactin-inducible protein, Zn-α-2-glycoprotein, galectin 3 binding protein and dermcidin, in complex biological samples.

Our studies on cell cultures have proved the inducible feature of β-defensin 2 and 3 during inflammation, and we have demonstrated that β-defensin 1 and 4 cannot be induced by classical proinflammatory cytokines. We have analyzed the levels of β-defensins in tears, and based on the results, the designed SRM assay is suitable for the quantitative analysis of β-defensins in body fluids typically of low sample volume. Given the facts that the composition of the AMP cocktail changes in pathological conditions and alterations in tears of patients with neurodegenerative diseases were demonstrated, we have analyzed the levels of β-defensin 1-4 in tears from patients with Alzheimer's disease, but their levels were under the detection limit of the mass spectrometer. In order to gain more insight into the eye-related protein changes of AD, a more detailed analysis was performed. Significant increase in the tear flow rate and protein concentration was observed in patients with Alzheimer's disease, additionally; reduced level of the major tear proteins was also demonstrated. The decreased level of the major tear proteins and the increased level of dermcidin were demonstrated using SRM-based targeted proteomics analysis. In order to test which proteins with a significant difference can be used as potential biomarkers, ROC analysis was performed and the combination of lipocalin-1, dermcidin, lysozyme-C and lacritin was shown to be potential biomarker. Beside tears, sweat is also a part of the chemical barrier, and considering that the protein composition of the normal sweat has not been examined in details, we have analyzed the sweat proteome using mass spectrometry. We have identified 95 proteins, 20 of them were not reported as sweat proteins previously. The highly abundant sweat proteins dermcidin, prolactin-inducible protein, clusterin, apolipoprotein D and serum albumin are essential parts of the chemical barrier.

Our results demonstrate the relevance and utility of targeted proteomics approaches in biomarker studies from human body fluids.

7. List of publications prepared by the Kenézy Life Science Library

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List of publications related to the dissertation

1. **Kalló, G.**, Emri, M., Varga, Z., Ujhelyi, B., Tőzsér, J., Csutak, A., Csősz, É.: Changes in the chemical barrier composition of tears in Alzheimer's disease reveal potential tear diagnostic biomarkers.
   PLoS One. 11 (6), e0158000, 2016.
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2. Csősz, É., Emri, G., **Kalló, G.**, Tsaprailis, G., Tőzsér, J.: Highly abundant defense proteins in human sweat as revealed by targeted proteomics and label-free quantification mass spectrometry.
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   DOI: http://dx.doi.org/10.1111/jdv.13221
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3. **Kalló, G.**, Chatterjee, A., Tóth, M., Rajnavölgyi, É., Csutak, A., Tőzsér, J., Csősz, É.: Relative quantification of human [béta]-defensins by a proteomics approach based on selected reaction monitoring.
   Rapid Commun. Mass Spectrom. 29 (18), 1623-1631, 2015.
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List of other publications

4. Csősz, É., Deák, E., **Kalló, G.**, Csutak, A., Tőzsér, J.: Diabetic retinopathy: proteomic approaches to help the differential diagnosis and to understand the underlying molecular mechanisms.
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Oral presentations:

Éva Csősz, Péter Lábiscsák, **Gergő Kalló**, Miklós Emri, Viktória Bácsik, Ildikó Tar, Mária Fera, József Tőzsér, Ildikó Márton: *Proteomics examination of OSCC-specific salivary biomarkers in a Hungarian population using Luminex-based multiplex assay and SRM-based targeted proteomics method*, FEBS3+ Meeting, Protorož, 2015. *

Gergő Kalló, Adrienne Csutak, Miklós Emri, Zsófia Varga, József Tőzsér, Éva Csősz: *Analysis of several antimicrobial and immunmodulatory proteins in tears of patients with Alzheimer's disease*, 8th Molecular Cell and Immune Biology Winter Symposium, Debrecen, 2015.

Éva Csősz, Adrienne Csutak, **Gergő Kalló**, Miklós Emri, Péter Lábiscsák, Ildikó Márton, Eszter Deák, Bernadett Jakob, Zsuzsanna Pató, Goran Petrovszki, József Tőzsér: *Body fluid analysis with targeted proteomics*, 8th Molecular Cell and Immune Biology Winter Symposium, Debrecen, 2015. *

Éva Csősz, Adrienne Csutak, **Gergő Kalló**, Miklós Emri, Péter Lábiscsák, Ildikó Márton, József Tőzsér: *Selected reaction monitoring as a versatile tool for biomarker discovery*, Annual Meeting of the Hungarian Biochemical Society, Debrecen, 2014. *

Éva Csősz, **Gergő Kalló**, Adrienne Csutak, József Tőzsér: *Development of SRM-based targeted proteomics methods for study of tears antimicrobial and immunmodulatory peptides*, 32nd Informal Meeting on Mass Spectrometry, Balatonszárszó, 2014. *

Gergő Kalló, Arunima Chatterjee, Éva Rajnavölgyi, József Tőzsér, Éva Csősz: *Quantification of human alpha and beta defensins in colonic epithelial cells by targeted MRM-based proteomics*, 7th Molecular Cell and Immune Biology Winter Symposium, Galyatető, 2014.

Éva Csősz, **Gergő Kalló**, Eszter Deák, József Tőzsér: *Go targetted - the power of targeted proteomics analyses in the study of antimicrobial and immunmodulatory peptides*, 7th Molecular Cell and Immune Biology Winter Symposium, Galyatető, 2014. *

Kalló Gergő, Arunima Chatterjee, Rajnavölgyi Éva, Tőzsér József, Csősz Éva: *Defenzinek mennyiségi meghatározása célzott tömegspektrometriás módszer segítségével*, A szív és érbetegségek interdiszciplináris kutatása a DE OEC-ben TÁMOP konferencia, Debrecen, 2013.

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Éva Csősz, Adrienne Csutak, **Gergő Kalló**, József Tőzsér: *Blood, sweat and tears – body fluid proteomics with emphasis to antimicrobial and immunomodulatory peptide type biomarkers*, Hungarian Molecular Life Sciences 2013 conference, Siófok, 2013.

Poster Presentations:

Gergő Kalló, Miklós Emri, Zsófia Varga, József Tőzsér, Adrienne Csutak, Éva Csősz: *Alterations in the chemical barrier components in tears of patients with Alzheimer's disease*, FEBS3+ Meeting, Protorož, 2015.

G. Kalló, T. Székely, J.A. Mótyán, Sz. Benkő, J. Tőzsér and É. Csősz: *Relative quantification of the human NOD-like receptor family CARD domain containing 5 (NLRC5) protein by targeted mass spectrometry approach*, 33rd Informal Meeting on Mass Spectrometry, Szczyrk, 2015.

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* Co-author presentations

8. Keywords

Body fluids, antimicrobial and immunomodulatory peptides/proteins, biomarker, quantitative proteomics, method development, tears, sweat, Alzheimer's disease

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