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

by Gergő Kalló

Supervisor: Éva Csősz, PhD

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY
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Contents

Abbreviations

Aβ: β-amyloid
AD: Alzheimer's disease
ALB: Serum albumin
AMP: Antimicrobial and immunomodulatory peptide/protein
AUC: Area under the curve
AZGP1: Zn-α2 glycoprotein
BLAST: Basic Local Alignment Search Tool
C18: Octadecyl carbon chain
CE: Collision energy
CI: Confidence Interval
CST4: Cystatin S
CT: Computed tomography
DCD: Dermcidin
DP: Declustering potential
ELISA: Enzyme-linked immunosorbent assay
ESI: Electrospray ionization
FCS: Fetal calf serum
FDA: Food and Drug Administration
FDR: False discovery rate
GAL3BP: Galectin 3 binding protein
GO: GeneOnthology
hBD: Human β-defensin
HPLC: High performance liquid chromatography
HR-SRM: High resolution Selected Reaction Monitoring
IGHA1: Ig α-1 chain C region
IGKC: Ig κ chain C region
IGLC: Ig λ chain C region
IL-1β: Interleukin 1β
iTRAQ: Isobaric tag for relative and absolute quantitation
KRT1: Keratin, type II cytoskeletal 1
KRT10: Keratin, type I cytoskeletal 10
LACRT: Extracellular glycoprotein lacritin
LC: Liquid chromatography
LC-MS: Liquid chromatography-mass spectrometry
LC-MS/MS: Liquid chromatography-tandem mass spectrometry
LCN1: lipocalin-1
LOD: Limit of detection
LogFC: Log2 fold change
LOQ: Limit of quantification
LPNA: Lipophilin A
LPNC: Lipophilin C
LTF: Lactotransferrin
LYZ: Lysozyme-C
MALDI: Matrix-assisted laser desorption/ionization
MR: Magnetic resonance
MRI: Magnetic resonance imaging
MS: Mass spectrometer
PAGE: Polyacrylamide gel electrophoresis
PET: Positron emission tomography
PIP: Prolactin-inducible protein
PRM: Parallel Reaction Monitoring

PRR4: Proline-rich protein 4

PTP: Proteotypic peptide

PV: Predictive value

Q: Quadrupole

QTOF: Quadrupole-time of flight tandem mass spectrometer

QTRAP: Quadrupole-ion trap hybrid mass spectrometer

ROC: Receiver-operating characteristic

SDS: Sodium dodecyl sulfate

SE: Standard error

SELDI: Surface-enhanced laser desorption/ionization

SIL: Stable isotope-labeled

SILAC: Stable isotope labeling with amino acids in cell culture

SRM: Selected Reaction Monitoring

SWATH: Sequential window acquisition of all theoretical mass spectra

TMT: Tandem mass tag

TNF-α: Tumor necrosis factor α

TOF: Time of flight

UPLC: Ultra performance liquid chromatography

ZG16B: Zymogen granule protein 16 homolog B

1. Introduction

Identification of new biomarkers specific for various pathological conditions is an important field in medical sciences. Biomarkers can be used for the detection of diseases and can be suitable for monitoring the progression of pathological conditions. Despite intensive research efforts and advances in analytical methods, the number of biomarkers with Food and Drug Administration (FDA) approval remains low compared to the number of discovered potential biomarkers. The so called "classical" sources of biomarker studies are tissue samples obtained by biopsy or autopsy which can give information about the molecular changes of the environment where the pathological phenomenon occurs. Another widely used material is serum which can give information about the global changes caused by diseases. In spite of the fact that biological samples obtained by biopsy are perfect sources for biomarkers, these types of samples cannot be used in screens because of the highly invasive sample collection and the possibility for infection and other complications. While the serum collection is not highly invasive, it is still uncomfortable for many patients.

Body fluids have emerging potential in biomarker studies because of their continuous availability and the non- or minimally-invasive sample collection possibility. Changes in the protein composition of body fluids such as tears, saliva, sweat, etc. may provide information on both local and systemic conditions of medical relevance. Body fluids contain a chemical barrier of the human body as part of the innate immune system. The protein content of body fluids is different from each other, but every body fluid contains high number of antimicrobial and immunomodulatory proteins in order to create an effective barrier against pathogens.

The advancing "omics" technologies like genomics, proteomics and metabolomics provide new possibilities for biomarker studies form the continuously available body fluids. Proteomic-based approaches for biomarker investigation include classical two dimensional electrophoresis, mass spectrometry and antibody-based protein quantification techniques. In the recent decade, mass spectrometry techniques have become an important part of the discovery and verification phases of biomarker studies.

In this work I will present Selected Reaction Monitoring (SRM)-based targeted mass spectrometry method development for quantification of antimicrobial and immunomodulatory proteins from cell extract, cell culture supernatant, tear and sweat and the utility of some of the studied proteins as potential biomarkers for Alzheimer's disease (AD).

2. Theoretical background

2.1 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 [1]. 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 like tears, sweat, saliva, nasal secretion, urine and intestinal mucus (**Figure 1**).

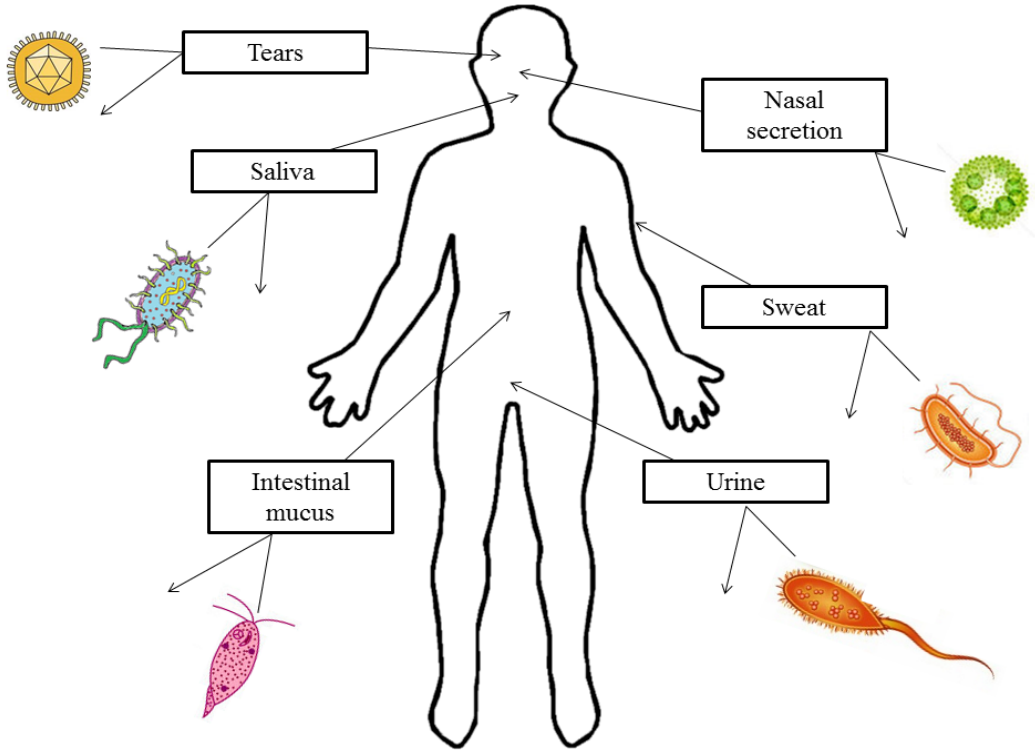


Figure 1: Chemical barriers of the human body.

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 a given body fluid [2].

Regarding the protein composition of the body fluids providing chemical barriers it was observed that the highly abundant proteins characteristic for each body fluid are part of the immune system and have protective roles. In case of tears, it was demonstrated that more than 90 % of the secreted proteins have a role in defense mechanisms [3]. 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. While AMPs are parts of the innate immune system, members of the adaptive immune system, such as secreted IgA, can be found in the chemical barriers as well. IgA has been shown to be capable of blocking pathogens from attaching to intestinal epithelial cells [4] while the different AMPs are responsible for pathogen killing, limiting their growth and modulate the immune reaction [2].

2.1.1 Prototypic AMPs in the chemical barriers

In the human body, the chemical barriers contain several prototypic AMPs, like defensins, dermcidin and LL-37 cathelicidin [2]. Prototypic AMPs adsorb onto the bacterial cell membrane by electrostatic attraction and insert into the membrane leading to the formation of channels and transmembrane pores or create extensive membrane ruptures [2].

Defensins are small cationic peptides produced by epithelial cells forming a very stable 3D structure because of their cysteine rich sequence [5]. The two subfamilies of human defensins (α- and β-defensins) show broad antimicrobial, antifungal and antiviral activities [2,6]. Human α-defensins 1–4 are expressed by neutrophils and have been shown to protect against mycobacterium and various viruses including herpes simplex virus 1 and 2 (HSV-1, HSV2), cytomegalovirus (CMV), and also influenza virus, however, their activity may depend on the lipid composition of the viral envelope [5,7]. α-defensins 1–3 also possess chemotactic activity for monocytes [5], α-defensins 5–6 are human enteric defensins constitutively expressed by Paneth cells and α-defensin 5 has been detected preferentially in the cervical mucosa and the oviduct [8]. Human β-defensins (hBDs) are coded on 11 genes but not all transcripts have been identified so far [9]. They can be considered as potential AMPs in epithelial cells providing a protective barrier against Gram-negative bacteria and Candida species [2]. Another important function of β-defensins is their chemotactic activity towards various immune cells [10]. While some defensins, like hBD1 show constitutive expression pattern, other members of this family found to be induced upon pathogenic

or inflammatory stimuli [11]. In addition to their antimicrobial and immune modulatory effects, some defensins have been identified as cancer-associated molecules with anti-tumor effects [12].

LL-37 cathelicidin is an α-helix type AMP of the body fluids mainly produced by epithelial cells and, similar to defensins, it exerts various antimicrobial activity against different type of pathogens [2]. In addition, cathelicidin has an important role in re-epithelialization during wound healing [13].

Dermcidin is the main skin AMP which is also present in tears and exerts broad spectrum antimicrobial activity [2,14]. Dermcidin is constitutively secreted by eccrine sweat glands and epithelial cells, and it's secretion cannot be further induced by skin injury or inflammation [15]. By post-secretory proteolytic processing dermcidin is cleaved to several truncated dermcidin-derived peptides which differ in length and net charge. Reduced levels of dermcidin-derived peptides were detected in sweat of patients with atopic dermatitis in association with an impaired cutaneous antimicrobial defense [16], while the increased expression of dermcidin has been demonstrated in lung, prostate and pancreatic cancer cells [17,18].

2.1.2 Highly abundant body fluid proteins are constituents of the chemical barriers

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 various defense functions. It has been shown that lactotransferrin found in all body fluids is an active agent against microbes and parasites, and has been implicated in protection against cancer [2]. Because of its iron sequestering activity, lactotransferrin has an important role in the prevention of bacterial colonization. Lysozyme-C is an ubiquitous hydrolytic enzyme with muraminidase activity required for the peptidoglycan degradation of the bacterial cell wall [2]. Besides its antibacterial activity, lysozyme-C has various functions including activity against several fungus species [19] and protection against HIV infection [20]. Lipocalins are a family of lipid binding proteins with protease inhibitor activity and by sequestrating iron, they can limit bacterial growth [21,22]. Extracellular glycoprotein lacritin is a secreted glycoprotein found in tears and saliva. The protein has various functions including effect on lacrimal gland secretion [23], epithelial cell proliferation [24] and corneal wound healing [25]. Additionally, the C-terminal fragment of extracellular glycoprotein lacritin has bactericidal activity [26]. Prolactin-inducible

protein is an aspartyl protease enzyme [27] which can be found in various body fluids as part of the host defense system. Beside the protease activity, prolactin-inducible protein can modulate immune reaction by binding to immunoglobulin G and Zn-α-2-glycoprotein [28,29] and its elevated expression have been associated with breast cancer [27,30].

2.2 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" [31]. 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 disease [32]. Consequently, these biomarkers are at the same time targets for drug design and therapy [33]. 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 [34]. 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 [32]. In spite of the availability of advanced imaging methods, there is a high demand for laboratory diagnostic assays using biological samples or body fluids collected by non- or minimally invasive 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 diseased 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. The composition of the AMP cocktail changes constantly, implying both qualitative and quantitative changes of the cocktail components,

in order to adapt to various conditions. 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 characteristic symptoms [35]. 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.

2.3 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 normal tear production rate is approximately 2 µl/min [36] and its typical protein concentration is 5-7 µg/µl [37]. The functions of the tear film are the lubrication of the eye, delivery of nutrients and maintaining of the refractivity of the cornea [38]. Besides these roles, tear creates an effective chemical barrier on the surface of the eye *via* secreted AMPs which provide protection against pathogens [39]. Currently, more than 1500 protein have been identified in tears by state of the art proteomics techniques [3,40,41]. Major tear proteins; such as lysozyme-C, prolactin-inducible protein, lactotransferrin, lacritin etc., are involved in the defense against pathogens [3], and their relatively high abundance makes these proteins the major tear proteins. 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 [42].

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. Based on localization, tear can reflect pathological conditions related to the anterior segment of the eye but it can provide information about the retinal or vitreal status as well [43]. More broadly, the analysis of tears can serve information related to systemic changes [44]. Analysis of tear protein profile can provide useful information on understanding the molecular mechanisms of ocular diseases; such as dry eye syndrome [45–48], blepharitis [49], diabetic retinopathy [50], keratoconus [51,52] etc., and can also reflect systemic diseases such as multiple sclerosis [53].

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 [54] therefore, tear is a potential source for biomarkers specific to neurological disorders as well.

2.4 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 [55]. Sweat is composed of more than 99% water, making it a very dilute body fluid [56]. Similarly to tear fluid, the abundant sweat proteins are part of the innate immune system; sweat protein content provides an effective defense against pathogens, and is involved in tissue regeneration after injury [2]. Some AMPs were shown to be expressed constitutively (e.g. dermcidin and RNase7) while others were found to be inducible upon pathogenic stimuli (e.g. LL-37 cathelicidin, hBD2 and hBD3) [2,55,57]. Besides these prototypic AMPs, the presence of lysozyme-C and lactotransferrin in the sweat has been reported as well [2,58].

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 [16,59,60]. It was proposed that reduced expression of proteins involved in the immune homeostasis can contribute the development of ectodermal dysplasia; therefore, these proteins can be considered as potential biomarkers for this disease. The sweat proteome has been analyzed in order to identify biomarkers for systemic diseases as well. The analysis of sweat from patients with schizophrenia has revealed 17 differentially expressed proteins which can be used as potential biomarkers to help establish the diagnosis of schizophrenia [61].

2.5 Saliva, the defense system of the oral cavity

Saliva is a complex mixture of organic and inorganic compounds secreted from major and minor salivary glands and from the gingival crevice [62]. Similar to sweat, saliva is a very dilute body fluid with 0.7-2.4 μg/μl protein concentration [63,64]. Saliva contains more than 2000 proteins, the most abundant ones are α-amylase, mucins, cystatins, proline rich peptides, and serum albumin [65]. Similarly to tears and sweat, the abundant salivary proteins are part of the immune system due to their antimicrobial activity, antioxidant function and protective role from microbial proteases. The continuous availability and non-invasive collection of saliva make it an excellent

source for biomarker studies. The protein composition of saliva has been analyzed by several workgroups, indicating its relevance to medical applications in oral diseases like oral squamous cell carcinoma [66] or bisphosphonate-related osteonecrosis of the jaw [67] and also in systemic diseases such as breast cancer [68] and autism spectrum disorder [69].

2.6 Nasal secretion as part of the host defense system

The nasal secretion has a protective role in the airways; nasal discharge contains a variety of AMPs such as lactotransferrin, lysozyme-C and several types of defensins [70]. Various studies revealed more than 450 proteins in the nasal mucus and many of them are related to the host defense system [71–73]. Compared to the other body fluids only few publications report nasal mucus as a source of biomarkers, by quantitative proteomics six biomarkers have been identified in patients with chronic rhinosinusitis [71]. With the help of state of the art proteomics methods, the easy-to-collect nasal discharge is predicted to be a very valuable source for further biomarker studies.

2.7 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 [74,75]. The physical barrier function is provided by the intestinal epithelial cell junctions and their well-regulated transport systems, while the chemical barrier function is based on the secreted AMPs by the epithelial cells and activated immune cells [76]. In the secreted AMP cocktail, the ubiquitous lysozyme-C and lactotransferrin are present [77,78], but the major constituents of this chemical barrier are prototypic AMPs, such as defensins and LL-37 cathelicidin [79]. In the small intestine hBD1 was described as a constitutively expressed AMP, as its expression was not regulated by proinflammatory cytokines [74]. The level of hBD4 can be upregulated as a result of bacterial infection but not by classical proinflammatory signals such as IL-1β [80]. On the contrary, hBD2 and hBD3 were described as inducible AMPs, which respond to stimulation caused by proinflammatory cytokines like IL-1β [74,81]. Inflammatory bowel diseases such as ulcerative colitis and Chron's disease are associated with increased levels of inducible β-defensins [82] leading to epithelial cell proliferation, immune cell migration and enhanced

production of proinflammatory cytokines [83]. Elevated level of hBD2 was observed in patients with irritable bowel disease and in ulcerative colitis indicating the activated AMP secretion in these diseases. Increased levels of hBD2 was also detected in stool samples of patients with inflamed digestive tract [84], whereas increased hBD3 levels were described in the colonic mucosa of patients with ulcerative colitis [85].

2.8 Urine as part of the chemical barrier

Urine is formed in the kidneys as a result of ultrafiltration of the plasma to eliminate waste products from the body. The protein concentration in urine under physiological conditions is very low, nearly 1000 times less compared to other body fluids such as plasma [86]. Urine contains more than 1500 proteins; unsurprisingly, many of these proteins are part of the defense system of the body [87]. Compared to the other body fluids, the AMP composition of urine is different; its distinct anatomical and physiological properties imply the presence of a different chemical barrier such as the urine specific uromodulin [88]. Because of the continuous availability, urine has become one of the most studied body fluids in clinical proteomics; changes in its proteome can reflect pathological conditions of the urogenital tract like polycystic kidney disease [89], urolithiasis [90], diabetic nephropathy [91] and bladder [92,93], prostate [94] and renal cancer [95]. Besides urogenital and kidney dysfunctions, urinary proteomics has a great potential in biomarker studies of coronary artery atherosclerosis [96], breast cancer [97] and sepsis [98].

2.9 Mass spectrometry

Mass spectrometry is an analytical technique which allows the separation of gas phase ions based on their mass/charge (m/z) ratio. The essential components of the mass spectrometers are the ion source, mass analyzer and a detector [99]. As the mass spectrometer can only detect gas phase ions, therefore, prior to any separation in the mass analyzer, molecules must be ionized and converted into gas phase using different ionization techniques. The two most commonly used ionization techniques in proteomics are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) [99]. Mass analyzers separate the ions originated from the ion source based on their m/z ratio. The basis of separation is specific for each type of analyzer; separation can be done based on the flight time (time of flight-TOF analyzers), stability of the ion trajectories (quadrupole, ion trap), electrostatic attraction (Orbitrap) etc. [100,101].

Tandem mass spectrometers containing two or more mass analyzers are available allowing specific fragmentation and further separation of fragment ions. The fragment ion spectra or MS/MS spectra contain information about the composition and the structure of the molecules allowing more sophisticated analyses like protein identification, detection of post-translational modifications and protein quantification [99,101,102]. Protein identification is critical in proteomics, however in many cases, the qualitative information is not enough to answer biological questions. Quantitative proteomics is mainly based on different mass spectrometry techniques allowing relative and absolute quantification of proteins. Absolute quantification determines the exact concentration of proteins, while in relative quantifications, only the relative change in protein quantity can be examined [103]. In most cases, relative quantification is sufficient to answer biological questions but sometimes the exact amount of the analytes is necessary.

The two major fields of protein quantification with mass spectrometry are the shotgun and targeted methods. In case of shotgun analysis there is no need for prior information regarding the protein to be studied, while in case of targeted analyses, information about the proteins of interest is indispensable. Protein quantification with shotgun techniques can rely on special labeling techniques or can be done without any kind of labeling. In proteomics, the two major labeling techniques are the metabolic labeling and chemical labeling [101]. Metabolic labeling refers to the incorporation of stable isotope-labeled amino acids into proteins in the sample of interest. Typically, this is accomplished by culturing cells in a medium in which one or two essential or conditionally essential amino acids have been replaced with their stable isotope-labeled forms

[104]. Stable isotope labeling by amino acids in cell culture (SILAC) is a widely used technique of quantitative proteomics, used to compare the protein amounts in two or more, typically cell culture samples, and hence is not suitable for body fluid analysis [105]. Chemical labeling is based on the attachment of synthetic chemical group(s) to the proteins. There are two types of labels: isotopic and isobaric. Isobaric labeling is widely used and relies on the attachment of the isobaric label to free amines of the N-terminus and lysine side chains of proteins or enzyme-digested peptides. The most widely used chemical labeling methods in quantitative proteomics are the Tandem mass tags (TMT) [106], isobaric Tags for Relative and Absolute Quantitation (iTRAQ) [107] and dimethyl labeling [108]. Besides the different labeling techniques available for shotgun proteomics, the easiest and nowadays one of the most widely used method is the label-free quantification technique.

The targeted proteomics techniques such as Selected Reaction Monitoring (SRM) [103], Parallel Reaction Monitoring (PRM) [109], High-resolution SRM (HR-SRM) [110] and hybrid acquisition methods such as sequential window acquisition of all theoretical mass spectra (SWATH) [111] make possible the relative or absolute quantification of proteins of interest.

2.9.1 Targeted proteomics using Selected Reaction Monitoring

Targeted proteomics has a high impact on medical sciences; in 2012 it was chosen as the *Method of the year* in Nature methods [112]. SRM is one of the most commonly used targeted mass spectrometry approach in proteomics and in biomedical analyses [103], 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 (**Figure 2**) [113,114]. 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 [114].

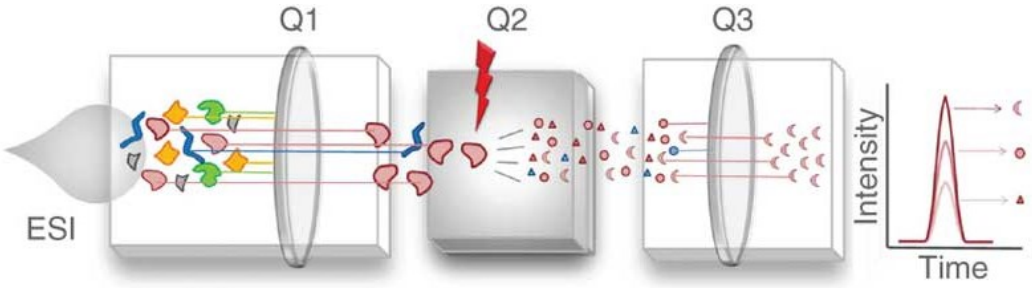


Figure 2: SRM scan mode of triple quadrupole-containing mass spectrometers. *ESI: electrospray ion source, Q1: first quadrupole, Q2: second quadrupole (collision cell), Q3: third quadrupole. [103]*

When functioning in a semi-quantitative setup, SRM experiments allow the relative quantification of specified analytes [115] but absolute quantification is also possible [116]. 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 [117]. 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 [114]. These SIL peptides are introduced into the samples in order to serve as internal controls during the experiments [114]. For relative quantification, lyophilized non-purified SIL peptides can be used to decrease the cost of the analyses, but in case of absolute quantification, non-frozen purified SIL peptides should be applied, making absolute quantification more expensive than relative quantification [117].

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 (ELISA, Western-blot) utilizing signal multiplication systems (secondary antibodies, conjugated enzyme's activity), although the dynamic range of SRM is usually wider than of antibody-based methods [118,119]. The sensitivity of SRM can be increased by the administration of specific enrichment techniques [120,121]. 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 prior information about the proteins to be studied and the limitation of this approach could be the restricted availability of unique, protein-specific peptides.

2.9.1.1 SRM assay design

The selection of the target proteins is the first step of SRM assay design. SRM has multiplex feature, many proteins can be analyzed in one SRM acquisition. From the selected proteins, characteristic peptides sequences have to be selected. These unique, protein-specific peptides with good MS response are called proteotypic peptides (PTPs) [114]. The peptides can be selected experimentally or by *in silico* methods. In case of experimental peptide selection, the proteins of interest are digested and MS/MS spectra of the peptides are registered resulting in MS/MS spectral library. Peptides with good MS response have to be selected for further analysis. In order to ensure the specificity of the SRM assay the peptides are subjected for sequence analysis using BLAST algorithm and only the unique protein-specific peptides are used for transition design [113]. The availability of MS/MS library allows the manual selection of SRM transitions for each PTP, the most intensive fragment ions have to be selected from SRM assay. Then the SIL counterparts of the PTPs can be ordered and the designed SRM assay can be validated. Using *in silico* methods, the sequences of the proteins of interest are retrieved from databases and *in silico* protease digestion has to be carried out. Then the peptides are subjected to sequence analysis with BLAST in order to find the unique, protein-specific peptides. From these unique peptides, peptides with good MS response can be selected by prediction using different algorithms [113]. Those peptides which are specific to the protein and a good MS response is predicted are considered as PTPs. Transition design for PTPs retrieved by *in silico* method can be done with different softwares; in most cases Skyline is used as a freely available, continuously updated software tool (www. brendanx-uw1.gs.washington.edu). The SIL counterparts of the selected PTPs have to be ordered and the SRM spectra of each transition have to be recorded. Using the recorded data, the best transitions can be selected. Optimization of the MS parameters is sometimes necessary; optimization of the collision energy (CE), the declustering potential (DP), the gas flow in the ion source etc. can result in better peak shape and higher intensity signal [114].

2.9.2 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 [99,122,123]. 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 obtained MS/MS spectra and these numbers are used for relative quantification of proteins. The high scan rate and accuracy provided by the high resolution mass spectrometers, preferably an Orbitrap, make relative quantification accessible, even in case of large sample number [124]. 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 [123]. Although no expensive labeling is needed, label-free quantification is less accurate than chemical or metabolic labeling; more technical replicates are thus needed which increase instrument usage time, making the label free quantification costs comparable to those of labeling techniques [125]. One of the drawbacks of label-free quantification is the need for a very stable and carefully controlled system to avoid potential errors caused by inappropriate chromatographic separation.

3. 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

4. Materials and methods

4.1 Sample collection

4.1.1 Cell cultures

For cell culture studies, we used human HT-29, SW-1116 and Caco2 colonic epithelial cell lines derived from various types of human colorectal adenocarcinomas. The tested cell lines were adherent and resembled the features of the human colon. HT-29 and SW-1116 cells were grown at 5% CO_2 and 37 °C in RPMI medium supplemented with 10% fetal calf serum (FCS), 100 μg/ml streptomycin, 100 U/mL penicillin and 2 mM L-glutamine. Caco2 cells were grown in 20% FCS containing RPMI medium supplemented with 100 U/mL penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1% non-essential amino acids at 5% CO_2 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 Aldrich) was added to collect the cells. The detached cells were washed twice with phosphate buffered saline (PBS) in order to avoid contamination by the cell culture supernatant, and lysis buffer (50 mM Tris-HCl, pH 8.3, 1 mM EDTA, 17 mM β-mercaptoethanol, 0.5% TritonX-100) was added to collect the cell lysates. The protein concentration of the samples was determined with the Bradford method [126].

4.1.2 Tear collection

In total, 26 donors were recruited, 14 patients with AD and 9 age- and sex-matched controls as well as 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, although none of them were placed under guardianship. All of the donors were patients of the University of Debrecen, Faculty of Medicine and the assessment of AD was done by a psychiatrist specialist. The diagnosis of AD was based on the NINCDS-ADRDA [127] and the DSM-IV-TR [128] criteria. Besides psychiatric and neurological assessment, patients underwent basic laboratory

testing (blood chemistry analysis, complete blood cell count, hepatic and renal function test, vitamin B12 and folate determination, thyroid function test) and CT or MR imaging of the brain in order to rule out other causes of dementia. Patients with a history of sudden onset, early extrapyramidal signs, early behavioral changes or focal neurological features were excluded.

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 [129]. Only controls without any signs of cognitive impairment were included in the study. Patients with systemic inflammation, autoimmune disorders or ophthalmological disorders were excluded.

In case of the AD group, the women: men ratio was approximately 1:2 (5 women and 9 men) and the mean age was 77 years (65-90 years), whereas, in the control group, the women: men ratio was approximately 1:1 (5 women, 4 men) and the mean age was 72 years (62-89 years). In case of the three healthy volunteers, the women: men ratio was 2:1 and the mean age was 28 years (26-33 years). 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 out without topical anesthesia using standard capillary collection technique [130]. Tears were collected using 60 µl glass capillary tubes (Hirschmann-Laborgeräte). The collection time was limited to a maximum of 5 min. The capillary tube rested in the lateral tear meniscus and care was taken to minimize contact with the bulbar conjunctiva or the lid margin. Tears were expelled from the capillaries into Eppendorf tubes and the samples were centrifuged at 4^{o}C with 1500 rpm. 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.

4.1.3 Sweat sample collection

Ten male and 10 female healthy young adults (between 22–35 years of age) 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 to 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 at 4^{o}C with 1500 rpm in order to get rid of skin cells and cellular debris and the clear sweat was pooled, concentrated in SpeedVac protein concentrator (Thermo Fischer Scientific) and re-dissolved in 220 µl 0.1 M ammonium bicarbonate buffer (pH 7.8). The protein concentration of the pooled sweat was determined by Bradford method [126], 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 [131] was performed according to the manufacturer's instructions (Pierce) to determine the total protein content.

4.2 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.

4.3 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.

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

The bands with significantly different intensities between AD and control samples were excised (approximately 6 mm x 2 mm, depending on the size of the band) and in-gel digested with trypsin. First a reduction was performed using 20 mM final concentration of dithiothreitol for one hour at 56^{o}C followed by alkylation with 55 mM final concentration of iodoacetamide for 45 minutes. The overnight trypsin digestion was carried out using 10 µg stabilized MS grade TPCK-treated bovine trypsin (ABSciex) at 37^{o}C. For reduction, alkylation and digestion the amount of the used chemicals was set in that way to cover completely the gel pieces (approx. 50 µl). The digested peptides were extracted and lyophilized and 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). 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. Collision induced dissociation (CID) spectra were acquired in Enhanced Product Ion mode at 4000 amu/s scan rate and rolling collision energy was applied with the maximum of 90 eV. Information Dependent Acquisition method was utilized; after the first mass scan (mass range 400–1700 amu), 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 (mass range 100–1900 amu).

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.

4.5 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 LC-MS^E [132], 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. The mass spectrometer was operated in V-mode of analysis with a typical resolving power of at least 10 000 full width half-maximum using positive nanoelectrospray ion mode. The reference sprayer was sampled with a frequency of 60 sec. Accurate mass LC-MS data were collected in an alternating low energy and elevated energy mode of acquisition [133]. The spectral acquisition time in each mode was 1.5 sec with a 0.1 sec interscan delay. In low energy MS mode, data were collected at constant collision energy of 4 eV in 50-2000 m/z mass range while in elevated energy MS mode, the collision energy was ramped from 15 to 35 eV during each cycle with one complete cycle of low and elevated energy data acquired every 3.2 sec. High energy MS ions were collected between 100 to 1500 m/z.

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 at 60000 resolution in the Orbitrap analyzer scanning 400–1600 m/z, followed by CID spectra of the 14 most intense ions were acquired in the linear ion trap. Dynamic exclusion was set to place any selected m/z on an exclusion list for 45 sec after a single MS/MS.

4.5.1 Data 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 as determined by a reversed database search. 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% false discovery rate (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 with the embedded ion accounting algorithm of the software (IdentityE) and searching a human database downloaded from UniPot to which the primary sequence of the T33V cytochrome c from Rhodobacter 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.

4.6 Sample preparation for SRM analysis

Proteins were denatured with 6 M final concentration urea (Bio-Rad) and were reduced with 10 mM final concentration dithiothreitol (Bio-Rad). The reduced samples were alkylated with 20 mM final concentration iodoacetamide (Bio-Rad) and diluted with 25 mM ammonium bicarbonate (Sigma Aldrich) to decrease the urea concentration to 1 M. Trypsin digestion was performed at 37 °C overnight by adding MS-grade modified trypsin (ABSciex) 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.

4.7 Development of SRM-based targeted proteomics method

Amino acid sequences of hBD1–4 (Accession numbers: P60022, O15263, P81534, Q8WTQ1), lipocalin-1 (P31025), lactotransferrin (P02788), extracellular glycoprotein lacritin (Q9GZZ8), lysozyme-C (P61626), lipophilin A (P60201), Ig λ-chain C region (P0CG04), prolactin-inducible protein (P12273), Zn-α-2-glycoprotein (P25311), galectin 3 binding protein (Q08380) and dermcidin (P81605) were retrieved from the UniProt database (www.uniprot.org) and were subjected to *in silico* trypsin digestion by the PeptideCutter software (http://web.expasy.org/peptide_cutter). Tryptic fragments with 100% cleavage probability were selected for BLASTp analysis (http://blast.ncbi.nlm.nih.gov) 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 (www. brendanx-uw1.gs.washington.edu). SIL crude peptides were obtained from JPT Peptide Technologies GmbH, Germany, while purified hBD2 SIL peptide was obtained from PepscanPresto, The Netherlands. The quality of the synthetic peptides was determined by MALDI-TOF analyzes. The SRM spectra of all singly charged "y" ions were recorded on a 4000 QTRAP mass spectrometer (ABSciex) 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.

4.8 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 IL1β-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 analyses. These configurations provide nearly 30 data points/chromatographic peak for hBDs, 16 data points/ chromatographic peak for tear proteins and approximately 20 data points/ chromatographic peak for the analysis of sweat proteins.

SRM data were analyzed by using the Skyline software, the AUC values for light, endogenous peptides, for heavy, synthetic SIL peptides and the light:heavy peptide 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 heavy 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.

4.9 Statistical analysis

Statistical analysis was performed with the SigmaPlot 12.0 software (Systat Software Inc.) 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 [134] 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 [135]. 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 [136]. 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 [137] were also carried out by the pROC software [138], the accuracy and the 95% Confidence Interval (CI) values were calculated for each protein alone and for their combinations.

5. Results

In this work proteomics approaches for the analysis of AMPs in body fluids were developed. Prototypic AMPs usually have low concentration in body fluids; therefore, cell cultures were used as a source of hBDs in order to develop a targeted proteomics approach. This method was further transferred to the analysis of body fluids.

5.1 SRM analysis of hBDs in cell culture and tear samples

Identification of hBDs can be performed by gene expression profiling, ELISA, Western blot or SELDI-TOF mass spectrometry, but to our best knowledge the targeted proteomics approach has not been used so far for the detection of hBDs. Although the classical antibody-based methods are widely used for the quantitative or semi-quantitative analyses of proteins and peptides, 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 method. 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.

When the study was performed protein data were available in the literature only for hBD1-4 based on the UniProt database thus, we have developed SRM-based targeted proteomics assay for these forms. hBD5 and hBD6 also exist in the UniProt database but when our study was performed no protein data have been published for these AMPs. 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 PTPs. BLASTp searches demonstrated the presence of peptides specific for the four studied hBDs, 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 [139]. All transitions were designed using Skyline and were analyzed on

4000QTRAP 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. The optimized SRM parameters for each hBD-specific peptide are listed in **Table 1**.

Protein	Peptide	Q1 (m/z)	Q3 (m/z)	Ion	Dwell Time (msec)	DP (eV)	CE (eV)
hBD1	IQGT***C***YR	449.220	599.261	y5	20	61.8	19.7
			542.239	y4	20	61.8	19.7
			441.191	y3	20	61.8	19.7
			338.182	y2	20	61.8	19.7
	IQGT***C***YR*	454.220	666.290	y5	20	61.8	19.7
			609.269	y4	20	61.8	19.7
			508.221	y3	20	61.8	19.7
			348.191	y2	20	61.8	19.7
hBD2	GIGDPVT***C***LK	551.284	1002.528	y9	20	69.2	25.5
			889.445	y8	20	69.2	25.5
			832.423	y7	20	69.2	25.5
			717.396	y6	20	69.2	25.5
			620.344	y5	20	69.2	25.5
			521.275	y4	20	69.2	25.5
			420.227	y3	20	69.2	25.5
			260.196	y2	20	69.2	25.5
	GIGDPVT***C***LK*	555.291	1010.543	y9	20	69.2	25.5
			897.459	y8	20	69.2	25.5
			840.437	y7	20	69.2	25.5
			725.411	y6	20	69.2	25.5
			628.357	y5	20	69.2	25.5
			529.289	y4	20	69.2	25.5
			428.242	y3	20	69.2	25.5
			268.211	y2	20	69.2	25.5
			283.186	y2	20	65.0	22.2

Table 1: Optimized SRM transition parameters of hBD1, hBD2, hBD3 and hBD4 specific peptides. *Sequences of hBD1-4 were retrieved from the UniProt database and after in silico trypsin digestion, the peptides were analyzed by BLASTp and the unique, protein-specific peptides were identified. SRM transitions were designed for the peptides using the Skyline software and the optimal parameters were determined with the help of the software. "C" in bold italics represent carbamidomethylated cysteines. DP: declustering potential, CE: collision energy. * indicates the stable isotope-labeled amino acids.*

Protein	Peptide	Q1 (m/z)	Q3 (m/z)	Ion	Dwell Time (msec)	DP (eV)	CE (eV)
hBD3	GIINTLQK	464.776	829.514	y7	20	65.0	22.2
			716.430	y6	20	65.0	22.2
			603.346	y5	20	65.0	22.2
			489.303	y4	20	65.0	22.2
			388.255	y3	20	65.0	22.2
			275.171	y2	20	65.0	22.2
	GIINTLQK*	468.784	837.258	y7	20	65.0	22.2
			724.444	y6	20	65.0	22.2
			611.360	y5	20	65.0	22.2
			497.713	y4	20	65.0	22.2
			396.269	y3	20	65.0	22.2
			283.186	y2	20	65.0	22.2
hBD4	I***C***GYGTAR	449.216	624.310	y6	20	63.9	21.3
			567.288	y5	20	63.9	21.3
			404.225	y4	20	63.9	21.3
			347.203	y3	20	63.9	21.3
			246.156	y2	20	63.9	21.3
	I***C***GYGTAR*	454.220	634.318	y6	20	63.9	21.3
			577.297	y5	20	63.9	21.3
			414.234	y4	20	63.9	21.3
			357.212	y3	20	63.9	21.3
			256.164	y2	20	63.9	21.3

Table 1*: Continued*

5.1.1 Linear dynamic range of hBD peptides

Determination of the range where the AUC is proportional to the amount of peptide introduced to the mass spectrometer 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 method 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 different peptides (**Table 2**). The LOQ was typically 250-fold dilution in cell culture supernatant and 500-fold dilution in tears.

Protein	Peptide	Linear dynamic range in cell culture supernatant	Linear dynamic range in tears
hBD1	IQGTCYR	250x-5x	500x-25x
hBD2	GIGDPVTCLK	250x-10x	500x-10x
hBD3	GIINTLQK	250x-5x	250x-25x
hBD4	ICGYGTAR	250x-5x	500x-25x

Table 2: Linear dynamic range of the hBD SIL peptides in cell culture supernatant and tear matrix. *Increasing amounts of SIL hBD peptides were added to cell culture and tear samples in the range of 1000 fold- to 5-fold dilution. After SRM analysis the logAUC values were plotted against the dilution and logistic regression was applied to determine the linear dynamic range. The values represent the dilution factors.*

Based on these results, the designed SRM method exhibits a broad dynamic range and allows the analyses within a wide range of changes, characteristic to biological systems.

5.1.2 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. Analyses were carried out using 10 μg digested samples.

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 (**Figure 3a**). Considering the secreted hBD1 levels, significant decrease was observed in stimulated HT-29 cells (**Figure 3d**).

Changes in the light:heavy peptide ratio showed significantly elevated hBD3 level in all stimulated cells compared to their unstimulated counterparts (**Figure 3b**), and these changes could be detected at the level of secreted hBD3 as well (**Figure 3e**).

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 (**Figure 3c and 3f**).

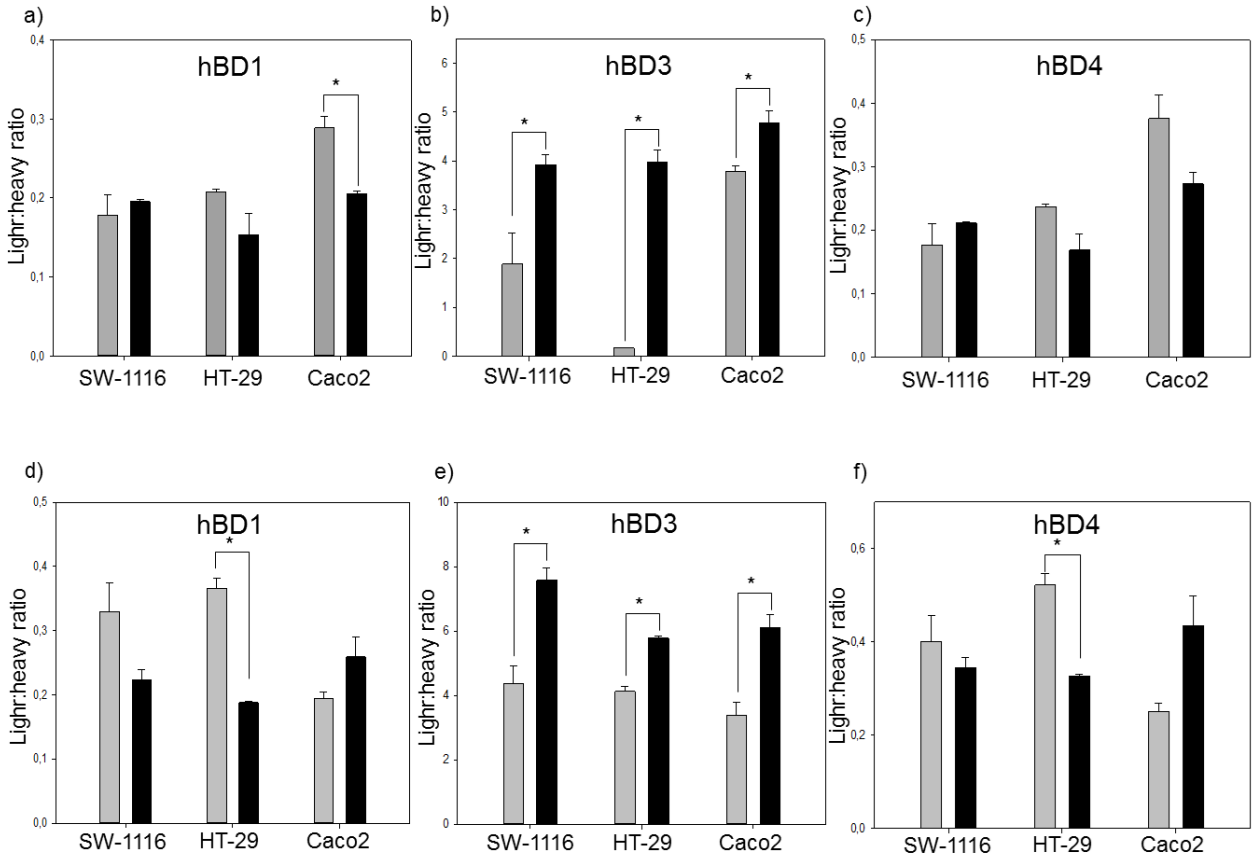


Figure 3: Determination of hBD1, hBD3 and hBD4 levels in colonic epithelial cells. *5×10^5/ml cells were stimulated using 10 ng/ml IL-1β. Cell lysates and cell culture supernatant samples were collected from control and IL-1β treated cell cultures and subjected to in-solution trypsin digestion. 10 µg digested samples were analyzed with SRM method and the light:heavy ratios were determined. (a) Determination of β-defensin 1 IQGTCYR levels in cell lysates. (b) Determination of β-defensin 3 GIINTLQK levels in cell lysates. (c) Determination of β-defensin 4 ICGYGTAR levels in cell lysates. (d) Determination of secreted β-defensin 1 IQGTCYR levels in cell culture supernatants. (e) Determination of secreted β-defensin 3 GIINTLQK levels in cell culture supernatants. (f) Determination of secreted β-defensin 4 ICGYGTAR levels in cell culture supernatants. The bars represent the mean values of three independent experiments with the standard error of the means; the grey bars show the values for the control group and the black bars correspond to the IL-1β-treated group; * indicates $p < 0.05$.*

In our experiments, we could detect hBD2 only in CaCo2 cells; 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 (**Figure 4**).

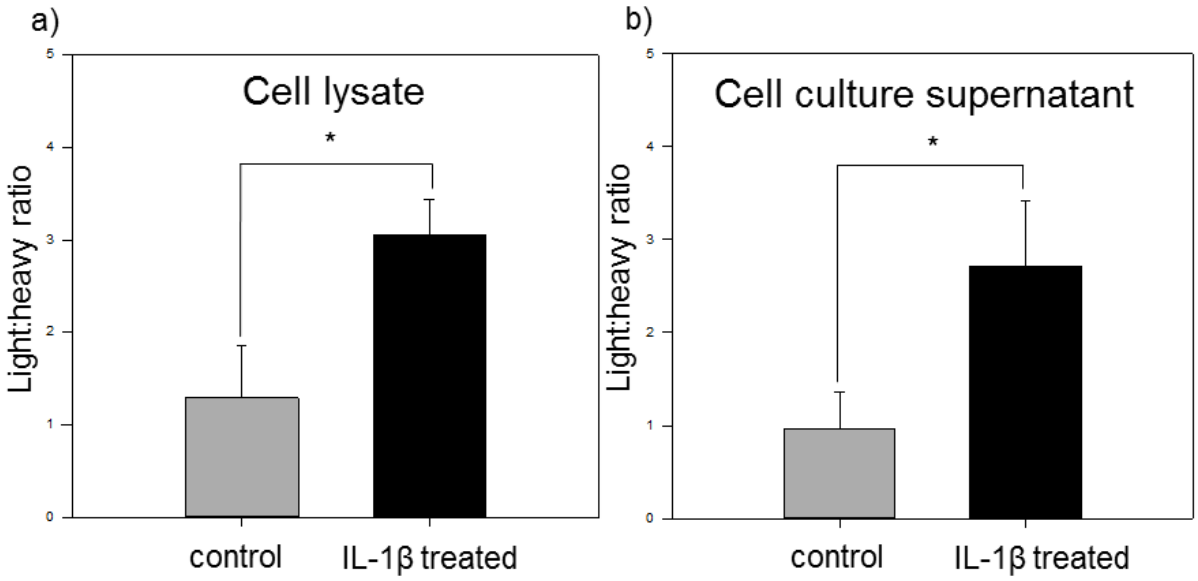


Figure 4: Analysis of hBD2 levels in Caco2 cell culture*. 5×10^5/ml CaCo2 cells were stimulated using 10 ng/ml IL-1β. Cell lysates and cell culture supernatant samples were collected from control and IL-1β treated cell cultures and subjected to in-solution trypsin digestion. 10 µg digested samples were analyzed with SRM method and the light:heavy ratios were determined. (a) Determination of β-defensin 2 GIGDPVTCLK levels in Caco2 cell lysates. (b) Determination of secreted β-defensin 2 GIGDPVTCLK levels in Caco2 cell culture supernatants. The bars represent the mean values of three independent experiments with the standard error of the means; the grey bars show the values for the control group and the black bars correspond to the IL-1β-treated group; * indicates p <0.05.*

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 [6].

5.1.3 Analysis of hBD levels in tears

In order to assess whether the newly developed SRM method could also be utilized for the analysis of tears, we collected tear samples from healthy volunteers and analyzed them. The quantification of hBD1, hBD2, hBD3 and hBD4 peptides was successfully performed by the SRM method (**Figure 5**). The level of hBD2 was the lowest and the level of hBD3 was the highest, while hBD1 and hBD4 had almost the same levels in tears.

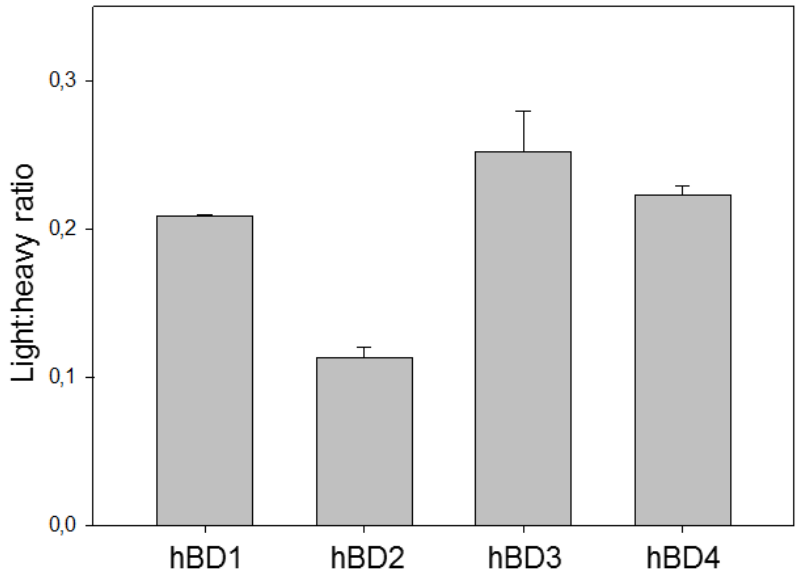


Figure 5: Examination of hBD1–4 levels in tears*. Tear samples were collected from three healthy individuals and in-solution trypsin digestion was carried out. 5 µg digested tear protein was subjected to SRM analyses and the light:heavy ratios were determined. The bars represent the mean values of three independent experiments with the standard error of means.*

The hBD levels in tear samples were found to be lower than those detected in the supernatants of colonic epithelial cells. While hBDs were secreted by epithelial cells, the fact that the levels of hBD1-4 secreted by colonic epithelial cells were different from those which were secreted by corneal epithelial cells indicating body fluid-dependent expression of hBDs [9].

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 (**Figure 6**) and data obtained by SRM analyzes showed the same tendency as the data obtained by ELISA analyses.

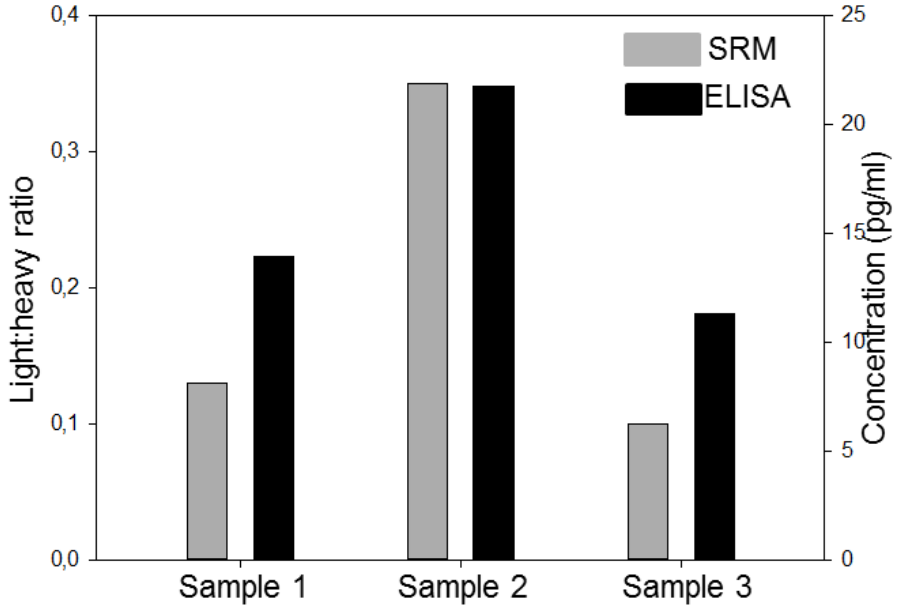


Figure 6: Comparison of tear hBD2 amounts determined by SRM and ELISA. *Tear samples collected from healthy individuals were divided into two parts. In one part of each of the samples the level of hBD2 was determined by ELISA, while the other part of the samples was subjected to in-solution trypsin digestion, analyzed by SRM method and the light:heavy ratio was determined. Grey bars represent the results of SRM analyses, while the black bars represent the results obtained by ELISA.*

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.

Based on these results, the developed SRM approach can be evaluated as an alternative quantification method to the antibody-based techniques, and could be especially useful in those cases when the determination of more than one hBD is needed and the sample volume does not permit the use of consecutive ELISA measurements.

5.2 Analysis of tear proteins of patients with AD

Several studies have shown that the pathology of AD can affect the eye. A variety of visual problems have been reported in patients with AD including loss of visual acuity, color vision etc. [140], and the presence of amyloid plaques in lens and retina of patients with AD was also demonstrated [141,142]. Considering that neurodegenerative disorders like Parkinson's disease can change the tear protein profile [54] and amyloid deposits have been found in the eye of patients with AD [143], we hypothesized that these changes in the microenvironment of the eye may lead to changes in the protein composition of tears, therefore, we wanted to investigate if the tear fluid could serve as a possible source for AD-specific biomarkers. The level of hBD1-4 was analyzed in tears of patients with AD using the newly developed SRM-based assay, but it was under the detection limit of the mass spectrometer. As far as hBD1-4 could not be detected in tears from patients with AD, 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.

5.2.1 Tear protein profile changes in AD

The amount of proteins in tears can be diagnostically relevant information. 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 (**Figure 7**).

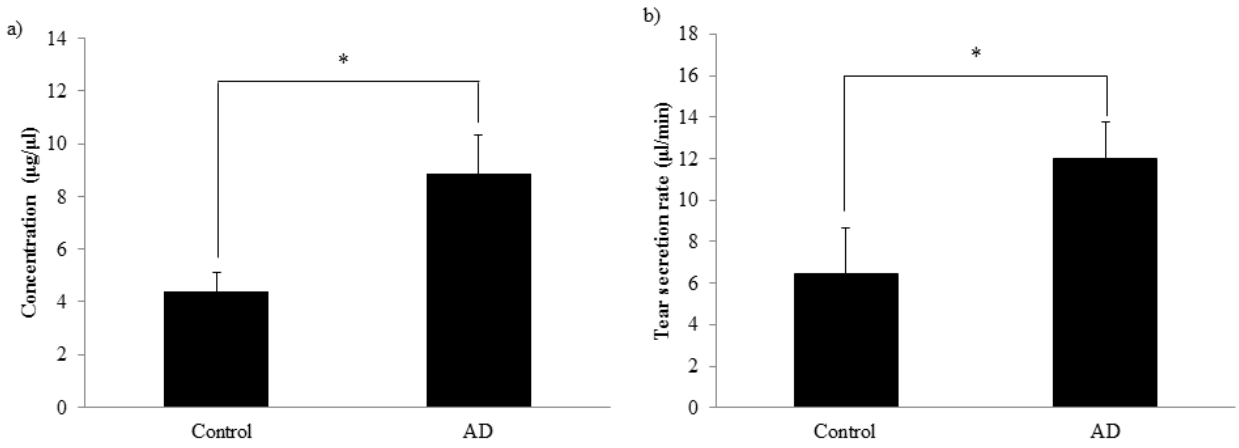
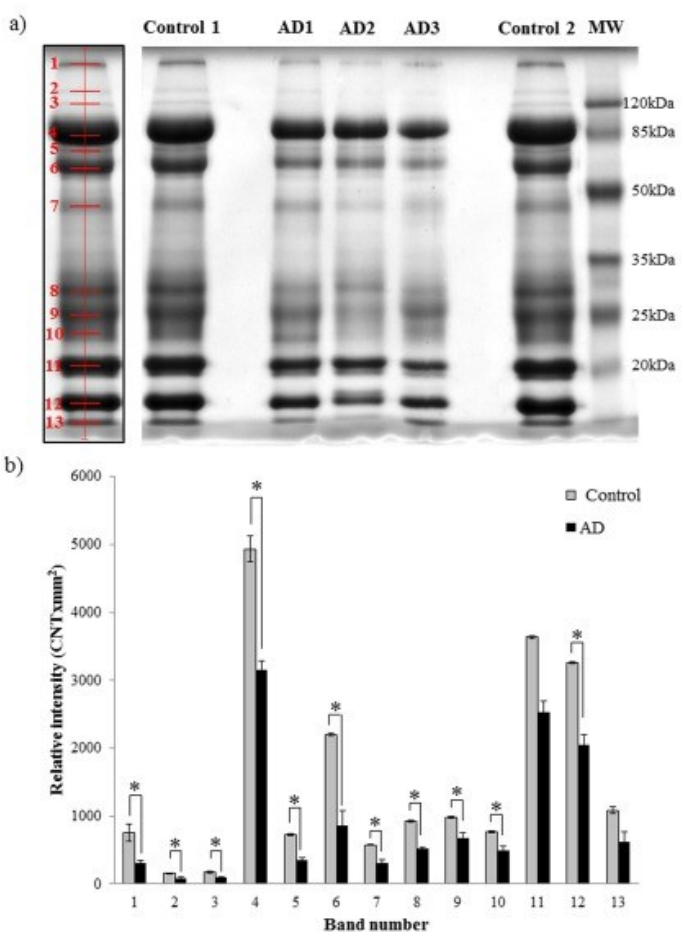


Figure 7*: *Protein concentration and flow rate of the collected tears. *Tears were collected from 37 eyes of 26 donors (14 patients with AD and 9 controls). The amount of the collected tear was divided by the collection time in minutes to calculate the flow rate. The total protein concentration of the tear samples was determined by Bradford method. The bars represent the mean values with the standard error of mean of a) total protein concentration measured by Bradford method, b) tear secretion rate. * indicates $p < 0.05$.*

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 (**Figure 8a**) indicated significant decrease in band intensity in AD samples in case of 11 bands (**Figure 8b**). The bands were excised and digested with trypsin followed by LC-MS/MS based protein identification. We have accepted as identified only those proteins, where at least two peptides with ≥95% confidence could be detected (**Figure 8c**).



c)

Band nr.	Identified proteins (symbol)	UniProt ID
1	-	-
2	LTF	P02788
3	LTF	P02788
4	LTF	P02788
5	LTF	P02788
	ALB	P02768
	IGHA1	P01876
6	LTF	P02788
	IGHA1	P01876
7	AZGP1	P25311
	KRT1	P04264
	LACRT	Q9GZZ8
8	KRT1	P04264
	KRT10	P13645
9	IGLC*	P0CG05/P0CG06
10	IGLC*	P0CG05/P0CG06
	IGKC	P01834
	ZG16B	Q96DA0
	PRR4	Q16378
11	LCN1	P31025
	PIP	P12273
12	LYZ	P61626
	LPNC	O75556
	PIP	P12273
	LPNA	O95968
	CST4	P01036
13	LYZ	P61626

Figure 8: Changes in the tear proteome in AD. *a) 20 µg of tear proteins originating from 3 patients with AD and 2 controls were subjected to SDS-PAGE on a 7cm 10% gel. The proteins were stained using Coomassie PageBlue (Fermentas). The left panel shows the representative band distribution detected with QuantityOne (BioRad) software. b) Densitometric analysis of gel bands using QuantityOne (BioRad) software. The bars show the mean values with the standard error of mean, grey bars indicate the control group while the black bars indicate the AD group. * indicates p <0.05. c) List of the identified proteins with the corresponding UniProt accession numbers. The gel bands were excised and in-gel trypsin digestion was performed. Protein identification was carried out by LC-MS/MS analyses. MS/MS spectra were searched against the UniProt database using ProteinPilot search engine. The criteria for protein identification was the detection of minimum two peptides w*ith ≥95% confidence *# indicates the presence of Ig λ but the identified peptides could not distinguish between Ig λ-2 chain C region and Ig λ-3 chain C region.*

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 [3]. 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 [16].

5.2.2 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 according to the image analysis (Figure 8), 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 [3,59] 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 [144,145] were included into the panel of analyzed tear proteins. The amino acid sequences of the selected proteins were utilized from the UniProt database and *in silico* digestion was revealed the potential tryptic peptides. The peptides were subjected to BLASTp analysis in order to identify the PTPs. 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. **Table 3** contains the SRM parameters of the studied peptide sequences.

Protein	Peptide	Q1 (m/z)	Q3 (m/z)	Ion	Dwell time (msec)	DP (eV)	CE (eV)
LCN1	VTMLISGR	438.752	676.381	y6	20	63.1	20.7
			432.257	y4	20	63.1	20.7
	VTMLISGR*	443.756	686.389	y6	20	63.1	20.7
			442.265	y4	20	63.1	20.7
	HVAYIIR	436.261	564.350	y4	20	62.9	20.6
			175.119	y1	20	62.9	20.6
	HVAYIIR*	441.265	574.359	y4	20	62.9	20.6
			185.127	y1	20	62.9	20.6
	GLSTESILIPR	593.346	498.340	y4	20	74.4	29.6
			272.172	y2	20	74.4	29.6
	GLSTESILIPR*	598.350	508.348	y4	20	74.4	29.6
			282.180	y2	20	74.4	29.6
LTF	***C***GLVPVLAENYK	681.858	737.383	y6	20	80.8	34.6
			553.262	y4	20	80.8	34.6
	CGLVPVLAENYK*	685.865	745.397	y6	20	80.8	34.6
			632.313	y5	20	80.8	34.6
	CLAENAGDVAFVK	697.343	920.484	y9	20	82.0	35.5
			735.404	y7	20	82.0	35.5
	CLAENAGDVAFVK*	701.350	928.498	y9	20	82.0	35.5
			743.418	y7	20	82.0	35.5
LACRT	QELNPLK	421.243	584.377	y5	20	61.8	19.7
			357.250	y3	20	61.8	19.7
	QELNPLK*	425.250	592.391	y5	20	61.8	19.7
			365.264	y3	20	61.8	19.7
	SILLTEQALAK	593.856	873.504	y8	20	74.4	29.6
			760.420	y7	20	74.4	29.6
	SILLTEQALAK*	597.863	881.518	y8	20	74.4	29.6
			768.434	y7	20	74.4	29.6
LPNA	QIFGDYK	435.721	629.293	y5	20	62.9	20.6
			310.176	y2	20	62.9	20.6
	QIFGDYK*	439.729	637.307	y5	20	62.9	20.6
			318.190	y2	20	62.9	20.6

Table 3: SRM transition parameters of examined tear proteins. *Sequences were retrieved from the UniProt database and after in silico trypsin digestion, the peptides were analyzed by BLASTp to identify the protein specific peptides. SRM transitions were designed for the unique peptides using the Skyline software and the optimal parameters for each peptide were determined with the help of the software. "C" in bold italics represents carbamidomethylated cysteines, while * indicates the stable isotope-labeled amino acids. DP: de-clustering potential, CE: collision energy.*

Protein	Peptide	Q1 (m/z)	Q3 (m/z)	Ion	Dwell time (msec)	DP (eV)	CE (eV)
IGLC	SYS***C***QVTHEGSTVEK	856.383	987.474	y9	20	93.5	44.5
			276.155	y2	20	93.5	44.5
	SYS***C***QVTHEGSTVEK*	860.390	995.488	y9	20	93.5	44.5
			284.170	y2	20	93.5	44.5
PIP	YTA***C***L***C***DDNPK	678.782	1092.445	y9	20	80.6	34.4
			748.293	y6	20	80.6	34.4
	YTA***C***L***C***DDNPK*	682.789	1100.459	y9	20	80.6	34.4
			756.307	y6	20	80.6	34.4
	TVQIAAVVDVIR	642.388	842.509	y8	20	77.9	32.4
			771.472	y7	20	77.9	32.4
	TVQIAAVVDVIR*	647.392	852.518	y8	20	77.9	32.4
			781.481	y7	20	77.9	32.4
AZGP1	DYIEFNK	464.724	650.351	y5	20	65.0	22.2
			537.267	y4	20	65.0	22.2
	DYIEFNK*	468.731	658.365	y5	20	65.0	22.2
			545.281	y4	20	65.0	22.2
	IDVHWTR	463.746	599.305	y4	20	64.9	22.2
			462.246	y3	20	64.9	22.2
	IDVHWTR*	468.750	609.313	y4	20	64.9	22.2
			472.254	y3	20	64.9	22.2
GAL3BP	LADGGATNQGR	530.263	575.290	y5	20	69.8	26.0
			175.119	y1	20	69.8	26.0
	LADGGATNQGR*	535.267	585.298	y5	20	69.8	26.0
			185.127	y1	20	69.8	26.0
	LASAYGAR	404.719	624.310	y6	20	60.6	18.8
			175.119	y1	20	60.6	18.8
	LASAYGAR*	409.723	634.318	y6	20	60.6	18.8
			185.127	y1	20	60.6	18.8
DCD	ENAGEDPGLAR	564.768	628.341	y6	20	72.3	27.9
			513.314	y5	20	72.3	27.9
	ENAGEDPGLAR*	569.772	638.350	y6	20	72.3	27.9
			523.323	y5	20	72.3	27.9
LYZ	GISLANWM***C***LAK	682.347	993.464	y8	20	80.9	34.6
			922.427	y7	20	80.9	34.6
	GISLANWM***C***LAK*	686.354	1001.479	y8	20	80.9	34.6
			930.442	y7	20	80.9	34.6
	WESGYNTR	506.728	826.369	y7	20	68.1	24.6
			697.326	y6	20	68.1	24.6
	WESGYNTR*	511.732	836.377	y7	20	68.1	24.6
			707.335	y6	20	68.1	24.6

Table 3*: Continued*

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 (**Table 4**).

Protein	Peptide	Linear dynamic range (dilution fator)
LCN1	VTMLISGR*	10000x-250x
	HVAYIIR*	10000x-5x
	GLSTESILIPR*	500x-25x
LTF	CGLVPVLAENYK*	500x-50x
	CLAENAGDVAFVK*	500x-50x
LACRT	QELNPLK*	10000x-500x
	SILLTEQALAK*	100x-10x
LYZ	GISLANWMCLAK*	5000x-25x
	WESGYNTR*	500x-100x
LPNA	QIFGDYK*	10000x-250x
IGLC	SYSCQVTHEGSTVEK*	10000x-100x
PIP	YTACLCDDNPK*	10000x-250x
	TVQIAAVVDVIR*	10000x-10x
AZGP1	DYIEFNK*	10000x-250x
	IDVHWTR*	10000x-100x
GAL3BP	LADGGATNQGR*	10000x-250x
	LASAYGAR*	10000x-500x
DCD	ENAGEDPGLAR*	10000x-250x

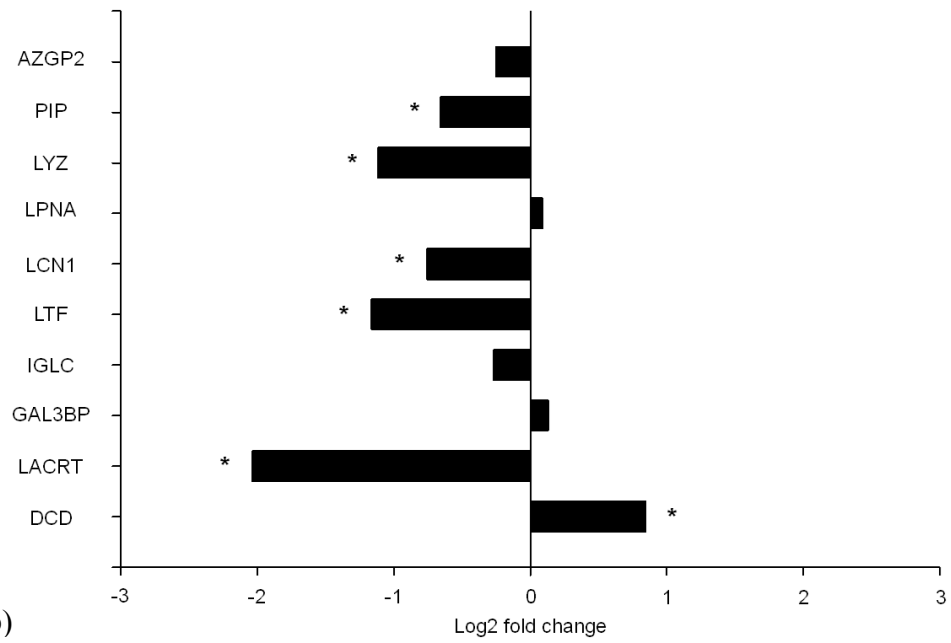
Table 4: Linear dynamic range of the SIL peptides in tear. *Increasing amounts of SIL peptides were added to tear samples in the range of 10000 fold- to 5-fold dilution. After SRM analysis the logAUC values were plotted against the dilution and logistic regression was applied to determine the linear dynamic range. The values represent the dilution range where the amount of peptide introduced into the mass spectrometer is proportional with the signal intensity. * indicates the stable isotope-labeled amino acids.*

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.

5.2.3 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 immunmodulatory proteins was analyzed using the developed SRM methods in 37 tear samples of 14 patients having AD and 9 controls (**Figure 9**).

a)



b)

Protein	Log2FC	SE	Tvalue	Adjusted p value
AZGP1	-0.25	0.17	-1.43	0.26
PIP	-0.66	0.14	-4.75	<0.0001
LYZ	-1.11	0.15	-7.29	<0.0001
LPNA	0.09	0.23	0.39	0.76
LCN1	-0.76	0.13	-5.79	<0.0001
LTF	-1.16	0.17	-6.66	<0.0001
IGLC	-0.27	0.23	-1.19	0.35
GAL3BP	0.13	0.19	0.68	0.59
LACRT	-2.04	0.18	-11.53	<0.0001
DCD	0.85	0.23	3.64	0.0006

Figure 9: Quantitative analysis of tear proteins by SRM. *5 µg of digested tear protein was analyzed by SRM and the results were examined using Skyline software and MSstats. Mixed effect variance analysis and p value correction was applied. a) The log2 fold change in case of the studied proteins in tears of patients with AD compared to controls. * indicates p≤0.05. b) Result of the mixed effect variance analysis. The log2 fold change (logFC), standard error (SE), Student T values (Tvalue) and the FDR corrected p-values are shown.*

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.

5.3.4 Identification of potential 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. A receiver operator characteristic (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 sensitivity, specificity, positive and negative predictive values and the area under the ROC curve (AUC) were calculated in each setting (**Table 5**). An AUC value close to 1.0 indicates a well-performing biomarker; whereas values close to 0.5 suggest a biomarker performing no better than random.

Proteins	Sensitivity (%)	Specificity (%)	PV+ (%)	PV- (%)	AUC	Accuracy	95% CI
LCN1	53.1	79.7	22.7	43.3	0.68	0.72	0.56-0.80
LTF	78.1	51.6	17.5	55.4	0.67	0.73	0.55-0.78
LYZ	90.6	50.0	8.6	52.5	0.68	0.67	0.57-0.79
PIP	71.9	56.2	20.0	54.9	0.60	0.67	0.48-0.72
LACRT	90.6	57.8	7.5	48.2	0.71	0.65	0.59-0.82
DCD	62.5	76.6	19.7	42.9	0.70	0.70	0.59-0.82
LCN1+DCD	53.1	89.1	20.8	29.2	0.74	0.76	0.63-0.85
LYZ+LACRT	90.6	59.4	7.3	47.3	0.72	0.64	0.61-0.83
LCN1+DCD+ LACRT	90.6	65.6	6.7	43.1	0.80	0.69	0.71-0.89
LCN1+DCD+LTF	53.1	89.1	20.8	29.2	0.74	0.76	0.62-0.84
LCN1+DCD+LYZ	59.4	82.8	19.7	36.7	0.75	0.76	0.64-0.86
LCN1+LTF+LYZ	87.5	50.0	11.1	53.3	0.72	0.71	0.61-0.83
LYZ+LACRT+ DCD	90.6	65.6	6.7	43.1	0.79	0.71	0.69-0.89
LYZ+LACRT+ LCN1	87.5	59.4	9.5	48.1	0.72	0.67	0.62-0.83
PIP+LACRT+DCD	93.8	60.9	4.9	45.5	0.79	0.68	0.69-0.97
DCD+LCN1+LTF+ PIP	62.5	76.6	19.7	42.9	0.74	0.72	0.64-0.84
LCN1+DCD+LYZ+ LACRT	81.2	76.6	10.9	36.6	0.80	0.72	0.71-0.89

Table 5: ROC analysis of possible tear biomarkers for AD. *The sensitivity was plotted against 1-specificity and the AUC of the ROC curve was determined. The positive predictive value (PV+), negative predictive value (PV-), accuracy and the 95% CI values calculated by the pROC software are indicated in case of each biomarker or biomarker combination.*

In all cases, the AUC value was above 0.6 and when the different combinations of two or more proteins were analyzed, the AUC value was above 0.7 indicating an additive effect of the different proteins in improving the test performance. 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.

5.3 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.

5.3.1 Sweat protein identification and label-free quantification

A sweat pool collected during sauna bathing from healthy adult volunteers was examined 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. The criteria of the identification was the registration of minimum two peptides identified at 0.1% FDR at peptide level and 1% FDR at the protein level. 20 of the identified proteins were considered novel; they were not reported in sweat previously (**Table 6**).

Nr.	Identified Proteins	Accession Number	Nr.	Identified Proteins	Accession Number
1	**78 kDa glucose-regulated protein**	**P11021**	23	Cluster of Keratin, type I cytoskeletal 10	P13645
2	Actin, cytoplasmic 1	P60709	24	Cluster of Keratin, type II cytoskeletal 1	P04264
3	Alpha-2-macroglobulin-like protein 1	A8K2U0	**25**	**Cluster of Serpin B3**	**P29508**
4	Alpha-enolase	P06733	**26**	**Complement C4 beta chain**	**B0UZ83**
5	Apolipoprotein D	P05090	27	Cystatin-M	Q15828
6	Arginase-1	P05089	28	Deoxyribonuclease-2-alpha	O00115
7	**Beta-glucuronidase**	**P08236**	29	Desmoglein-1	Q02413
8	Beta-hexosaminidase subunit beta	P07686	**30**	**Dipeptidyl peptidase 1**	**P53634**
9	Bleomycin hydrolase	Q13867	**31**	**Dipeptidyl peptidase 2**	**Q9UHL4**
10	Calpain-1 catalytic subunit	P07384	32	Epididymal secretory protein E1	P61916
11	Carboxypeptidase A4	B7Z5J4	33	Eukaryotic translation initiation factor 6	B7ZBH1
12	Caspase-14	P31944	34	Fatty acid-binding protein, epidermal	Q01469
13	Catalase	P04040	35	Filaggrin	P20930
14	Cathepsin B	P07858	36	Filaggrin-2	Q5D862
15	Cathepsin D	P07339	37	Fructose-bisphosphate aldolase	J3KPS3
16	Cathepsin L2	O60911	38	Galectin-3-binding protein	Q08380
17	CD59 glycoprotein	P13987	39	Gamma-glutamylcyclotransferase	O75223
18	**Cluster of Ig alpha-1 chain C region**	**P01876**	40	Ganglioside GM2 activator	P17900
19	Cluster of Ig gamma-1 chain C region	P01857	41	Gelsolin	P06396
20	Cluster of Ig lambda-2 chain C regions	P0CG05	42	Glutaminyl-peptide cyclotransferase (Fragment)	B5MCZ9
21	Cluster of Isoform 2 of Clusterin	P10909-2	43	Glyceraldehyde-3-phosphate dehydrogenase	P04406
22	Cluster of Isoform 2 of Dermcidin	P81605-2	**44**	**Ig kappa chain C region**	**P01834**

Table 6: Sweat proteins identified by LC-MS/MS*. 4.2 µg pooled sweat protein samples were first reduced and alkylated, followed by trypsin digestion overnight at 37°C. 240 ng digests were analyzed and protein identification was carried out using Thermo Proteome Discoverer software. List of the identified proteins with the corresponding UniProt accession numbers is shown in alphabetical order. Proteins in bold are novel proteins not identified in human sweat so far.*

Nr.	Identified Proteins	Accession Number	Nr.	Identified Proteins	Accession Number
45	Interleukin-36 gamma	Q9NZH8	**71**	**Phospholipase B-like 1**	**Q6P4A8**
46	Isoform 11 of Titin	Q8WZ42-11	72	Polymeric immunoglobulin receptor	P01833
47	Isoform 1B of Desmocollin-1	Q08554-2	**73**	**Probable carboxypeptidase PM20D1**	**Q6GTS8**
48	Isoform 2 of Annexin A2	P07355-2	74	Pro-cathepsin H	P09668
49	Isoform 2 of Carboxypeptidase E	P16870-2	75	Prolactin-inducible protein	P12273
50	**Isoform 2 of Inositol monophosphatase 2**	**O14732-2**	76	Protein DJ-1	K7ELW0
51	**Isoform 2 of NAD(P)H-hydrate epimerase**	**Q8NCW5-2**	**77**	**Protein S100-A8**	**P05109**
52	Isoform 2 of Sulfhydryl oxidase 1	O00391-2	78	Protein S100-A9	P06702
53	Isoform 2 of Triosephosphate isomerase	P60174-1	79	Protein-glutamine gamma-glutamyltransferase E	Q08188
54	Kallikrein-7	P49862	80	Purine nucleoside phosphorylase	P00491
55	Keratin, type I cytoskeletal 9	P35527	**81**	**Putative gamma-glutamyltranspeptidase 3**	**A6NGU5**
56	Keratin, type II cytoskeletal 6A	P02538	82	Ribonuclease T2	D6REQ6
57	Ly6/PLAUR domain-containing protein 3	O9527	83	Secretoglobin family 1D member 2	O95969
58	**Lysosomal alpha-glucosidase**	**P10253**	**84**	**Serotransferrin**	**P02787**
59	Lysosomal protective protein	P10619	**85**	**Serpin B8**	**P50452**
60	Lysosomal Pro-X carboxypeptidase	B7Z7Q6	86	SERPINB12 protein	Q96P63
61	Lysosome-associated membrane glycoprotein 1	P11279	87	Serum albumin	P02768
62	Lysozyme C	P61626	88	Sialidase-2	Q9Y3R4
63	Malate dehydrogenase, mitochondrial	P40926	89	Suprabasin	Q6UWP8
64	Malic enzyme	P48163	90	Thioredoxin	P10599
65	**Melanotransferrin**	**P08582**	91	Thrombospondin-1	P07996
66	**Neutrophil defensin 1**	**P59665**	**92**	**Thymidine phosphorylase (Fragment)**	**C9JGI3**
67	N-sulphoglucosamine sulphohydrolase	P51688	93	Tissue alpha-L-fucosidase	P04066
68	Paragranulin (Fragment)	K7EKL3	94	Transaldolase	P37837
69	Peroxiredoxin-1	Q06830	95	Zinc-alpha-2-glycoprotein	P25311
70	Phosphatidylethanolamine-binding protein 1	P30086			

Table 6: *Continued*

The amounts of proteins identified from human sweat were determined based on the spiked 850 fmol T33V *Rhodobacter capsulatus* cytochrome c internal standard. Among the identified proteins, 15 proteins could be quantified; dermcidin was the most abundant protein in the sweat having 1.14 pmol/µg concentration (**Figure 10**).

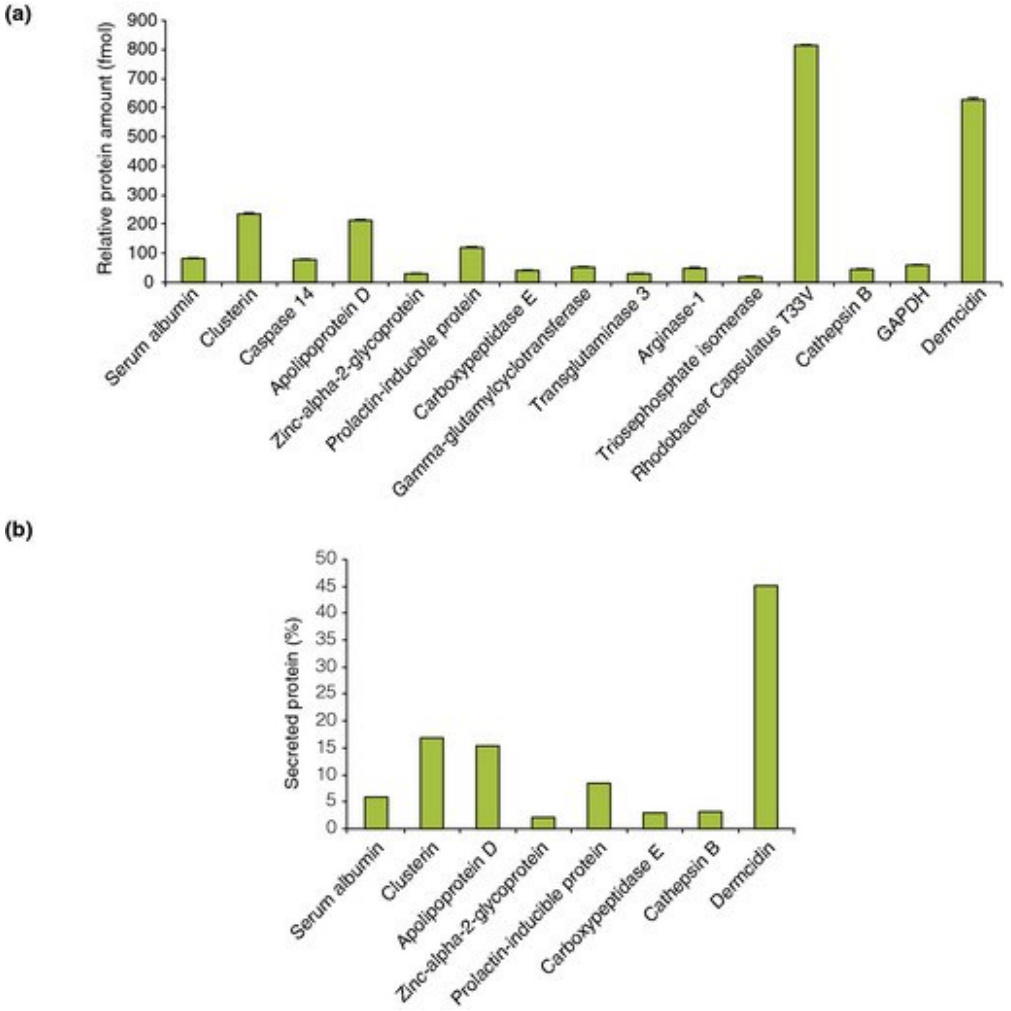


Figure 10: Sweat protein amounts determined by label-free quantification*. 4.2 µg pooled sweat protein samples were first reduced and alkylated, followed by trypsin digestion overnight at 37°C. 600 ng digested sample to which 850 fmol of a T33V Rhodobacter cytochrome c digest was added as an internal standard was analyzed by label-free mass spectrometry. Based on the normalized signal intensities the relative amount of each protein could be calculated. The percentage of the secreted proteins were calculated using the previously determined relative amounts. (a) Proteins quantified in the sweat. (b) Percentage of the eight most abundant secreted sweat proteins. The bars show the amount of sweat proteins representing the mean values of three parallel experiments. Error bars indicate standard error of means.*

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% (**Figure 10b**).

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.

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

In order to validate the label-free quantification results, 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. The parameters of the SRM experiments are listed in **Table 7**.

Protein	Peptide	Q1 (m/z)	Q3 (m/z)	Ion	Dwell Time (msec)	DP (eV)	CE (eV)
Dermcidin	ENAGEDPGLAR	564.768	628.341	y6	20	72.3	27.9
			513.314	y5	20	72.3	27.9
	ENAGEDPGLAR*	569.772	638.349	y6	20	72.3	27.9
			523.323	y5	20	72.3	27.9
PIP	YTA***C***L***C***DDNPK	678.782	1092.445	y9	20	80.6	34.4
			748.293	y6	20	80.6	34.4
	YTA***C***L***C***DDNPK*	682.788	1100.459	y9	20	80.6	34.4
			756.307	y6	20	80.6	34.4
	TVQIAAVVDVIR	642.387	842.509	y8	20	77.9	32.4
			771.472	y7	20	77.9	32.4
	TVQIAAVVDVIR*	647.391	852.517	y8	20	77.9	32.4
			781.481	y7	20	77.9	32.4

Table 7: SRM parameters of dermcidin and PIP specific peptides. *Sequences were retrieved from the UniProt database and after in silico digestion, BLASTp was used to identify the protein specific peptides.*

*SRM transitions were designed for the unique peptides using the Skyline software and the optimal parameters for each peptide were determined. Amino acids in italics represent carbamidomethylated cysteines. DP: declustering potential, CE: collision energy. * indicates the stable isotope-labeled amino acids.*

SIL peptides were added right before the analysis and the relative amount of dermcidin and PIP in the sweat pool was determined in two parallel experiments (**Figure 11**).

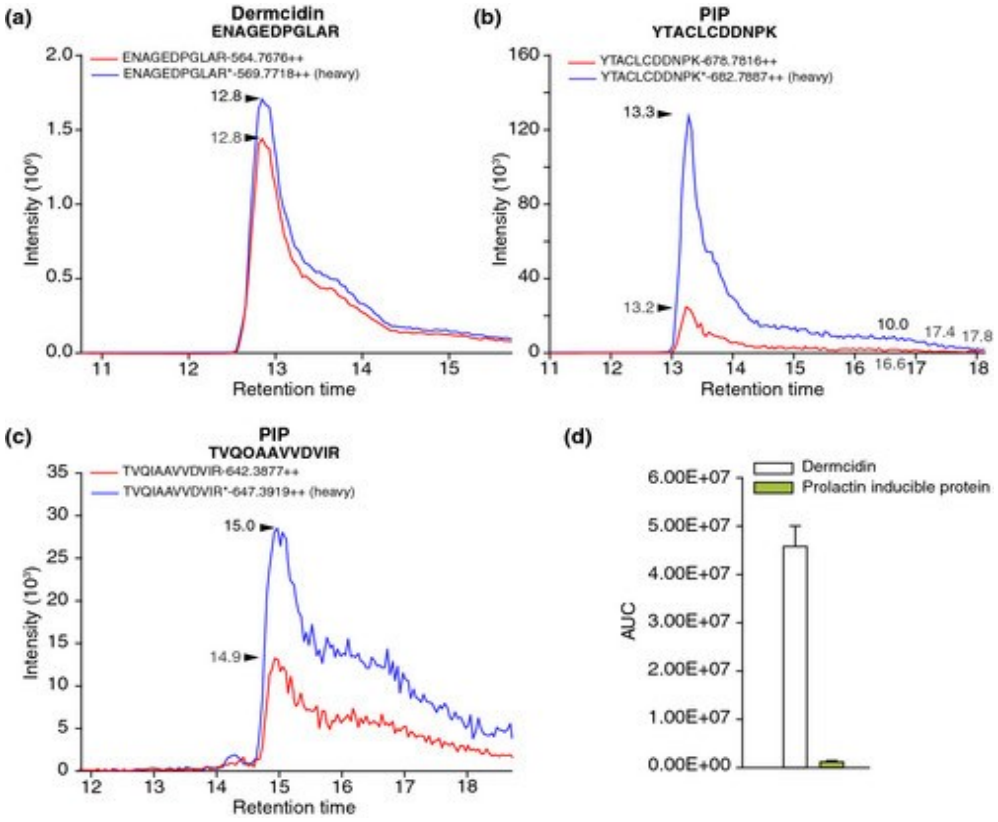


Figure 11: Relative amount of dermcidin and PIP determined by SRM-based approach. *Sweat samples were subjected to in-solution trypsin digestion and 10 µg digests were analyzed by SRM. (a–c) Representative SRM spectra for dermcidin and PIP-specific sequences (red spectra) and their SIL counterparts (blue spectra). (d) Relative amount of dermcidin and PIP. The bars represent the mean AUC/µg of total protein obtained from two parallel experiments. Error bars indicate standard error of means.*

Both the SRM-based targeted proteomics experiment and the label-free quantification support the evidence that dermcidin is the most characteristic sweat protein.

5.3.3 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 proteins revealed their possible implication in protecting the skin from invading pathogens (**Table 8**), as part of a chemical barrier whose alterations may lead to an increased rate of skin infections [2,146–152].

Protein	Accession number	Function	References
Dermcidin	P81605	Defense response to bacteria and fungi	[2]
Clusterin	P10909	Modulator of MMP9 activity	[144]
PIP	P12273	Binding to different species of bacteria, IgG, IgG-Fc and CD4-T cell receptor	[145-147]
Apolipoprotein D	P05090	Tissue regeneration Prevents lipid damage caused by free radicals	[148, 149]
Serum albumin	P02768	Transport, binding, antioxidant role	[150]

Table 8: ***The function of highly abundant sweat proteins***.

Proteins listed in **Table 8** are essential to the formation of the chemical barrier of the skin, therefore the probable function of the highly abundant sweat proteins are the modulation of immune responses, tissue regeneration and defense against oxidative stress and pathogens [153].

6. 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 [47,61]. 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 [154]. 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 [155]. 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 (cell lysate, cell culture supernatant, tears). 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, this cost-effectiveness 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 [156]. Members of the hBD family can be induced upon pathogenic or inflammatory stimuli or can be constitutively expressed [6]. 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 [6,157]. Data suggest that hBD1 is constitutively expressed and cannot be induced by inflammatory stimulus [158]. 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 [80]. 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 [80], 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 [47], diabetic retinopathy [50], in addition to systemic pathophysiological processes [54]. Tear samples collected from healthy volunteers were analyzed with the developed SRM method in order to determine whether or not the method can 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 [156]. 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 [9]. 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 [156], 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 immunmodulatory 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 (Aβ) peptides, the microtubule-associated protein tau and α-synuclein, are involved in the pathogenesis of AD [159,160]. 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 [141,142]. 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 [140]. 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 [140–142]. 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 [2]. The down-regulated proteins are produced by the lacrimal glands being involved in the first line defense of the eye [3]. 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 [161]. 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. The AUC values were above 0.65 except for one protein, indicating an acceptable biomarker, moreover, the performance was increased when different combinations of the proteins were tested, showing their additive effect on the test performance. 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 [162].

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 (based on our data, above 5 µl/min) 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 (CT, MRI, PET), neuropsychological testing and cerebrospinal fluid analyses ($A\beta_{42}$, total tau and p-tau levels). 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 [163]. Little is known about the causes and consequences of qualitative and quantitative changes of the human sweat proteins, moreover, the composition of the normal 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 [153]. Data obtained by label-free quantification mass spectrometry revealed that 91% of the secreted sweat proteome is made up of six highly abundant proteins. The most prominent protein was dermcidin, accounting for 46% of the secreted sweat proteins, followed by clusterin (17%), apolipoprotein D (15%), PIP (8%) and serum albumin (6%) [153]. 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 [153].

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.

Dermcidin and its peptide derivatives with broad spectrum of antimicrobial activity were shown to be the main sweat protein/peptides secreted. Their activity is not altered by low pH and high salt concentrations – conditions typically observed in sweat – making them the principal skin antimicrobial peptides [2]. Secreted form of clusterin belongs to the extracellular chaperones, and it is present in almost all body fluids. The major functions of these proteins are the maintenance of fluid-epithelial interface homoeostasis, and the prevention of the onset of inflammatory conditions [146,164]. Recent data suggest that clusterin can interact with matrix metalloproteinases inhibiting their enzymatic activity, and it is possible that the protein is able to reduce keratinocyte damage and inflammation of the skin [165]. The function of PIP is not clear; it can bind different bacteria, and was identified practically in all body fluids which are exposed to microbes, such as sweat, saliva

and tears. The protein was detected in mice at an early embryonic stage before the development of the immune system, and in human amniotic fluid, most probably providing protection against bacteria before the development and maturation of the immune system [29]. Apolipoprotein D is a multi-ligand-binding member of the lipocalin family, which is able to transport hydrophobic molecules. Studies proved that it plays a role in preventing lipid peroxidation; especially in the central nervous system, protecting the cells from oxidative stress [151,166]. Its presence in the sweat indicates that it has protective roles as a component of the first line of defense, preventing the skin lipids from damage caused by peroxidation. The presence of serum albumin in the sweat was demonstrated by other studies as well [167], yet the origin of this protein is still unclear. Most probably it originates from the blood as seen in the case of tears [42]. Previous experiments demonstrated that the N-terminal DAHK peptide of serum albumin has a potent antioxidant activity [152], indicating a possible antioxidant function of this protein in human sweat.

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. Nowadays, the focus on tissue samples and biopsies has shifted to the non-invasively collectable body fluids. 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.

7. Keywords/kulcsszavak

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

Testfolyadék, antimikrobiális és immunmodulátor peptidek/fehérjék, biomarker, kvantitatív proteomika, módszerfejlesztés, könny, verejték, Alzheimer-kór

8. 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 a 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 examined the sweat proteome using mass spectrometry analyses. 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.

9. Összefoglalás

A disszertáció a kémiai barrier részét képező antimikrobiális és immunmodulátor fehérjék mennyiségi változásainak vizsgálatára fókuszál epitél sejtkultúrában valamint könny és verejték mintákban célzott proteomikai módszer alkalmazásával. Vizsgálatainkhoz SRM alapú célzott tömegspektrometriás módszert fejlesztettünk β-defenzin 1-4, lipokalin-1, laktotranszferrin, extracelluláris glikoprotein lakritin, lizozim-C, lipofilin A, Ig λ-lánc C régió, prolaktin indukált fehérje, Zn-α-2-glikoprotein, galektin 3 kötő fehérje és dermcidin fehérjék szemi kvantitatív meghatározására komplex biológiai mintákban.

Sejtkultúrákon végzett kísérleteink során igazoltuk a β-defenzin 2 és 3 fehérjék expressziójának indukcióját gyulladásos jel hatására, emellett bizonyítottuk, hogy a β-defenzin 1 és 4 nem indukálódik klasszikus gyulladásos stimulusra. A β-defenzinek mennyiségét könnymintákban vizsgálva megállapítottuk, hogy az SRM módszer alkalmas β-defenzinek vizsgálatára kis mennyiségű biológiai mintákból is. Mivel bizonyított, hogy a kémiai barrier patológiás esetekben megváltozik, emellett bizonyították a könnyfehérjék mennyiségi változását neurodegeneratív betegségekben, megvizsgáltuk a β-defenzin 1-4 mennyiségét Alzheimer-kóros betegektől származó könnymintákban. A β-defenzinek mennyisége a kimutatási határ alá esett, így a könnymintákat további vizsgálatoknak vetettük alá. Szignifikáns növekedést tapasztaltunk az Alzheimer-kóros betegek könny szekréciós rátájában és a könny fehérje koncentrációjában, emellett megfigyeltük a fő könnyfehérjék mennyiségének csökkenését a kontroll csoporthoz képest. SRM kísérletekkel igazoltuk a fő könnyfehérjék mennyiségének csökkenését, valamint a dermcidin szint emelkedését. ROC analízisek segítségével kimutattuk, hogy a lipokalin-1, lizozim-C, dermcidin és lakritin fehérjék kombinációja biomarkerként használható lehet az Alzheimer-kór diagnózisában. A könny mellett a verejtékben lévő AMPk is a kémiai barrier részét képezik és mivel a verejték normál fehérjetartalmát részletesen nem tanulmányozták, tömegspektrometriás módszerek segítségével meghatároztuk a normál verejték proteomot. Vizsgálatainkban 95 fehérjét azonosítottunk ezekből 20 fehérjét eddig még nem azonosítottak a verejték alkotójaként. Az azonosított abundáns fehérjék a dermcidin, prolaktin indukált fehérje, kalszterin, apolipoprotein D és a szérum albumin a kémiai barrier fontos alkotórészei.

Eredményeink alátámasztják a célzott proteomika módszerek felhasználhatóságát és fontosságát a testfolyadékokból történő biomarker kutatások területén.

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

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Candidate: Gergő Kalló
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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.
   IF: 2.226

DEBRECENI EGYETEM KENÉZY ÉLETTUDOMÁNYI KÖNYVTÁRA EGYETEMI ÉS NEMZETI KÖNYVTÁR

Address: 1 Egyetem tér, Debrecen 4032, Hungary Postal address: Pf. 39. Debrecen 4010, Hungary
Tel.: +36 52 410 443 Fax: +36 52 512 900/63847 E-mail: publikaciok@lib.unideb.hu, ¤ Web: www.lib.unideb.hu

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.
J. Proteomics. S1874-3919 (16), 30280-30289, 2016.
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