Published in final edited form as:

J Proteome Res. 2012 April 6; 11(4): 2127–2139. doi:10.1021/pr2007957.

A Proteomic Analysis of Eccrine Sweat: Implications for the Discovery of Schizophrenia Biomarker Proteins

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Abstract

Liquid chromatography tandem mass spectrometry (LC-MS/MS) and multiple reaction monitoring mass spectrometry (MRM-MS) proteomics analyses were performed on eccrine sweat of healthy controls, and the results were compared with those from individuals diagnosed with schizophrenia (SZ). This is the first large scale study of the sweat proteome. First, we performed LC-MS/MS on pooled SZ samples and pooled control samples for global proteomics analysis. Results revealed a high abundance of diverse proteins and peptides in eccrine sweat. Most of the proteins identified from sweat samples were found to be different than the most abundant proteins from serum, which indicates that eccrine sweat is not simply a plasma transudate, and may thereby be a source of unique disease-associated biomolecules. A second independent set of patient and control sweat samples were analyzed by LC-MS/MS and spectral counting to determine qualitative protein differential abundances between the control and disease groups. Differential abundances of selected proteins, initially determined by spectral counting, were verified by MRM-MS analyses. Seventeen proteins showed a differential abundance of approximately two-fold or greater between the SZ pooled sample and the control pooled sample. This study demonstrates the utility of LC-MS/MS and MRM-MS as a viable strategy for the discovery and verification of potential sweat protein disease biomarkers.

Keywords

Sweat; Proteome; Schizophrenia; Biomarkers; LC-MS/MS; MRM

Introduction

Although sweat is less complex than blood in terms of protein composition, it represents an attractive source for the discovery of proteins potentially modulated due to disease-related

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Supporting Information Available: Supplemental A – List of all proteins identified from the 1st set of pooled sweat samples by LC-MS/MS analysis. Supplemental B – List of all proteins identified from whole serum by LC-MS/MS analysis. Supplemental C – List of all proteins identified from the 2nd set of pooled sweat samples by LC-MS/MS analysis. Supplemental D – List of all peptides, transition ions, and collision energies chosen for MRM analysis of the second set of patient and control pooled sweat samples.

This material is available free of charge via the Internet at <http://pubs.acs.org>

biochemistry. Sweat is produced primarily for temperature regulation. However, because sweat glands are controlled by the sympathetic nervous system, sweat production is subject to psychological stimuli. Eccrine sweat glands are distributed over the entire body and produce a fluid that is largely water and salts.¹ In contrast, apocrine sweat glands are associated with hair follicles and generate an oily fluid consisting of fatty compounds. Compared with other physiological fluids, there have been relatively few studies characterizing sweat proteins, and very few mass spectrometry (MS)-based proteomics analyses of this fluid.² Sweat is an attractive biological fluid source of biomarkers because it can be obtained less invasively than fluids such as serum, and constitutes a rich source of proteins and peptides.

SZ is a severe, chronic and debilitating brain disorder that affects approximately 51 million people worldwide.³ SZ is characterized by distortions in thought and perception. Research aimed at the discovery of biomarkers for SZ has been carried out for more than 20 years.⁴ The niacin skin flushing test, a potential skin test for the disease based on methyl nicotinate triggering production of prostaglandin D2 from macrophages in the skin and resulting vasodilatation, is one of the earliest attempts toward a diagnostic test. Although many studies have been conducted during this time to identify a significant target or set of targets that could be used in a reliable diagnostic test for SZ, no such test has come to fruition.⁵

In order to eliminate the possibility of patient opposition or discomfort related to blood collection, a less invasive sweat collection technique was used in this study to obtain a biomarker source fluid. This technique involved the Macroduct™ forearm sampling device. This device is clinically approved for the collection of eccrine sweat for the diagnosis of cystic fibrosis through measurement of osmolality and sodium concentrations.⁶

Due to the potential value of sweat as a source of disease biomarkers, we conducted an in-depth proteomic analysis of eccrine sweat in order to obtain an improved characterization of the sweat proteome. A liquid chromatography – tandem mass spectrometry (LC-MS/MS) - based approach was used to perform an initial global proteomic analysis of healthy control and schizophrenic (SZ) patient sweat samples. A total of 150 unique proteins were identified in this manner, most of which are not among the most abundant proteins found in whole serum. In order to establish the feasibility of using sweat as a source of disease-related biomarkers, we compared the pooled sweat of healthy controls to an age-matched, pooled population of patients with schizophrenia. Using a separate, independent sample set, we conducted the first attempt at a comparative analysis of SZ patient and control sweat samples using LC-MS/MS and spectral counting, followed by MRM-MS to verify selected proteins/peptides as candidate biomarkers.

Materials and Methods

Subjects

Subjects with SZ were identified by a team of trained psychiatrists at Loma Linda University, California. These subjects included cases of new-onset SZ and cases of established SZ. In addition, control subjects were selected based on an extensive evaluation to ensure a well-matched control group. Age-, race-, and gender-matched control subjects constituted the control population. Individuals with diabetes, rheumatological or dermatological diseases were excluded. Diagnosis of SZ was established by Structured Clinical Interviews to establish the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (SCID-DSM-IV) or DSM-IV Text revision (TR) and SCID-DSM ratings plus longitudinal monitoring. The Birchwood Social Functioning Scale was used to determine the level of social function in SZ patients. Control subjects were screened with psychiatric research interviews to exclude individuals with either current or past history of SZ, schizo-

affective disorder, depression, bipolar disorder, obsessive compulsive disorder, generalized anxiety disorder, and substance abuse or dependence. Diets of subjects and controls were not regulated. A subjective determination was made by the examining psychiatrist of any unusual or specific body odor.

The average age of controls and patients was approximately twenty-two years old. This cohort included samples from non-smoking, smoking, male and female controls and patients. Disease stages of patients in this study were classified as early-onset, moderate and established. The samples that fit the criteria for the best match of patient and control subjects were selected for analyses. Table 1 shows the list of SZ patients that were used in all of the mass spectrometric analyses in this study. It includes pools that patients belong to, age, gender, ethnicity, available patient laboratory tests, and medications that patients were on during the time of sweat collection. Table 2 is a list of the medications used by the patients during the time of sweat collection. It also includes medication-induced risk of metabolic syndrome, potential medication-related changes in prolactin levels in blood, potential heart rate changes induced by medication, possible extra pyramidal symptoms associated with medication, and any potential anticholinergic effects of medications.

Sweat collection

The volar forearm is rich in eccrine sweat glands. After the selected forearm area was cleansed with alcohol and rinsed with distilled water, sweat was collected with the Macroduct™ Sweat Stimulation and Sweat Collection System (WESCOR, Inc., Logan, UT). The Macroduct™ system consists of the Webster Sweat Inducer, the Macroduct™ Sweat Collector and the Sweat-Chek Analyzer, which are used for cystic fibrosis (CF) diagnosis. Sweat is stimulated using pilocarpine, according to the 1959 pad adsorption method of Gibson and Cooke iontophoresis⁷ resulting in a sample of undiluted pure eccrine sweat. On average, 50–60 µL of sweat were collected from each subject during the 30-minute collection interval. After a sufficient volume of sweat had accumulated, a sweat dispenser, or blunt needle on a tuberculin syringe, was connected to the open end of the tubing. The tubing was uncoiled from the Macroduct™ Sweat Collector and severed at the point of attachment. The sweat was then transferred from the tubing to microcentrifuge tubes, which were placed immediately on dry ice.

The relationship between patient and physician was a key factor in comfort level for patients during sweat collection as it would be for collection of other biological fluids. Hence, assurance of patient comfort and understanding of procedure was made a top priority during collection.

None of the patients were experiencing psychosis during the time of collection. No subject was ever forced to have their sweat collected. Sweat collection took place without any interruptions. Both adolescents and adults were fully aware of the study and what was taking place. At times, some of the adolescents exhibited slight apprehension but the psychiatrist and clinical assistants made sure that everyone was comfortable during collection. Collection did not proceed until all subjects and any parents involved consented, and all subjects were relaxed. All apparatuses and the collection procedure were demonstrated and discussed with subjects before collection. Many subjects, particularly adolescents, were intrigued by the procedure. During the step of the procedure in which pilocarpine is placed in the electrode and applied to the skin with running current, the psychiatrist and clinical assistants constantly asked patients about discomfort level and toleration. There were no complaints of pain, and the current contacting the skin was never at a level of discomfort that caused patients to react. After electrodes containing pilocarpine were removed, everyone including controls and patients, experienced slight irritation in the form of red circles. This would disappear within minutes to a few hours depending on the individual's

sensitivity level. There were never any reports of long-term irritation, burns, blisters, or other adverse reactions. Overall, patients and controls were comfortable with the psychiatrist, clinical assistants, and collection procedure.

Sample Preparation

The total cohort consisted of 78 subjects (55 controls and 23 patients). Total protein concentration was determined to be 0.10 – 0.25 $\mu\text{g}/\mu\text{L}$ by protein microarray analysis of the individual sweat samples. A patient pooled sample and a control pooled sample were created by combining eight individual sweat samples for each pool. Both pooled samples were constructed to yield a total protein concentration of 0.1 $\mu\text{g}/\mu\text{L}$. These 8 patient and 8 control samples were chosen specifically to form the pooled samples due to the fact that they contained the largest volume and protein concentration among all of the 78 subject samples. The remaining 62 subject samples were more limited in their volume and/or protein concentration. Ten nanograms of bovine β -casein (Sigma Aldrich, St Louis, MO) were added to each pooled sample to serve as an internal protein standard. This initial set of two pooled samples was analyzed by LC-MS/MS only, and was completely consumed after LC-MS/MS analysis. A second sample set was constructed, consisting of a new patient pooled sample and new control pooled sample. Each of these new pooled samples consisted of 4 new individual samples that were different from the ones used in the first sample set. Again, each pooled sample was constructed to yield a total protein concentration of 0.1 $\mu\text{g}/\mu\text{L}$. These 4 patient and 4 control samples were chosen specifically, as they contained the largest volume and protein concentration among the remaining 62 subject samples. As an internal standard, recombinant human S100A6 protein was added to each pooled sample; 130 ng in the patient pooled sample and 13 ng in the control pooled sample. The second set was analyzed by LC-MS/MS and MRM-MS. Table 1 shows to which pool each of the individual samples belong, along with which analyses were performed on each pool.

All pooled samples were prepared for mass spectrometric analyses by: (1) reduction with 10 mM dithiothreitol in 8 M urea at 50°C for 20 minutes, (2) alkylation with 50 mM iodoacetamide at room temperature in the dark for 20 minutes, (3) overnight enzymatic digestion with 0.5 μg sequencing grade trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate (NH_4HCO_3), pH 8 at 37°C. Digestions were quenched with the addition of 2 μL glacial acetic acid, followed by the addition of 500 fmol of angiotensin II. Digest samples were then desalted with C-18 Zip Tips (Millipore, Billerica, MA) and dried in a vacuum concentrator.

The first set of pooled samples was reconstituted in 5 μL of 0.1% formic acid (FA), followed by the addition of 100 fmol angiotensin I. The second set of pooled samples was reconstituted in 40 μL of 0.1% FA. Half the volume (20 μL) of these pooled samples was removed and 100 fmol of angiotensin I was added. These samples were analyzed by LC-MS/MS only. To the remaining 20 μL of the second set of pooled samples, 100 fmol of a yeast enolase tryptic digest (Michrom Bioresources, Auburn, CA) was added. These samples were analyzed by MRM-MS.

Mass Spectrometry

LC-MS/MS: The sample protein digests were analyzed by online LC-MS/MS using an LTQ-Orbitrap (Thermo Fisher Scientific, Waltham, MA) mass spectrometer. Samples were loaded onto a 0.2 mm $\times$ 50 mm Magic C-18 AQ reversed-phase column (Michrom Bioresources) with a Micro A/S autosampler (Thermo Fisher Scientific) fitted with a 2 μL sample loop. The LC pump was a Surveyor (Thermo Fisher Scientific) operated at 90 $\mu\text{L}/\text{min}$, the output of which was split to generate a column flow rate of 2 $\mu\text{L}/\text{min}$. A Thermo nanospray-ESI source was used. After sample injection, the column was washed for 5

minutes with mobile phase A (0.1% formic acid), and peptides were eluted using a linear gradient of 0% mobile phase B (0.1% formic acid, 80% acetonitrile) to 45% mobile phase B in 120 min. The LTQ Orbitrap mass spectrometer was operated in a data-dependent mode in which each full MS scan (60,000 resolving power) was followed by eight MS/MS scans where the eight most abundant molecular ions were dynamically selected and fragmented by collision-induced dissociation (CID) using a normalized collision energy of 35%. The “FT master scan preview mode”, “Charge state screening”, “Monoisotopic precursor selection”, “Dynamic Exclusion” (30 seconds), and “Charge state rejection” were enabled so that only the 1+, 2+ and 3+ ions were selected and fragmented by CID. The first set of pooled samples was analyzed in quadruplicate. The second set of pooled samples was analyzed once.

MRM-MS: Sweat sample protein digests were analyzed with a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific). Samples were loaded onto a 0.2 mm × 50 mm Magic C-18 AQ reversed-phase column (Michrom Bioresources) from an Accela autosampler (Thermo Fisher Scientific) fitted with a 10 µL sample loop. The LC pump was an Accela (Thermo Fisher Scientific) operated at 100 µL/min, and the output was split to generate a column flow rate of 2 µL/min. A Michrom Bioresources ADVANCE nanospray source was used. After sample injection, the column was washed for 5 minutes with mobile phase A (0.1% formic acid), and peptides were eluted using a linear gradient of 0% mobile phase B (0.1% formic acid in acetonitrile) to 45% mobile phase B in 60 min. Q1 and Q3 resolution was set to 0.4 FWHM and 0.7 FWHM, respectively. Q2 contained 1.5 mTorr of argon. The scan width was 0.002 m/z, and dwell time was 5 msec. With a total of 304 transitions, this yielded a cycle time of 1.5 seconds. (See Supplement A for a list of the peptides and transitions.) Collision energies (CE) were calculated according to the formula: $0.034 \times \text{precursor ion mass } m/z + 3.314$. Patient and control pooled samples from the second set were each analyzed in triplicate. Prior to, and after each pooled sample single analysis, an analysis of 2.5 fmol of a yeast enolase tryptic digest was performed to evaluate instrument performance. Two yeast enolase peptides, with 5 transitions each, were monitored by MRM (TFAEALR; y₂-y₆, and AADALLK; y₃-y₇). Since 100 fmol of a yeast enolase tryptic digest was spiked into the 2nd set of pooled samples during sample preparation, these same 2 yeast enolase peptides (with 5 transitions each) were also monitored during each pooled sample analysis.

Bioinformatic Analysis—Tandem mass spectra were searched against the human NCBI Sep2006; 34191 sequences protein database using SEQUEST version SRF v.5 (Bioworks software from Thermo Fisher, version 3.3.1) with full tryptic cleavage constraints, a maximum of 2 missed cleavage sites, static cysteine carbamidomethylation (+57 Da), and variable methionine oxidation (+16 Da). The mass tolerance for precursor ions was 10 ppm, and the mass tolerance for fragment ions was 0.2 Da. The SEQUEST search results were filtered using the following criteria: minimum Xcorr of 1.9, 2.2, and 3.5 for 1+, 2+, and 3+ ions, respectively, *Cn* greater than 0.1 and precursor ion mass deviation less than or equal to 3 ppm. False positive rates for peptide identifications were estimated using a reverse database approach. Proteins identified by at least two unique peptides, each with a false positive rate of less than 1%, were considered to be present in the sample.

SEQUEST MS/MS results were then used to perform a spectral counting analysis using Scaffold version 2_06_02 (Proteome Software, Portland, OR). Utilizing the same SEQUEST parameters described previously, combined with a minimum of two peptides identified and minimum protein and peptide confidence levels of 95%, Scaffold results were reported as the total number of assigned MS/MS spectra for each protein identified in patient and control pooled samples.

All MRM results were loaded into the Pinpoint program version 1.0 (Thermo Fisher Scientific) for data analysis. Data were divided into 3 groups; (1) a pooled patient sample group (triplicate analyses), (2) a pooled control sample group (triplicate analyses), and (3) a standard sample group (seven analyses of yeast enolase). Peak width tolerance was 30 seconds, alignment error tolerance was 0.5 minutes, and minimum signal threshold was 100. The fragment ion peak areas for all transitions were summed, and the CV calculated for each peptide detected in all sample groups analyzed.

Gene Ontology—To determine gene ontological annotations for selected proteins, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID) (located at <http://david.abcc.ncifcrf.gov/david/>). DAVID is a Web-based application that allows users to access a relational database of gene functional annotations.^{8,9}

Results and Discussion

Sweat proteome characterization

Pooling of the samples was deemed necessary due to the limited amount of volume and/or protein abundance in each individual sample. Pooling the samples provided us with greater protein abundances that would help enhance our ability to identify and quantitate more proteins. In addition, the pooling of control and patient samples allowed for easy generation of two separate comparative sample sets that were equal in their total protein amount.

From the first set of pooled control and pooled patient sample analyses, approximately 760 unique peptides and a total of 150 unique proteins were identified using a minimum of 2 peptides identified per protein (see Supplemental A). This list was generated using Scaffold as specified in the Materials and Methods section. When using 1 peptide identified per protein, approximately 850 unique peptides and 220 unique proteins were identified (data not shown). Table 3 shows the number of proteins identified in the corresponding number of LC-MS/MS analyses of the 4 technical replicates. From the pooled control sweat sample, 110/131 (84%) of the proteins identified were found in at least 2 of the replicate analyses, and 21 proteins were identified in only 1 of the replicate analyses. The remaining 19 proteins were identified in only the pooled patient sample analyses. From the pooled patient sweat sample, 101/121 (83%) of proteins identified were found in at least 2 of the replicate analyses, and 20 proteins were identified in only 1 of the replicate analyses. The remaining 29 proteins were identified in only the pooled control sample analyses. The Venn diagram in Figure 1 shows an overlap of 102 identified proteins between the pooled control sample and the pooled patient sample (84%), demonstrating high reproducibility for sample preparation and mass spectrometric analyses. Examples of MS/MS spectra of 1 peptide, each corresponding to the top 3 proteins identified in sweat (prolactin-induced protein, dermcidin, and caspase 14) from the first set of patient and control pooled sample analyses, are shown in Figure 2. These proteins were identified from doubly-charged peptides with SEQUEST Xcorr values of 4.07, 3.74, and 5.04, respectively.

The list of 150 proteins identified from this first set of pooled sweat samples was compared to proteins identified from an LC-MS/MS analysis of trypsin-digested whole serum (from previous experiments; Supplemental B). Only 5 proteins are found to be in common between sweat and serum (Figure 3). These proteins are: albumin, alpha-2-glycoprotein 1, clusterin isoform 1, apolipoprotein D precursor, and gelsolin isoform b. While albumin is the most abundant protein in serum, it is clearly not the most abundant protein found in sweat. These results suggest that sweat may not simply be a plasma transudate and contains a unique set of proteins.

Figure 4 presents the results of a gene ontology analyses for molecular function classifications of identified sweat and serum proteins. The most significant differences between serum and sweat proteins are in the categories of catalytic activity, enzyme regulation and structural molecular activity. There is a higher abundance of proteins related to catalytic activity in sweat than in serum (35% vs. 22%) and a considerably higher abundance of structural molecular activity in sweat than in serum (5.5% vs. 0.8%). However, there is a much higher percentage of enzyme regulating proteins in serum than in sweat (20% vs 5%). Gene ontology analyses were also performed for cellular components (Figure 5) and biological processes (Figure 6). Proteins coded by genes for cellular components; those related to the cytoplasm, intracellular organelles, and cytoskeleton, were all found in higher abundance in sweat, whereas proteins coded by genes of the extracellular region were in higher abundance in serum. The remaining components analyzed showed fairly even ratios between sweat and serum. A few differences found between sweat and serum, in the classification of biological processes, include a slightly higher abundance of proteins in sweat that are related to metabolic processes, developmental processes, and multicellular organismal processes.

Since the first set of pooled samples had been completely consumed following LC-MS/MS analysis, no further analyses could be performed using these particular samples. Consequently, a second independent set of patient and control pooled samples was created using 4 different individual samples each. This new, second set of pooled samples was analyzed by LC-MS/MS and MRM-MS. Supplemental C provides a list of all the proteins identified from this 2nd set of pooled patient and pooled control samples by LC-MS/MS. Approximately 950 unique peptides and a total of 185 unique proteins were identified using a minimum of 2 peptides identified per protein. This list was generated using Scaffold as described in the Materials and Methods section. When using 1 peptide identified per protein, approximately 970 unique peptides and 195 unique proteins were identified (data not shown).

Figure 7A presents a Venn diagram showing the overlap of the proteins identified from the 2nd set of pooled patient and control samples, and the 1st set of pooled patient and control samples. There was an overlap of 122 proteins (81%). Of the 28 proteins found only in the 1st set of samples, 15 proteins were identified with only 2 assigned spectra, 6 proteins were identified with 3 assigned spectra, and 3 proteins were identified with 4 assigned spectra. The remaining 4 proteins were identified with 5, 6, 7, and 19 assigned spectra. This suggests that most of the 28 proteins that were found in the 1st set of samples and not in the 2nd set of samples were lower in abundance. The protein of highest abundance identified in the 1st set (19 assigned spectra) and not in the 2nd set was suprabasin. A possible reason for this could be that one or more of the individual samples used to construct the 1st set of samples had a large amount of this protein present. The Venn diagram in Figure 7B shows the overlap of the proteins identified from the 2nd set of pooled patient and control samples, and whole serum. Again, as with the 1st set of pooled samples, there are only 5 proteins in common between the 2nd set of samples and whole serum. These proteins are: albumin, alpha-2-glycoprotein 1, clusterin isoform 1, gelsolin isoform b, and hypothetical protein LOC649897. All but hypothetical protein LOC649897 are the same proteins that serum had in common with the 1st set of pooled samples. Because the first and second set of pooled sweat samples were created from different individual patient and control samples, these results demonstrate that sweat proteins can be collected and analyzed in a reproducible manner.

Candidate biomarker selection and verification

Following LC-MS/MS analysis of the 2nd set of pooled sweat samples, a list of 30 proteins was generated for MRM analysis. Using Scaffold results, proteins showing a relative

difference of 30% or more (+ or –) between patient and control, were chosen for MRM-MS. Relative difference was calculated as [(number of Patient spectra – number of Control spectra) / (average number of spectra for patient and control) × 100]. Included in these 30 proteins, are 3 proteins that showed little or no relative difference (glyceraldehyde-3-phosphate dehydrogenase, kallikrein 11, and keratin 9), so we could also verify by MRM that they were not differentially expressed. Table 4 shows a list of these 30 proteins, along with the spectral counting results from Scaffold. At least one peptide and 5 transitions for each peptide were chosen for MRM based on the data obtained from LC-MS/MS. (See Supplemental D for complete list of peptides and transitions.)

A 2.5 fmol yeast enolase digest was analyzed by MRM prior to and after each pooled sample single analysis to evaluate instrument performance throughout the entire MRM analysis of the pooled sweat samples. A total of 7 individual yeast enolase digest analyses were performed, Figure 8 shows how the enolase digest was incorporated into the workflow. The summed fragment ion peaks for the 2 peptides monitored (TFAEALR and AADALLK) from the yeast enolase digest, yielded CVs of 7% and 8% respectively across all 7 analyses. This result demonstrates excellent instrument performance and reproducibility throughout the MRM analyses.

As discussed in the Materials and Methods section, a 100 fmol aliquot of the yeast enolase tryptic digest was spiked into each pooled sample following tryptic digestion and desalting (see Figure 8). In addition, the protein S100A6 was spiked into each pooled sample prior to tryptic digestion in a 10:1 ratio (patient:control). Table 5 shows the MRM results for the enolase peptides (TFAEALR and AADALLK) spiked into the second set of pooled samples. All CVs for the summed fragment ion peaks are less than 15% and, as expected, an approximate 1:1 patient:control sample abundance ratio was determined. Table 6 shows the MRM results for the S100A6 protein spiked into the second set of pooled samples. Two peptides from this protein were monitored by MRM (ELTIGSK, y_2 - y_6 and LMEDLDR, y_2 - y_6). All CVs are 20% or lower, and each peptide shows close to the expected 10:1 ratio (patient:control). The results from these standards demonstrate high performance and reproducibility of the MRM methods used.

MRM data was imported into Pinpoint Software (Thermo Fisher Scientific) for analysis. Results from the 30 proteins chosen for MRM are shown in Figure 9. The area under the curve for all transitions from a particular peptide was summed and then averaged for the 3 replicate analyses. In order for a peptide to be considered verified, it had to: (1) have all 5 transitions detected and (2) yield a CV of lower than 20% for both pooled samples. In cases where more than 1 peptide was chosen from a particular protein for MRM analysis, if one peptide met these 2 criteria, the results of the second peptide were also reported, even if it did not meet these criteria. Of the 30 proteins analyzed, 19 were considered verified, and the ratio between the patient pool and the control pool was reported (Table 7). Of the 8 proteins analyzed with 2 peptides (alpha-2-glycoprotein, annexin 5, calmodulin-like skin protein, caspase-14, corneodesmosin, keratin 10, peroxiredoxin-1, and thioredoxin), 6 of them showed excellent agreement in the ratio of proteins between the patient pool and the control pool. Only peroxiredoxin-1 and caspase-14 showed a slight discrepancy in ratios between each of their 2 respective peptides. These results demonstrate that the MRM technique is consistent in quantitating relative abundances between sample groups.

Only 2 of the 19 verified proteins (dermcidin and keratin 10) showed no differential abundance between patient and control pooled samples. The remaining 17 proteins showed a differential abundance of approximately 2-fold or higher (Table 7). Of the 17 differentially abundant proteins, 16 showed a higher abundance in the patient pool compared to the control pool. Only 1 protein (prolactin-binding protein) showed a higher abundance in the

control pool compared to the patient pool. There was not always agreement between spectral counting and MRM-MS when determining differential abundances between the patient pool and the control pool. Of the 19 proteins verified by MRM (Table 7), 12 proteins showed a differential abundance of patient > control by spectral counting (> +30% relative abundance; Table 4), and 5 proteins showed a differential abundance of control > patient (> -30% relative abundance). The remaining 2 proteins (G3PDH and kallikrein 11) showed no differential abundance, with relative abundances of 14% and 0%, respectively.

When comparing the 12 proteins that showed patient > control with spectral counting to MRM-MS results, all 12 were in agreement; however, in some cases the magnitude of the relative differential abundances differed greatly. The largest differences were seen with peroxiredoxin, thioredoxin, and S100A7. Spectral counting showed peroxiredoxin having a total of 16 MS/MS spectra for the patient pool, and 10 MS/MS spectra for the control pool. MRM-MS data showed a differential abundance of approximately 6:1 to 9:1 patient:control. Thioredoxin had a total of 25 MS/MS spectra for the patient pool, and 11 MS/MS spectra for the control pool. MRM-MS data showed a differential abundance of approximately 9:1 to 10:1 patient:control. S100A7 showed a total of 6 MS/MS spectra for the patient pool and 2 MS/MS spectra for the control pool. MRM-MS data showed a differential abundance of approximately 9:1 patient:control.

When comparing the 5 proteins that showed control > patient with spectral counting to MRM-MS results, only 1 protein (prolactin-induced protein), was in agreement. The magnitude of the differential abundance was in agreement as well, with spectral counting and MRM-MS showing approximately 2:1 control:patient. Of the remaining 4 proteins showing control > patient with spectral counting, 2 showed patient > control by MRM-MS (alpha-2-glycoprotein zinc and prostatic binding protein). Prostatic binding protein not only showed patient > control with MRM-MS, but also a large differential abundance of approximately 7:1 patient:control. This is in sharp contrast to spectral counting results. The last 2 proteins showing control > patient with spectral counting (dermcidin and keratin 10) show an approximate ratio of 1:1 patient:control by MRM-MS.

The 2 proteins showing little or no differential abundance by spectral counting (G3PDH and kallikrein 11) showed differential abundances of 7:1 and 3:1 patient:control, respectively in MRM-MS.

Scaffold results from the LC-MS/MS analysis of the S100A6 protein spiked into the 2nd set of pooled sweat samples shows 12 peptides identified from the patient pooled sample, and no peptides identified from the control pooled sample (Table 4). Therefore, no quantitative data can be obtained from this analysis of S100A6. This only demonstrates that the protein is more abundant in the patient pooled sample. However, MRM-MS analysis of S100A6 from the 2nd set of pooled sweat samples shows a differential abundance of approximately 12:1 to 14:1 patient:control (Table 7). These results are very close to the actual 10:1 patient:control ratio of protein spiked into the pooled sweat samples.

The discrepancies between spectral counting and MRM-MS are not surprising. Spectral counting provides only a qualitative measure or estimate of relative abundance, while MRM-MS is more quantitative by using the area under the curve of specific peptide ion fragments. Thus, these MRM-MS results more accurately measure the protein relative abundances between the patient pooled sample and the control pooled sample.

This first broad-based proteomics analysis of sweat provided an improved characterization of the proteome of this unique physiological fluid, and demonstrated significant differences compared with the serum proteome. Sweat, a fluid initially thought to contain only water and salts, has been shown to be a fluid of much greater diversity and a rich source of

proteins. Our global proteomics analysis yielded an extensive list of proteins heretofore not associated with eccrine sweat. In total, 185 sweat proteins were identified in our study as compared to the prior literature, in which only 10 proteins were discovered.²

Eccrine sweat is not simply a plasma transudate, but contains a unique set of proteins that may have a different origin. When comparing the full list of proteins identified in serum and sweat, very little overlap appears between the two. Only 6 proteins are found to be in common between serum and sweat (albumin; clusterin isoform-1; gelsolin isoform b; serine (or cysteine) proteinase inhibitor, clade A; alpha-2-glycoprotein-1 zinc; and apolipoprotein D precursor).

After LC-MS/MS analyses, using spectral counting, we were able to qualitatively identify several sweat proteins that were differentially abundant between a SZ pooled patient sample and a control pooled sample (Table 4). By MRM-MS analyses, we were able to verify differential abundances of selected proteins (Table 7), which were initially determined by spectral counting. By using the S100A6 protein as a spiked internal standard, we were able to demonstrate that MRM-MS is capable of a more reliable or semi-quantitative measure of peptide abundance (vs. that obtained by spectral counting) and therefore inferred differential protein abundance. Additionally, 8 proteins were analyzed by MRM-MS using 2 peptides each. There was excellent agreement between each set of 2 peptides in 6 of the 8 proteins analyzed, showing consistency in the quantitative ability of MRM-MS. Excellent instrument reproducibility was demonstrated by the low CVs achieved from the yeast enolase digest spiked into the pooled samples just prior to MRM-MS analysis, as well as the S100A6 protein spiked into the pooled samples prior to trypsin digestion (Tables 6, 7).

A differential abundance of approximately 2-fold or greater was calculated by MRM-MS for 17 sweat proteins (Table 7). Some of these merit discussion. Neuroprotective protein DJ-1, is a protein that when mutated has been found to be linked to Parkinson's disease.¹⁰ Annexin-5, normally found in the anterior pituitary gland, is a calcium-binding protein related to apoptosis.¹¹ Bleomycin hydrolase is a neutral cysteine protease that's been found to be genetically linked to an increased risk for Alzheimer's disease. This protein plays a role in secretion of amyloid precursor protein.¹² It is important to note that Alzheimer's disease and schizophrenia both share similar psychiatric symptoms which suggests similar cerebral pathophysiologies.¹³ Thioredoxin (TRX) is a marker for oxidative stress and has been found in serum samples from first-episode schizophrenic patients. This protein is a redox regulator that scavenges reactive oxygen radicals with TRX-dependent peroxiredoxin, which was also found in differential abundance in SZ patient sweat.¹⁴ An increase in TRX and peroxiredoxin in serum and sweat of individuals with schizophrenia suggests a link between excessive production of free radicals resulting in oxidative stress and the pathophysiology of the disease. Caspase 14 is an apoptosis-related cysteine peptidase. Studies on postmortem SZ brain tissue indicate altered neuronal apoptosis in the pathophysiology of SZ. It is too early to suggest a link between caspase 14, which was found in higher abundance in SZ patient versus control sweat. Nonetheless, with a larger sample set and further studies, it might be possible to validate caspase 14 as a SZ biomarker and continue to study the link between this caspase and an apoptosis-related pathophysiology of SZ.¹⁵ One protein worth mentioning that was not validated by MRM-MS, but found by LC-MS/MS is synaptophysin. Synaptophysin is a glycoprotein present in the membrane of neuronal presynaptic vesicles in brain, vesicles of adrenal medulla, retina, spinal cord, endocrine cells and neuromuscular junctions.¹⁶ This serves as an example to show how proteins that originate from distant locations from sweat glands, such as those listed above, may be detected in eccrine sweat.

It is important to note that during the time of sweat collection, each of the SZ patients included in this study were on one or more prescription medications. In addition, two of the

SZ patients were using recreational drugs (Table 1). Some of these medications may have anticholinergic effects, the possibility of metabolic syndrome, antipsychotic-induced prolactin elevation, increased heart rate, and extra pyramidal symptoms (Table 2). For instance, drugs such as Risperidone, Olanzapine, Quetiapine, Clozapine, Paroxetine, and Bzotropine all have anticholinergic effects and inhibit sweat production. Pilocarpine, on the other hand, has the opposite effect and acts as a cholinergic agent in the parasympathetic nervous system. As discussed in the materials and methods section, pilocarpine was used to induce sweat production through the Macroduct Sweat Collector™.

Prolactin elevation has been observed in the blood of patients taking antipsychotics listed in Tables 1 and 2. However, prolactin levels in sweat were found to be lower in the patient pool relative to the healthy control pool in our study (Table 7). This result reminds us, once again, that sweat and blood, although they may be biologically linked, can have very different components and can be potentially associated with different biological processes.

Despite the fact that these drugs may have had an effect on the proteome of the SZ patients, this project was not meant to be a clinical analysis of drug effects on SZ patient fluids and was beyond the scope of this study. Instead, it was meant to establish the feasibility of using sweat as a source of disease-related protein biomarkers. The correlation between drug effects and the eccrine sweat proteome would involve a much larger and strictly controlled patient and control cohort.

The sweat proteome analysis may reveal diagnostic biomarkers that could be useful in a variety of diseases beyond SZ, including cancer and autoimmune diseases. It is of interest that certain cutaneous malignancies such as cutaneous T-cell lymphomas (CTCL) could be queried with this type of approach, where sweat sampling could identify markers of therapeutic response.

Conclusion

In this work we demonstrate that the eccrine sweat proteome is significantly different compared with the serum proteome. Eccrine sweat is a rich source of functionally important cellular proteins that may reflect the physiologic state of diverse tissues including those of the central nervous system. This feasibility study indicates that the relative abundance of sweat proteins is in the sensitivity range acceptable for MRM quantitation of candidate disease biomarkers. Candidate sweat proteins that were differentially abundant in SZ versus control samples in this study constitute the starting set for a further exploration of sweat biomarkers that may be potentially useful in the early diagnosis and monitoring of SZ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding provided by the Stanley Medical Research Institute, grant # 07R-1664. We would like to thank Mark Simpson from the NCI, for the S100A6.

References

- (1). Schwartz IL, Thaysen JH, Dole VP. Urea excretion in human sweat as a tracer for movement of water within the secreting gland. *J. Exp. Med.* 1952; 97(3):429–437. [PubMed: 13052810]
- (2). Kalbacher H, Baechle D, Mueller C, Garbe C, Stevanovic S, Mueller M, Mueller G, Beck A, Dihazil H, Hermann T, Tolson J, Schitteck B, Steffen H, Cansier A, Flad T. Cathepsin D is

present in human eccrine sweat and involved in the postsecretory processing of the antimicrobial peptide DCD-1L. *J. Biol. Chem.* 2006; 281(9):5406–5415. [PubMed: 16354654]

- (3). Winograd-Gurvich C. Negative symptoms: A review of schizophrenia, melancholic depression and Parkinson's disease. *Brain Res. Bull.* 2006; 70:312–321. [PubMed: 17027767]
- (4). Prilipko LL. Biological studies of schizophrenia in Europe. *Schizophr. Bull.* 1986; 12(1):83–100. [PubMed: 2421395]
- (5). Ward PE, Sutherland J, Glen EMT, Glen AIM. Niacin skin flush in schizophrenia: a preliminary report. *Schizophr. Res.* 1998; 29(3):269–274. [PubMed: 9516668]
- (6). Carter EP, Barrett AD, Heeley AF, Kuzemko JA. Improved sweat test method for the diagnosis of cystic fibrosis. *Arch. Dis. Child.* 1984; 59(10):919–922. [PubMed: 6497428]
- (7). Gibson LE, Cooke RE. A test for concentration for electrolytes in sweat in cystic fibrosis in the pancreas utilizing pilocarpine by iontophoresis. *Pediatrics.* 1959; 23(3):545–549. [PubMed: 1363369]
- (8). Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 2009; 4:44–57. [PubMed: 19131956]
- (9). Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol.* 2003; 4(5):P3. [PubMed: 12734009]
- (10). Canet-Aviles RM, Wilson MA, Miller DW, Ahmad R, McLendon C, Bandyopadhyay S, Baptista MJ, Ringe D, Petsko GA, Cookson MR. The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. *Proc. Natl. Acad. Sci. U.S.A.* 2004; 101(24):9103–9108. [PubMed: 15181200]
- (11). Koopman G, Reutelingsperger CP, Kuijten GP, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood.* 1994; 84(5):1415–1420. [PubMed: 8068938]
- (12). Lefterov IM, Koldamova RP, Lazo JS. Human bleomycin hydrolase regulates the secretion of amyloid precursor protein. *FASEB J.* 2000; 14(12):1837–1847. [PubMed: 10973933]
- (13). White KE, Cummings JL. Schizophrenia and Alzheimer's disease: clinical and pathophysiologic analogies. *Compr. Psychiatry.* 1996; 37(3):188–195. [PubMed: 8732586]
- (14). Zhang XY, Chen da C, Xiu MH, Wang F, Qi LY, Sun HQ, Chen S, He SC, Wu GY, Haile CN, Kosten TA, Lu L, Kosten TR. The novel oxidative stress marker thioredoxin is increased in first-episode schizophrenic patients. *Schizophr. Res.* 2009; 113(2–3):151–157. [PubMed: 19540723]
- (15). Glantz L, Gilmore J, Lieberman J, Jarskog F. (2006) Apoptotic mechanisms and the synaptic pathology of schizophrenia. *Schizophr. Res.* 2009; 81(1):47–63. [PubMed: 16226876]
- (16). Wiedenmann B, Franke WW. Identification and localization of synaptophysin, an integral membrane glycoprotein of M_r 38,000 characteristic of presynaptic vesicles. *Cell.* 1985; 41(3):1017–1028. [PubMed: 3924408]

Figure 1. Venn diagram showing overlap between the numbers of proteins identified from the 1st pooled control sweat sample and the 1st pooled patient sweat sample by LC-MS/MS analyses on the LTQOrbitrap (Thermo).

Figure 2.

MS/MS spectra of peptides derived from the top 3 proteins found in sweat samples.

(A) TVQIAAVVDVIR from Prolactin-Induced Protein with a precursor m/z of 642.39²⁺.

(B) DAVEDLESVGK from Dermcidin preprotein with a precursor m/z of 581.28²⁺.

(C) RDPTAEQFQEELEK from Caspase 14 precursor with a precursor m/z of 860.41²⁺.

Figure 3. Venn diagram showing overlap between the number of proteins identified by LC-MS/MS analyses on the LTQ-Orbitrap (Thermo) from the 1st pooled sweat samples and whole serum.

Figure 4.
Gene ontology analysis for molecular functions of proteins identified in serum (A) or sweat (B).

Figure 5.
Gene ontology analysis for cellular components of proteins identified in serum (A) or sweat (B).

Figure 6.
Gene ontology analysis for biological processes of proteins identified in serum (A) or sweat (B).

Figure 7.

Venn diagrams showing overlap between the numbers of proteins identified by LC-MS/MS analyses on the LTQ-Orbitrap (Thermo) from (A) the 1st set of pooled sweat and control samples, and the 2nd set of pooled sweat and control samples; and (B) the 2nd set of pooled sweat and control samples, and whole serum.

Figure 8.

Workflow showing the use of the yeast enolase tryptic digest in order to monitor sample and instrument performance for the MRM analyses of the 2nd set of pooled samples. **A)** Sample preparation showing the introduction of the yeast enolase tryptic digest spiked into the 2nd set of patient and control pooled samples. **B)** List of the samples run for MRM analyses of the 2nd set of pooled samples. In between each pooled sample analysis, an MRM analysis of a yeast enolase digest, alone, was performed to continually monitor instrument performance.

Figure 9.

MRM results for sweat peptides/proteins chosen from Scaffold list after LC-MS/MS of second set of pooled samples (Table 4). Each graph shows the summed areas for all transitions, averaged over 3 injections for each separate peptide analyzed. Error bars indicate 1 standard deviation. (A) Alpha-2-glycoprotein zinc, (B) Annexin 5, (C) Arginase, (D) Bleomycin hydrolase, (E) Calmodulin-like Skin Protein, (F) Caspase 14, (G) Corneodesmosin, (H) Cystatin A, (I) Dermcidin, (J) Desmoglein, (K) DJ-1, (L) Glycerol-3-phosphate dehydrogenase, (M) Kallikrein 11, (N) Keratin 10, (O) Peroxiredoxin-1, (P) Prolactin-induced Protein, (Q) Prostatic-binding Protein, (R) S100A7, (S) Thioredoxin, (T) S100A6 [spiked protein standard]. See Table 5 for all %CVs.

Table 1

List of SZ patients that were used to construct sample pools for mass spectrometric analyses. Patient information provided includes: pools patients belong to, age, gender, ethnicity, available patient labs, and medications patients were on during time of sweat collection. Newly recruited patients did not have labs available during time of sweat collection.

Patient I.D.	Age	Gender	Ethnicity	Current Medications	Available Labs
Pool 1 (for Orbitrap LC-MS/MS protein ID. only)					
22	46	Male	Caucasian/Romanian Decent	Risperidone Quetiapine	No Current Labs
23R *	18	Female	Hispanic	Clozapine	CBC, Chem, LFTs, TSH, UA, UDS, EKG- All Normal
23L *	18	Female	Hispanic	Clozapine	CBC, Chem, LFTs, TSH, UA, UDS, EKG - All Normal
25	25	Male	African American	Paroxetine Olanzapine	No Current Labs
27	18	Male	African American	Olanzapine	No Current Labs
32	21	Male	Filipino/Asian	Risperidone Benzotropine	No Current Labs
051L	20	Male	Caucasian	Risperidone Benzotropine	CBC, Chem, LFTs, TSH, UA, UDS- Normal + Cannabis
043L	17	Male	Caucasian	Risperidone Benzotropine	No Current Labs
Pool 2 (for Orbitrap LC-MS/MS and MRM)					
103R	14	Female	Caucasian	Olanzapine Esitalopram	CBC, Chem, LFTs, TSH, UA, UDS- All Normal
104R	16	Female	Caucasian	Risperidone	CBC, Chem, LFTs, TSH, UA, UDS- All Normal
107L	18	Male	Caucasian	Olanzapine Lorazepam	CBC, Chem, LFTs, TSH, UA, UDS- All Normal
041R	17	Male	Native American	Olanzapine	CBC, Chem, LFTs, TSH, UA, UDS- Normal + Methamphetamine, Cannabis

* 23R and 23L were sweat samples collected from the same SZ patient, where R denotes collection from the right forearm, and L denotes collection from the left forearm.

Table 2

List of medications used by patients during time of sweat collection. This table also includes medication-related risk of metabolic syndrome, any medication related changes in prolactin levels in blood, potential heart rate changes induced by medication, any extra pyramidal symptoms associated with medication, and any potential anticholinergic effects of medications.

MEDICATION	METABOLIC SYNDROME	PROLACTIN LEVELS	HEART RATE	EXTRA PYRAMIDAL SYMPTOMS	ANTICHOLINERGIC EFFECTS
ATYPICAL ANTIPSYCHOTICS					
RISPERIDONE	Elevated lipids, cholesterol, blood sugar	Significant Elevation	Increase	EPS: tremor, cogwheeling, dystonia, blurred vision, urinary retention, galactorrhea	Mild
OLANZAPINE	Elevated lipids, cholesterol, blood sugar	Minimal Elevation	Increase	EPS: akathisia	Severe
QUETIAPINE	Elevated lipids, cholesterol, blood sugar	Minimal Elevation	Increase	EPS: akathisia	Severe
CLOZAPINE	Elevated lipids, cholesterol, blood sugar	Minimal Elevation	Increase	Minimal to no EPS: dystonia may occur	Severe
ANTIDEPRESSANTS-SSRI					
PAROXETINE	Minimal Metabolic Syndrome	No Elevation	No Change	Minimal to none	Severe
ESCITALOPRAM	None	No Elevation	No Change	Minimal to none	None
BENZODIAZEPINE	None	No Elevation	No Change	None	None
LORAZEPAM	None	No Elevation	No Change	None	None
ANTICHOLINERGIC MEDICATION					
BENZTROPINE	None	No Elevation	Increase	Reduction of EPS	Severe

Table 3

First set of pooled control and patient sweat samples showing the number of proteins identified in the corresponding number of LC-MS/MS analyses of the 4 technical replicates.

Pooled Control Sweat Sample		Pooled Patient Sweat Sample	
# Proteins Identified	# Replicates Found In	# Proteins Identified	# Replicates Found In
70	4	70	4
21	3	19	3
19	2	12	2
21	1	20	1
19	0 ^a	29	0 ^b

^aThese 19 proteins were identified in the pooled patient sweat sample

^bThese 29 proteins were identified in the pooled control sweat sample

Table 4

List of proteins chosen for MRM analysis based on spectral counts from LC-MS/MS analysis of the second set of patient and control pooled samples.

Proteins	Number of Assigned MS/MS Spectra		
	Patient Pool ^a	Control Pool ^b	% Relative Difference ^b
Alpha-2-glycoprotein 1 Zinc	62	92	−39%
Annexin 5	20	12	50%
Arginase	62	45	32%
Aspartate Aminotransferase	11	2	138%
Bleomycin Hydrolase	32	3	166%
Calmodulin-like 3	15	2	153%
Calmodulin-like Skin Protein	20	14	35%
Caspase 14	204	79	88%
Corneodesmosin	53	6	159%
Cystatin A	22	10	75%
Dermcidin	69	97	−34%
Desmoglein	44	30	38%
DJ-1	11	2	138%
Filamin B	11	8	32%
G3PDH	23	20	14%
Kallikrein 11	7	7	0%
Keratin 10	43	139	−105%
Keratin 1B	4	15	−116%
Keratin 2	43	79	−59%
Keratin 6A	9	23	−88%
Keratin 9	15	16	−6%
Mitochondrial Malate Dehydrogenase	12	2	143%
Nucleobindin 1	2	4	−67%
Peroxiredoxin	16	10	46%
Prolactin-induced Protein	173	385	−76%
Prostatic Binding Protein	20	40	−67%
Pyruvate Kinase 3	23	4	141%
S100A6 (spiked standard)	12	0	200%
S100A7	6	2	100%
Thioredoxin	25	11	78%
Triosephosphate isomerase	15	5	100%

^a Patient pool = total number of assigned MS/MS spectra for each protein identified

^b Control pool = total number of assigned MS/MS spectra for each protein identified

^b % Relative difference = [(patient – control) / Average number of spectra for patient and control] × 100

Table 5

MRM results showing the CV and ratio of the summed fragment ion peak areas for all transitions from yeast enolase peptides spiked into the second set of pooled samples. CVs are calculated for injection replicates.

Enolase Peptide	CV Patient Pool (n=3)	CV Control Pool (n=3)	Patient Pool: Control Pool
TFAEALR	10%	4%	1.2 :1
AADALLK	4%	14%	1.1 :1

Table 6

MRM results showing the CV and ratio of the summed fragment ion peak areas for all transitions from S100A6 peptides spiked into the second set of pooled samples. CVs are calculated for injection replicates.

S100A6 Peptide	CV Patient Pool (n=3)	%CV Control Pool (n=3)	Patient Pool: Control Pool
ELTIGSK	13%	20%	12.4 :1
LMEDLDR	3%	18%	14.5 :1

Table 7

Abundance ratios and %CV of each peptide as determined from MRM analysis of the second set of patient pooled and control pooled samples. Proteins listed met the criteria for MRM verification as described in the results sections. Bleomycin hydrolase is listed as *inf.* due to the fact that there was no signal detected in the control pooled sample. Protein S100A6, spiked into each pooled sample at a ratio of 10:1 (patient:control), is shown in bold.

Protein	Peptide	Patient Pool: Control Pool	Patient CV	Control CV
Alpha-2-glycoprotein	YSLTYIYTGLSK	1.9 : 1	3.0%	2.5%
	AYLEEECPATLR	1.7 : 1	0.5%	2.5%
Annexin 5	VLTEIIASR	4.6 : 1	6.5%	17.5%
	GTVTDFPGFDER	5.1 : 1	15.5%	12.5%
Arginase	GGVEEGPTVLR	2.9 : 1	1.0%	3.0%
Bleomycin Hydrolase	DGEAVWFGCDVGK	inf.	5.0%	n/a
Calmodulin-like Skin Protein	VNYEEFAR	2.1 : 1	2.0%	6.5%
	AGLEDLQVAFR	2.0 : 1	3.0%	4.0%
Caspase 14	FQQAIDSR	2.7 : 1	9.0%	12.0%
	LENLFEALNNK	5.4 : 1	1.0%	2.5%
Corneodesmosin	IILQPCGSK	2.5 : 1	3.5%	4.0%
	GSPGVPSFAAGPPISEGK	3.0 : 1	10.0%	24%
Cystatin A	TQVVAGTNYIYK	2.8 : 1	3.5%	9.0%
Dermcidin	ENAGEDPGLAR	1.0 : 1	2.0%	8.5%
Desmoglein	YQGTLISIDDLQR	3.6 : 1	5.0%	4.0%
DJ-1	DGLILTSR	4.0 : 1	1.0%	1.5%
G3PDH	VPTANVSVVDLTCR	6.7 : 1	5.5%	6.0%
Kallikrein 11	LLCGATLIAPR	2.6 : 1	3.0%	9.0%
Keratin 10	YENEVALR	1.0 : 1	4.0%	1.0%
	ALEESNYELEGK	0.97 : 1	14.5%	11.5%
Peroxiredoxin 1	GLFHDDK	6.2 : 1	4.0%	12.0%
	LVQAFQFTDK	9.3 : 1	6.0%	14.0%
Prolactin-induced Protein	TVQIAAVVDVIR	0.57 : 1	4.5%	6.5%
Prostatic-binding Protein	LYTLVLTPDAPSR	7.2 : 1	3.5%	15.5%
S100A7	GTNYLADVFEK	9.2 : 1	10.0%	21.0%
Thioredoxin	VGEFSGANK	8.9 : 1	5.5%	20.0%
	TAFQEALDAAGDK	10.6 : 1	2.0%	5.0%
S100A6 (spiked standard)	ELTIGSK	12.4 : 1	6.5%	18.0%
	LMEDLDR	14.5 : 1	1.5%	9.0%