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Searching for the Non-Invasive Biomarker Holy Grail: Are Urine Proteomics the Answer?

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Abstract

Recently, biobehavioral nursing scientists have focused attention on the search for biomarkers or biological signatures to identify patients at risk for various health problems and poor disease outcomes. In response to the national impetus for biomarker discovery, the measurement of biological fluids and tissues has become increasingly sophisticated. Urine proteomics in particular, may hold great promise for biobehavioral-focused nursing scientists for examination of symptom- and syndrome- related research questions. Urine proteins are easily accessible secreted proteins that provide a direct and indirect window into bodily functions. Advances in proteomics and biomarker discovery provide new opportunities to conduct research studies with banked and fresh urine to benefit diagnosis, prognosis, and evaluate outcomes in various disease populations. This paper provides a review of proteomics and a rationale for specifically utilizing urine proteomics in biobehavioral research. It addresses as well some of the specific challenges involved in data collection and sample preparation.

Keywords

irritable bowel syndrome; proteomics; mass spectrometry; urine

A. Introduction

For the past three decades biobehavioral nursing scientists have focused their attention on the search for biomarkers or biological signatures to identify patients at risk for health problems and poor disease outcomes (Lovejoy et al., 1987; Thompson & Voss 2009). More recently this search has led to exploration of molecular biomarkers that can potentially predict patient responses to interventions and therapies. For example, a number of nursing scientists have utilized targeted genetic analysis to predict disease risk as well as to understand patient and family member responses to genetic risk factors (McQuirter et al., 2010; Metcalfe et al., 2010).

A biomarker is a measure of a normal biological process, a pathological process, or the response of the body to a therapy. In addition, a biomarker can be used to objectively identify a patient group or subgroup with a disorder, define a treatment responsive population, and/or provide a target for future intervention studies. A specific marker may also offer information about the mechanism of action of a drug or therapy, its efficacy, its safety, and its metabolic profile. In response to the National Institutes of Health impetus for candidate biomarker discovery, the measurement of biological fluids and tissues has become increasingly sophisticated. Building on the work of interdisciplinary colleagues, biobehavioral nursing scientists have further expanded their research from system measures, e.g., heart rate, blood pressure, to embrace newer technologies to understand symptom experiences (Mitchell et al., 2008; Jarrett et al., 2007) as well as recovery from major adverse events (e.g., stroke/depression [Kohen et al., 2008], traumatic brain injury [Chuang, 2010]).

One biomarker discovery approach that may hold promise for biobehavioral-focused scientists is the examination of the proteome (proteomics). The term proteomics defines the global analysis of cellular proteins with mass spectrometry (MS) based techniques, image analysis, reverse-phase protein array, amino acid sequencing, and/or bio-informatics to identify and quantify a large number of proteins. Proteomics is focused on cellular or secreted proteins, both in terms of their structure as well as the functional interaction among proteins. Proteomic science uses both qualitative and quantitative comparison of proteomes to understand further biological function (Breedlove & Bosenhart, 2005). In an early review of genomics and proteomics, Kasper (2007) noted that the study of proteomics increased markedly following the completion of the Human Genome Project in 2003. Advances in high throughput genomics and proteomics have increased the potential for new biomarkers yet the actual output is still quite limited, with most work to date focusing on cancer detection. Furthermore the use of proteomics in biobehavioral science development remains virtually unexplored.

The purposes of this review are to 1) present a brief over-view of proteomics including measurement issues; 2) provide a rationale for utilizing urine proteomics in biobehavioral research; and 3) use a case study to exemplify some of the methodological challenges involved in data collection and sample preparation.

B. Proteomics

B.1. Proteomics in biomedical research

Prior to the advent of the proteomics era, proteins in biological fluids were studied using enzyme activity experiments, antibody detection, and micro-sequencing technology (Kopetzki et al., 1994, Klugman et al., 2008, Mir et al., 2009). However, many of these approaches were laborious and ultimately inefficient. The development of mass spectrometry (MS) technologies along with enzyme linked immunoabsorbent assay (ELISA) combined with a variety of sample and protein preparation strategies increased the ability to quantify even low abundant proteins in body fluids including blood, cerebrospinal fluid, and urine. Although these methods and measures have become more accessible and feasible, it still remains to be determined how scientists will utilize proteomics in their protocols to answer questions focused on the biology of symptom reports.

The use of body fluids for protein and peptide determinations continues to evolve (Schmidt & Aebersold, 2006). It is well established that alterations (i.e., upregulation and downregulation) of protein quantity occur in response to a number of disease processes including cancer and inflammation (Decramer et al., 2008; Apweiler et al., 2009). With improved analytical approaches for qualitative and quantitative measurement, proteins are

increasingly being tested as potential biomarkers for gastrointestinal, renal, liver, prostate, and breast cancer (Chua et al., 2009; Carey, 2010; Hoshida et al., 2010; Pejic et al., 2010). Protein levels and the interrelationships among proteins are influenced by a variety of factors including diet, hormone status, and physical activity, as well as disease state and medication history. Recently Schmidt et al. (2009) suggested that researchers take a more hypothesis-driven approach to utilizing MS and related technologies.

Proteomics has achieved expansive growth in recent years and is still rapidly growing, fueled by innovative experimental approaches, improvements in sensitivity, resolution, and accuracy of mass analyzers. However, the identification and measurement of proteins in body fluids including blood, urine and cerebrospinal fluid may not necessarily reflect changes occurring in a particular body system (e.g., heart, lungs, brain) or coincide with the clinical phenotype (fatigue, dyspnea, cognitive dysfunction). As Simpson (2009) notes for proteomics to be viable as a biomarker they must have high sensitivity (be positive for those with the disease or disorder) and high specificity (negative for those without the disease or disorder).

Determining whether protein levels are sensitive to pharmacologic and nonpharmacologic therapies, thus allowing them to be used as outcome measures, is in its infancy. In a recent paper, Pitteri and colleagues (2009) described the use of serum proteomics to examine outcomes in women in the Women's Health Initiative Study treated with estrogen plus progesterone and women treated with estrogen alone (Pitteri et al., 2009). Serum proteins involved in metabolic pathways related to coagulation, metabolism, osteogenesis, inflammation, and blood pressure regulation were upregulated in response to estrogen and estrogen plus progesterone therapy. Such findings open the door for both further mechanistic and therapeutic studies utilizing proteomic approaches.

B.2. Protein identification by Mass Spectrometry

A proteome reflects, not the entire genome of possible open reading frames present in each cell, but rather only those proteins expressed by a cell in a given environment or state. Quantitative proteomics is defined as comparison of relative changes in different proteomes (e.g., disease vs. normal), and it is an important component in the emerging proteomic sciences. Qualitative proteomics would be discriminating the proteome based on their MS characteristics. Qualitative proteomics refers to the identification and characterization of whole proteins by the generation of peptide-based mass spectra, the data analysis with matching algorithms, and finally the validation with immune-affinity techniques or mass spectrometry.

Two methods can be employed to generate qualitative and quantitative profiles of complex protein mixtures. The first and more traditionally used is a combination of one- or two-dimensional gel electrophoresis (1-DE or 2-DE) with mass spectrometry (MS). These methods, 1DE-MS or 2DE-MS, utilize protein "spot" intensities on gels. Using the 2DE Fluorescence system it is possible to separate up to three different samples within the same 2-D gel. With this approach, an internal standard is present in every gel and thus the gel is 'normalized' to a given internal control. Since the proteins from the different sample types (e.g., healthy/diseased, infected/non-infected) are run on the same gel they can be directly compared. While resolving power of 2DE-MS is arguably its best feature, it is limited by loading capacity problems which ultimately affect sensitivity. The second method is a more recently developed technique called *shotgun proteomics* (Goo & Goodlett, 2010). There are multiple variations on the shotgun proteomic theme (Gilmore, 2010). Many of these approaches involve protease digestion of a complex protein sample to make peptides that are in turn analyzed by tandem mass spectrometry (MS/MS) to identify the proteins from which they were derived. This peptide-based approach circumvents the fundamental decrease in

fragmentation efficiency that accompanies increasing molecular weight of proteins. One important limitation of standard shotgun methods is the requisite proteolysis of proteins to peptides of which only some are detected in the mass spectrometer while many others are not. This loss of protein sequence information means that shotgun proteomic experiments typically produce low protein sequence coverage.

B.3. Protein quantification by mass spectrometry

A few popular methods of proteomic quantification used in current research include: first, the aforementioned combination of gel electrophoresis and MS. In this approach 1-DE or 2-DE are used to distinguish differentially expressed proteins based on staining intensity of the gels and proteins identified by tandem MS (MS/MS). Protein identification may also be carried out via simple matrix-assisted laser desorption/ionization-time of flight MS (MALDI-TOF MS) peptide mass fingerprinting. Second, stable-isotope labeling is routinely used in protein quantification. This method introduces pairs of chemically, metabolically, or enzymatically identical “mass” tags that may be separated and then corresponding proteins identified by MS/MS of the labeled peptides. Third, label-free quantification is a method in which protein quantity is inferred from the number of spectra produced for all peptides from a given protein. This approach has recently become popular for its ease of use.

Currently large scale MS assays are expensive and may be beyond the reach of researchers who pose exploratory questions related to symptom candidate biomarkers. However, several multidimensional strategies for protein isolation have been developed that when combined with MS can potentially provide a stream-lined approach (Adachi et al., 2006; Goo et al., 2010). Such methodologies were designed to enrich, fractionate, and quantitate proteins for MS analysis and ultimately biomarker discovery.

B.4. Proteomics data processing

As pointed out by Founds (2009) in a recent review of systems biology, proteomic analyses much like genomic analyses yields massive volumes of data. As such, computational and pathway modeling programs are critical to the data analysis and interpretation. In a typical Trans-Proteomic Pipeline (www.systemsbio.org), the acquired MS/MS data are searched for protein identification against a database (i.e., International Protein Index [IPI] human protein database) using SEQUEST (Goo et al., 2010). Analytical programs such as PeptideProphet and ProteinProphet, which compute a probability of each identification being correct, are used for statistical analysis. Only proteins identified by more than one unique peptide sequence are typically used in the data analysis (Goo et al., 2010).

B.5. Verification of proteomic data

The potential for false positive results with MS requires verification. Initially proteins of interest identified by MS can be further verified by an orthogonal method such as Western blot analysis followed by a large-scale verification using enzyme-linked immunosorbent assay (ELISA) if an antibody or an ELISA kit is available. Many potentially promising biomarker candidates have been identified in human disease research with the help of proteomics in recent years. However, most of these studies have been limited to the “discovery” stage with putative biomarkers still awaiting verification or they have already failed confirmation as true markers when subjected to larger follow-up studies. These results demonstrate the relative ease of putative biomarker discovery coupled to difficulties in validation in current research (Goo & Goodlett, 2010).

C. Urine Proteomics

C.1. Urine as a biological fluid for biomedical research

Clinical researchers have long been interested in measures, including biomarkers that can be collected non-invasively, with minimal discomfort and subject burden. At the same time, such measures need to represent the biological mechanism or phenomena of interest. Urine collection is viewed as one potential route for sample collection. Multiple investigators have utilized urine samples to measure steroid hormones such as cortisol (Heitkemper et al., 1996; Hu, Jiang, Zeng, Chen, & Zhang, 2010; James et al., 2008), ovarian hormones (Woods et al., 2010), and peptides such as melatonin (Thomas 2010; Hu et al., 2010).

Because of its accessibility and ease of collection, urine has become one of the more attractive biofluids for proteomics (Julian et al., 2009; Moons KG, 2010; Goo & Goodlett, 2010). Urine is rich in a variety of proteins that are either filtered or secreted into, or shed by the urinary tract. Although urine contains fewer proteins than plasma, the urine proteome is complex and variable. Currently a urine biomarker network links genomic profiles from 127 diseases to 577 proteins detectable in urine (Dudley, 2009). The majority (> 80%) of these putative protein biomarkers are linked to multiple as opposed to single disease conditions. This observation is in agreement with a recent development that a disease phenotype can be better stratified by a panel of biomarkers rather than by using a single biomarker.

Urine proteomics were originally thought to be a venue for biomarker discovery solely for renal or uro-epithelium disorders. More recent discovery efforts have examined their potential for providing insights into mechanisms of health problems such as cancer and inflammation that originate outside the urinary tract system (Sobhani, 2010). Emerging data suggest that disorders involving infection, structure alterations, and coagulopathy are reflected by proteomic alterations in urine.

Similar to serum proteomics, urine proteomics may provide clues to disease etiology and pathophysiologic mechanisms as well as responses to therapeutic interventions. For example, in a clinical study Buhimschi and colleagues (2008) reported that urine proteomic approaches could be used to successfully predict preeclampsia. Women who developed preeclampsia displayed abnormal urinary profiles more than 10 weeks before clinical manifestation appeared. Using tandem MS/MS and de novo sequencing they identified the biomarkers as nonrandom cleavage products of SERPINA1 and albumin. The 21 amino acid C-terminus fragment of SERPINA1 was highly associated with the presence of severe preeclampsia. The investigators concluded that urine proteomics identified a proteomic fingerprint (group of proteins) which could be potentially useful in the screening for preeclampsia. In addition they found this approach could distinguish preeclampsia from other hypertensive proteinuric diseases.

Urine proteomic markers have correlated also with coronary artery disease (Zimmerli et al., 2008), cancer identification (True et al., 2010), and increased risk for organ transplant rejection (Sigdel et al., 2008). For example, Snow and colleagues employed 2-D fluorescence difference gel electrophoresis (2-D DIGE) and MS urine proteomics followed by validation with Western blot using samples from 60 children with obstructive sleep apnea (OSA). Sixteen differentially expressed proteins were identified in the urine from children with OSA and seven of these proteins were verified by immunoblot analysis. In particular, urine uromodulin, urocortin-3, orosomucoid-1, and kallikrein levels were found to be predictive of OSA. Whether these proteins are linked directly with OSA or represent a more generalized inflammatory response is an intriguing question. It should be noted that thus far, prospective disease-specific protein biomarkers have been found in only a small subset of the urine proteomes.

C.2. Methodological challenges in urine sample collection

The first challenge is the limited information on what constitutes the normal urine proteome and variability among normal urine samples. Currently there are over 1500 proteins that have been identified in normal/healthy human urine (Adachi et al., 2006). Similar to serum proteomics there are established reference profiles of urine proteins (Jai, 2010). Researchers are just beginning to understand how endogenous variables, e.g., urine composition, pH, ion concentrations, as well as peptides and proteins within the urine itself can influence protein levels.

Uromodulin is the most abundant and best characterized protein in urine of healthy people. It binds several low molecular weight proteins and plasma peptides that enter the tubular filtrate. Uromodulin levels are viewed as potential biomarker for urinary tract cancers that involve uro-epithelial tissues. However, exercise, variations in diet, and circadian rhythms all influence uromodulin levels indicating that standard operating procedures for urine collection must be carefully considered in urine proteomic studies (Afkarian et al., 2010). For example, should first void urines be used or only 24 hour sample collections? Should physical activity be limited? Should all samples be collected from participants who have fasted or maintained a constant dietary intake?

Unique to the use of urine as a biofluid is the additional question of standardization across samples. The concentration of a given protein in urine is dependent on glomerular filtration rate (Waikar et al., 2010). This may be accounted for in urine proteomics by measurement of creatinine and expression of protein values as per mg of creatinine. Another approach is to normalize the samples with a well characterized urine polypeptide specific to the disease or condition (e.g., creatinine for prostate cancer [Christenson et al., 2008] or uromodulin for OSA [Snow et al., 2010]).

C.3. Urine proteomics in symptom research

Although observations support the use of proteomics beyond a single disease to include those disorders which share common metabolic pathways, it is unclear whether urine proteomics can be used to identify biomarkers for functional disorders or symptoms. Potential urine proteomic markers that could be linked to symptoms such as fatigue, insomnia, and pain include proteins involved in the production and secretion of inflammatory modulators (e.g., cytokines) thought to play a role in these symptom expressions (Haack et al., 2007).

D. Case Report: Abdominal Pain Exemplar

D.1. Irritable Bowel Syndrome (IBS)

In 2009 our interdisciplinary team received ARRA funding for a study titled '*Pathways to Abdominal Pain in Irritable Bowel Syndrome* (5RC2NR011959). Irritable bowel syndrome (IBS) is a chronic functional gastrointestinal (GI) disorder characterized by abdominal pain associated with alterations in bowel function (constipation, diarrhea). The severity of symptoms ranges from mild to severe and disabling. Current theories of IBS etiology suggest inflammatory as well as visceral sensory disturbances (Drossman et al., 2002). There is no known single biomarker that is presently predictive or diagnostic for IBS. Given the heterogeneity of the symptoms (pain, constipation, diarrhea), it is likely multiple pathophysiologic factors may be at play. The lack of a biomarker that can be used to identify patients with IBS or those who are at risk (e.g., post gastroenteritis) may contribute to the number of diagnostic procedures performed and as well as the clinical burden of this common condition (Halpert, 2010).

To date, proteomic methods have not been utilized in human IBS studies. Using an animal model of IBS (abdominal withdrawal reflex in response to bowel distention) Ding and colleagues (2010) examined colonic tissue proteomics. Of the 13 differentially expressed proteins found, 8 were upregulated and 5 were downregulated in tissues from the 'IBS' rats as compared to non-IBS rats. The authors noted that all of the differentially expressed proteins were associated with inflammation and nerve regulation. Whether the expression patterns can be found when a non invasive sample (urine) in humans is used remains to be determined.

The focus of our project was to examine previously collected urine specimens from adult women for specific proteins that could potentially distinguish subgroups of patients with IBS as well as to provide insights into the pathophysiology of increased visceral pain. Thus, our hypothesis was that urine proteomic approaches could be employed to identify biomarker signatures for outcomes of clinical trials as well as shed light on mechanisms involved in this complex disorder.

D.2. Urine sample processing and IBS symptom clustering

Urine samples were previously collected as part of a study (NR04142) (Jarrett et al., 2009) of adult women with and without IBS. Women provided a first morning voided specimen at multiple, predetermined time points. Menstrual cycle phase was accounted for with the use of a daily symptom diary for 28 days. The proteomic analysis of stored samples was approved by the University Human Subjects Review Committees. Subject recruitment and study design were previously described (Jarrett et al., 2009).

To explore the feasibility of conducting proteomic analysis on stored urine samples, the first task was to determine if proteins were intact without degradation in samples kept at -80°C for a variable number of years. Preliminary analyses comparing protein quantities of two fresh urine samples to those of stored samples indicated no significant qualitative differences in number of proteins identified.

The next step was to determine the specific samples to be pooled for this feasibility 'shotgun' study of the urine proteome. For the purposes of this project IBS subjects were classified based on symptom profiles derived from both prospective 28 day-diary and recall questionnaire data. In particular, data derived from measures of abdominal pain, constipation, diarrhea and psychological distress were used. In the daily diary, women reported GI and psychological distress symptoms on a 0 – 'not present' to '4' severe scale as previously described (Cain et al., 2009). Based on the natural clustering of symptoms we selected four symptom groups: 1) constipation (n=19), 2) diarrhea + high pain (n=13), 3) diarrhea + low pain (n=19), and 4) high pain + high psychological (n=12) distress. This symptom classification matched our earlier work in which we described IBS bowel pattern subgroup (diarrhea, constipation) differences in heart rate variability (Jarrett et al., 2007) and stress hormone level (cortisol, catecholamine) especially during sleep (Burr et al., 2010). The fifth group (controls, n=10) was composed of women who did not have a history of IBS but also reported no or low GI symptom frequency on their daily diary.

Individual subject samples were screened for menstrual cycle phase to avoid using samples collected during menses. In addition, all samples were tested for hemoglobin (presence of RBCs) and excluded if hemoglobin greater than 0.03mg/dL was found. Out of 103 urine samples that were tested seven were positive. Of the 96 remaining samples without the presence of blood, 50 were used for proteomics study. In each group, 3mL of urine was collected from 10 subjects to make a 30mL total pooled sample (Figure 1). Investigators including the operator of the MS were blinded to the match between the symptom subgroup and sample until MS analyses were complete.

D.3. Proteomic analysis of IBS urine samples

Mass spectrometry was performed to qualitatively and quantitatively catalog proteins in the pooled samples. Peptide digestion products were analyzed by electrospray ionization on a linear ion trap Velos mass spectrometer (Thermo Scientific Corp., San Jose, CA). For each liquid chromatography-tandem MS (LC-MS/MS) approximately 0.5µg of peptides were loaded on the column and eluted in acetonitrile gradient. To maximize protein identification without protein fractionation, data-independent PACIFIC (Panchaud et al., 2009) method which acquire data every 37.5 *m/z* units was employed. Each experiment was done in duplicate (Figure 1).

More than 800 proteins were identified by MS of urine from each IBS subgroup. Of these more than 200 proteins showed ± 2 fold differences in protein quantity compared to the normal control group.

The selection of the initial candidate proteins for verification was based on the current hypothesized models (e.g., heightened pain sensitivity, inflammation) of IBS pathophysiology as well as the good agreement between the two replicate MS datasets. To select proteins for follow up verification study with Western blot consideration was given to the potential risk for the protein to serve as a biomarker in IBS. In our initial examination we found the protein gelsolin, was up regulated in both of the diarrhea+ high pain and high pain +high psychological distress subgroups as compared to the control sample, possibly representing a pain biomarker for IBS (Figure 2.A.). Gelsolin is an actin regulator and an inhibitor of apoptosis. In this latter role, it appears to stabilize mitochondrial membranes. The pooled MS findings were next verified using Western blot to determine if group differences in gelsolin levels could be replicated (Figure 2.B.). Moving forward, the next step will be verifying the expression level of gelsolin as well as other proteins in the urine from individual IBS and control subjects that make up the pooled sample by using ELISA. Ultimately, these results will need to be verified in a larger cohort. However, transition to these steps is dependent in part on the availability of suitable antibodies. In some cases an antibody is not adequately specific to differentiate different isoforms of the protein. It should be pointed out that one of the limitations of this feasibility study was that individual samples were not consistently randomized from a subgroup sample. This occurred because one subgroup only contained 10 subjects who met the clinical phenotype criteria. In addition, at this point we can only speculate as to the potential role of gelsolin in pathways responsible for pain sensitivity. To date, it has been noted that the levels are altered in the plasma and cerebrospinal fluid of patients who are HIV+ suggesting a role in inflammation (Pottiez, Haverland, & Ciborowski, 2010).

E. Future Directions

Proteomics is a rapidly growing field with potential for advancing nursing research. It remains to be determined, however, whether urine proteomics will yield useful biomarkers for clinical and mechanistic studies of chronic disorders such as IBS. In order for urine proteomic measures to be viewed as a biomarker it is critical that the values are representative of an established scientific framework or body of evidence that elucidates the physiological, toxicologic, pharmacologic, or clinical significance of the test results. Its utility as an approach to biomarker discovery will be closely linked to how it can be used clinically to predict symptom occurrence and response to therapies. Sufficiently trained nursing scientists and well established collaborations between proteomic core facilities and individual investigators are the cornerstones to integrate proteomics approaches into the research repertoire of nursing scientists.

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Figure 1. Schematic presentation of proteomics sample preparation and mass spectrometry workflow

For each group 30ml urine was collected by pooling 3ml of urine from 10 subjects. Only the first morning and those not containing blood samples were included in the study. The pooled samples were centrifuged briefly to remove cell debris and impurities and then concentrated by reducing volume using 3kDa cut-off filter. Urine protein was isolated and purified by trichloroacetic acid precipitation. Protein was then subjected to trypsinization for mass spectrometry (MS). MS data were acquired in duplicates and quantification was carried out by spectral counting. MS quantification was validated either by Western blot or ELISA analyses.

A. **B.** **Figure 2.**

Expression levels of gelsolin in four IBS subgroups and the control group. A) Spectral counts were used to represent fold changes in IBS subgroups in comparison to the control group. B) Protein expression level was measured by Western blot using specific antibody to GSN. The sample order is the same as in Fig 1.A.