

## Peptidomics of Urine and Other Biofluids for Cancer Diagnostics

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**BACKGROUND:** Cancer is a leading cause of death worldwide. The low diagnostic sensitivity and specificity of most current cancer biomarkers make early cancer diagnosis a challenging task. The comprehensive study of peptides and small proteins in a living system, known as “peptidomics,” represents an alternative technological approach to the discovery of potential biomarkers for the assessment of a wide variety of pathologies. This review examines the current status of peptidomics for several body fluids, with a focus on urine, for cancer diagnostics applications.

**CONTENT:** Several studies have used high-throughput technologies to characterize the peptide content of different body fluids. Because of its noninvasive collection and high stability, urine is a valuable source of candidate cancer biomarkers. A wide variety of preanalytical issues concerning patient selection and sample handling need to be considered, because not doing so can affect the quality of the results by introducing bias and artifacts. Optimization of both the analytical strategies and the processing of bioinformatics data is also essential to minimize the false-discovery rate.

**SUMMARY:** Peptidomics-based studies of urine and other body fluids have yielded a number of biomolecules and peptide panels with potential for diagnosing different types of cancer, especially of the ovary, prostate, and bladder. Large-scale studies are needed to validate these molecules as cancer biomarkers.

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Cancer is a major clinical problem worldwide. Accounting for approximately 1 in every 4 deaths, cancer represents the second leading cause of death in developed countries, after cardiovascular diseases. It is estimated that more than 1.6 million new cancer cases are diagnosed every year in the US (1). Highly heterogeneous and with only a few effective therapeutic strategies, cancer represents a challenging medical condition for both healthcare professionals and governments.

The possibility of detecting cancer at early stages, before it spreads to anatomically distant tissues, has long interested physicians and scientists, because early diagnosis is a key factor for successful treatment outcomes. For instance, the overall 5-year survival rate for ovarian cancer is <40%, whereas the rate increases to 90% if it is detected in its early stages (2). Consequently, finding new tools, such as endogenous biomolecules, that could help identify patients with early-stage disease is highly desirable. As stated by the WHO in 1968, the ideal biomarker for a disease should be measurable via a simple, reliable, and affordable method and have a high diagnostic sensitivity and specificity. The biomarker should be present in higher-than-normal concentrations during early disease stages, and its concentration should reflect the extent or severity of the disease. Defining the target population for whom the test would be applied is also of major concern.

Unfortunately, only a few cancer biomarkers have entered routine use. Even fewer have been approved for population screening or diagnosis (3). One of the most frequently used cancer biomarkers is prostate-specific antigen (PSA).<sup>6</sup> Despite its widespread measurement, many issues relating to overdiagnosis and overtreatment have arisen because serum PSA also increases in benign prostatic hyperplasia and other nonmalignant diseases (4). Similarly and despite being considered the best biochemical marker for breast cancer, carbohydrate antigen 15.3 is also increased in other tumors, such as pancreatic and colorectal cancers, as well as in a number of benign pathologies. The lack of diagnostic sensitivity for this antigen, the serum concentrations of

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<sup>6</sup> Nonstandard abbreviations: PSA, prostate-specific antigen; CSF, cerebrospinal fluid; LC-MS/MS, liquid chromatography–tandem mass spectrometry; CA125, carbohydrate antigen 125.

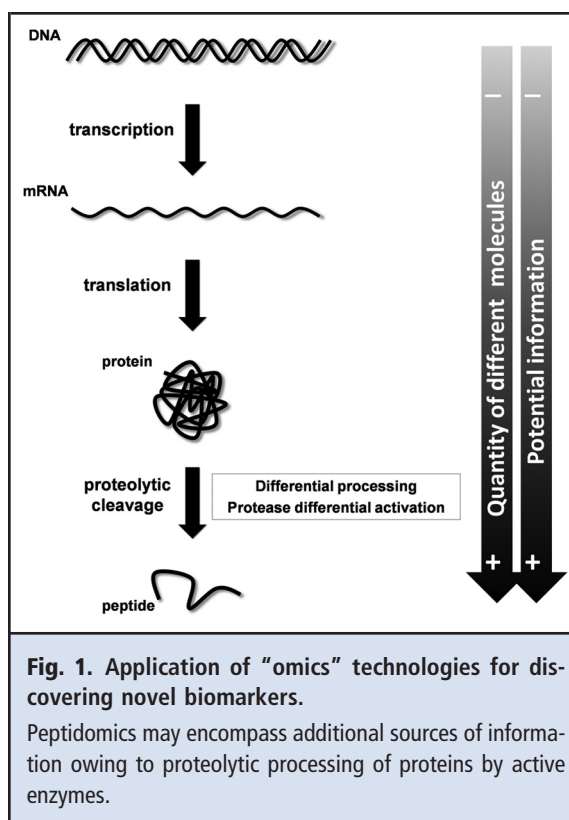
which barely increase in early-stage malignancy, is also an important limitation (5). Clearly, there is an urgent need to discover and validate new biomarkers with better performance characteristics.

High-throughput technologies that generate massive quantities of data have become known as “omics,” a suffix derived from “genomics,” the comprehensive study of genes and other DNA sequences (i.e., the genome)—hence transcriptomics, proteomics, metabolomics, epigenomics, and peptidomics. Three steps are essential in the process of developing a biomarker (3): (a) the discovery of candidate molecules in defined patient groups, (b) validation of the biomarkers for their capacity to assist in disease assessment, and (c) implementation in the clinical setting.

This review focuses on the current situation of cancer biomarker discovery through peptidomics. The emphasis is on urine, but this review also covers investigations of other body fluids. Technological aspects of peptidomics and their applications to different types of cancer are also reviewed.

### Proteomics and Peptidomics

Since the dawn of the genomics and transcriptomics era, numerous efforts have been directed toward discovering biomarkers that could help in the diagnosis, prognosis, or monitoring of different diseases. The main limitation of nucleic acid–based approaches is that recognition of an inherited predisposition to disease is usually not sufficient to identify the biological processes and mechanisms by which they operate (6). This limitation can be partially alleviated with proteomics. A complete analysis of the protein content of a cell, tissue, or organism comprises all of the layers of information gathered from the genome and the transcriptome, plus posttranslational modifications (e.g., phosphorylation, glycosylation). Proteomics is the large-scale study of the full complement of proteins in a living system—their structures, their physicochemical properties, and their functions. Proteomic technologies have the potential to detect dynamic changes in the production of proteins via these technologies’ integration of the proteome’s genetic and epigenetic features (7). The proteome is hence much more complex than the genome or the transcriptome, and it appears to reflect actual cellular processes more accurately than the genome or the transcriptome. Proteins are the effectors of biochemical actions (Fig. 1). From a pathophysiological point of view, genetic analyses can predict the risk of developing a disease, whereas proteomic approaches have a capacity both to show when the risks become evident as a disease and to facilitate monitoring of the therapeutic response. Both the concentra-

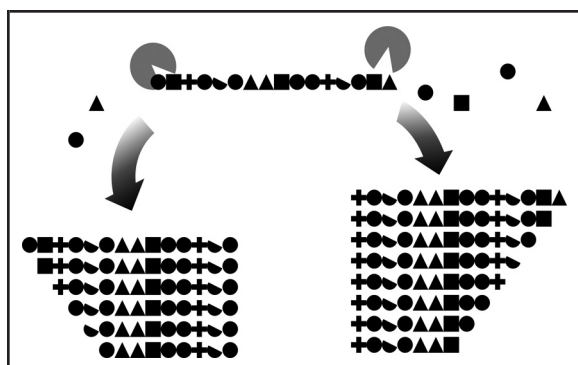


tions of proteins and their posttranslational modifications may be altered during disease progression (8).

The peptidome constitutes the low molecular weight proteome. The term “peptidomics,” a term coined in 1996, is the systematic and comprehensive analysis of the small proteins and endogenous peptides of biological samples at a defined time. Peptidomics typically encompasses polypeptides  $\leq 20$  kDa, although no clear limit has been established. It is interesting that results obtained with the first proteomic methodologies appeared to indicate that the peptide content samples was too simple and easily cleared by the kidney to carry useful information. That turned out not to be the case, for research efforts with peptidomics have already yielded positive results.

Most peptides in biological systems are not synthesized as such, but rather are derived from precursor proteins via proteolytic cleavage by endogenous peptidases in a specific or nonspecific way (Fig. 2), e.g., the activation of some zymogens of the coagulation cascade or the maturation of insulin. Other peptides are generated in situ and then traverse the endothelial vasculature, if they are sufficiently small to enter the blood passively or the wall becomes permeable owing to disease conditions (9, 10).

Proteolytic processing has been theorized to be necessary to facilitate metabolic variation so that indi-



**Fig. 2.** Generation of a series of peptides from a core peptide produced via endopeptidase activity.

Amino- and carboxypeptidases can further cleave the core peptide, generating numerous fragments, which can then be identified by mass spectrometry (12, 66).

viduals and species may better adapt to exogenous stimuli (11). Actually, peptides in body fluids are believed to be due to an imbalance between the activities of proteases on the one hand and the actions of protease inhibitors on the other. In this way, endogenous proteases are differentially regulated in the contexts of many physiological and pathologic phenomena (12). Therefore, it is reasonable to hypothesize that studying protease activity and regulation can lead to improved detection and a deeper understanding of the molecular mechanisms of some diseases.

Peptides also play central roles in healthy physiological processes (13). That is the case for many cytokines, growth factors, and some neuropeptides for which proteome mapping studies have revealed no precursor protein (14), suggesting that they are synthesized as such in the central nervous system and are not breakdown products of precursor proteins. Even peptides processed from large proteins usually show biological functions and activities different from their parent molecules (7). Given that a high percentage of proteins undergo proteolytic cleavage, identifying and characterizing their breakdown products might be of interest, because they could be even more informative than the precursor protein (10). The study of differential protease activities could be an inviting field for the application of peptidomics to medicine. For example, neoplastic processes are involved in the transformation and proliferation of certain cell types and thus alter the concentrations and activities of specific proteins and enzymes, such as proteases. Therefore, not only do proteins in the system (proteomics) become altered, but their metabolic products (peptides), which should be regarded as an extension of the proteome, also change.

**Table 1.** Examples of current peptide biomarkers used in clinical diagnosis.

Biomarker	Fluid	Disease
NT-pro-BNP <sup>a</sup>	Blood (serum)	Heart failure, ventricular dysfunction
Pro-GRP	Blood (serum)	Neuroendocrine tumors, small cell lung cancer
$\beta$ -CTX	Blood (serum)	Bone turnover
PINP	Blood (serum)	Bone turnover
Pancreatic polypeptide	Blood (serum)	Neuroendocrine tumors
Osteocalcin	Blood (serum)	Osteoporosis
$\beta_2$ -Microglobulin	Blood (serum)	Renal disease and inflammation
Calcitonin	Blood (serum)	Medullary thyroid carcinoma
Cystatin C	Blood (serum)	Renal failure
C-peptide	Blood (serum), urine	Diabetes mellitus
VIP	Blood (plasma)	Pancreatic tumor
ANF	Blood (plasma)	Heart failure
NTX	Urine	Bone turnover
$\beta$ -Amyloid (1–42)	CSF	Alzheimer disease

<sup>a</sup> NT-pro-BNP, N-terminal end of the pro-brain natriuretic peptide; pro-GRP, pro-gastrin-releasing peptide;  $\beta$ -CTX, cross-linked collagen type I C-terminal telopeptide; PINP, procollagen type I N-terminal propeptide; VIP, vasoactive intestinal peptide; ANF, atrial natriuretic factor; NTX, collagen type I N-terminal telopeptide.

### Peptidomics of Body Fluids

The peptidome constitutes a still mostly unexplored source of biological information, and it might provide useful biomarkers for disease assessment. Peptides in body fluids are proxies for protein synthesis, processing, and degradation. Worth mentioning is that some peptide biomarkers have already entered the clinic, although none of them were discovered with contemporary peptidomic methods (15) (Table 1). The peptides most widely known are the aminoterminal propeptide of brain natriuretic peptide (which is measured in serum for assessing heart failure) and C-peptide (for monitoring endogenous insulin production in diabetes patients). Collagen N-terminal telopeptides are measured in measured in urine as biomarkers of bone turnover (16). Thus, body fluids represent attractive sources to mine for informative proteins and peptides (Table 2).

#### PEPTIDOMICS OF BLOOD

Blood fluid (serum or plasma) is regarded as the most valuable specimen for biomarker elucidation (17), because blood is the transportation medium for most tissue-derived molecules in the organism. Therefore,

**Table 2. Characteristics of body fluids for peptidomics.**

Body fluid	Invasiveness of sample-collection procedure	Relative peptide stability
Serum	Minimally invasive	Unstable
Plasma	Minimally invasive	Unstable
Urine	Noninvasive	Stable
CSF	Invasive	Not known
Saliva	Noninvasive	Unstable
Ascitic fluid	Invasive	Unstable
Pleural fluid	Invasive	Unstable
Tears	Noninvasive	Not known

this biofluid can reveal the pathophysiological states of a broad spectrum of tissues and organs. Compared with healthy cells, disease-affected cells within tissues could differentially harbor peptides and proteins eventually released into the interstitial fluid and later into the bloodstream. The high protein content of serum makes it an attractive fluid for peptidomics; however, proteins and peptides are present in serum over a wide and dynamic range of concentrations— $>10$  orders of magnitude (7). This fact represents a considerable analytical challenge, because a few high-abundance proteins (albumin, immunoglobulins, transferrin,  $\alpha_1$ -antitrypsin, haptoglobin) hamper the identification of low-abundance molecules, which are more likely to be biomarker candidates (18). Given that the composition of blood reflects the metabolic state of the entire body as it transports molecules released from virtually any tissue or organ, pathophysiological changes in any single organ could easily be missed. Because the clotting time has a substantial effect on the polypeptide content of serum, plasma is often used instead (19).

Nevertheless, a few studies have demonstrated the applicability of serum peptidomic profiling to a range of medical conditions, although most of these studies have not been validated for biases and artifacts. Shen et al. (20) explored the plasma peptidome (the entire collection of protein breakdown products) in the quest for breast cancer biomarkers and detected increased concentrations of cancer-relevant protein products, including extracellular matrix components, innate immune system molecules, proteases, and protease inhibitors. In another study, Villanueva et al. (13) compared the peptidomes of patients with metastatic thyroid carcinoma with those of age- and sex-matched controls and obtained a 12-peptide signature for identifying malignancy that had a 95% diagnostic sensitivity and 95% specificity. These and similar approaches

have been challenged for bias and artifacts (21, 22) (<http://www.jci.org/eletters/view/26022>).

#### PEPTIDOMICS OF CEREBROSPINAL FLUID

Cerebrospinal fluid (CSF) is considered an outstanding source of biomarkers for neurologic diseases. A colorless fluid produced in the choroid plexus in the brain, CSF provides mechanical protection, nutrient supply, waste product removal, and metabolite transportation. Via continual interactions, CSF contains molecules that can reflect many of the processes of the central nervous system (14). Proteomic efforts have been aimed at characterizing the CSF peptidome to discover potential biomarkers for neurodegenerative conditions, neuropsychiatric disorders, traumatic brain injury, brain tumors, and aging-related conditions (23, 24). Wijte et al. used an enhanced mass spectrometry-based approach for both free peptides and peptides bound to proteins to evaluate postmortem CSF from patients with Alzheimer disease and identified a series of candidate peptides for its diagnosis, such as VGF, nerve growth factor, and C4 complement precursor (25). Additional verification studies remain to be done.

An analysis by Zougman et al. (14) revealed 391 peptides derived from 91 different proteins, and a more recent study yielded 626 unique peptide sequences of  $<5$  kDa that were derived from 104 proteins (26). The enrichment of CSF with small peptides might be due to a higher rate of filtration from plasma compared with components of higher molecular weight.

One of the major disadvantages of CSF studies is the invasive nature of the collection procedure, making it inappropriate for general screening of presumably healthy individuals or all patients with neuropathologies.

#### PEPTIDOMICS OF SALIVA

Saliva is a multifunctional body fluid secreted by the major salivary glands (parotid, submandibular, and sublingual glands) and other glands distributed in the oral cavity. It lubricates the oral cavity and participates in digestion and preventing infections (27). Saliva contains bacteria, cellular debris, crevicular fluid, and serum components, and as an alternative sample for noninvasive collection, saliva has promising, unique features (28). Proteolytic degradation occurs as soon as proteins enter the oral cavity and continues after a saliva sample is collected. This process leads to great variation in the peptide profile and thus limits the reproducibility of peptidomic analyses. Other preanalytical variables, such as sex, age, diet, and circadian rhythms, can also play important roles in the peptide composition of saliva (29). Despite these shortcomings, peptidomic analyses of saliva have been used to assess a variety of pathologies, including Sjögren syndrome, xerostomia, and diabetes (30). Studies of oral cancer

(31, 32) have identified a number of peptides that are overproduced in patients with squamous cell carcinoma. Hu et al. (31) reported a combination of 5 proteins that could be used to detect oral cancer with 90% diagnostic sensitivity and 83% specificity.

#### PEPTIDOMICS OF TEARS

Tears are a complex extracellular fluid that can be assessed noninvasively. As with plasma, the concentrations of proteins and peptides in tears span several orders of magnitude (33), with low interday but remarkably pronounced interindividual variation (34). de Souza et al. (35) identified 491 proteins in tears, and a more recent study yielded 1543 proteins (33). The most comprehensive peptidomics study of tears to date characterized 30 endogenous peptides, most of which were derived from proline-rich protein 4, a protein of unknown function produced at high concentrations in lacrimal acinar cells (36). As a body fluid, tears have a composition that reflects the pathophysiological state of the underlying tissues and organs and has proved useful for assessing both ocular and systemic pathologies, such as dry eye, meibomian gland dysfunction, and Sjögren syndrome (37, 38).

#### Urine as a Source of Biomarkers

Urine is formed in the kidney by ultrafiltration of the plasma for the elimination of metabolic waste products. Because urine is stored in the bladder for several hours before elimination, proteolytic degradation by endogenous proteases has largely been considered to have been completed by the time of voiding (39). Recent findings, however, have demonstrated the presence of a wide spectrum of proteases in urine (40) that might generate various combinations of different endogenous peptides, depending on how long the urine resides in the bladder. Proteolytic degradation of a urine sample can be minimized if second morning urine samples are used, because the time between the first and second voiding can be easily monitored and standardized. Despite such variation, urine is regarded as stable body fluid, especially compared with blood, in which proteases are known to be activated during and after blood drawing and thereby generate a considerable number of breakdown products that may skew proteomic and peptidomic approaches (39, 41).

Urine has long been an attractive body fluid for study, not only in biomarker research but also in clinical diagnosis, mainly because it can be obtained noninvasively in large amounts. Currently, several routinely analyzed biomolecules serve as highly discriminating markers. For example, measurements of urine catecholamines and their metabolites help in as-

sessing pheochromocytoma and in evaluating albuminuria as an index of glomerular function.

Some factors require consideration when planning experiments with urine. Urine shows high daily biological variation, reflecting the effects that many endogenous and exogenous factors have on its production and composition. Diet, exercise, and water intake are 3 major factors influencing the quality and the comparability of samples. Urine contains components released not only from the kidneys and the bladder but also from many other organs, and biological processes can have profound impacts on its fluctuating content (42). Even cardiovascular, autoimmune, and infectious diseases affect the presence and concentrations of some protein molecules (43).

#### URINARY PEPTIDOME

Despite containing very small amounts of proteins, urine samples from healthy and diseased individuals are attractive for exploring proteomic disease. In 1997, Heine et al. (44) reported the presence of 13 proteins in human urine. Since then, many other investigators have assessed the human uroproteome and have drawn different, methodology-dependent conclusions. The initial approaches with 2-dimensional electrophoresis identified 1400 spots (45), a number that increased when liquid chromatography was introduced. Adachi et al. (46) stated that urine from healthy donors contains at least 1543 different proteins, mostly extracellular and membrane bound. This finding led the authors to suggest the possibility of specific transport pathways for lysosomal and plasma membrane proteins for reaching the urine. The lower protein content of urine compared with plasma reduces the possibility for high-abundance proteins to mask potential biomarkers. Studies have also demonstrated urine to be highly enriched for small peptides (47); healthy individuals and patients with Fanconi syndrome contain a >100-fold enrichment of molecules <10 kDa, compared with higher molecular weight polypeptides, perhaps because the former pass freely through the glomerulus (48). On the other hand, low-abundance and low-mass peptides can become bound to large carrier proteins that act as harvesters in the circulation (49). The major constituents of the urinary peptidome appear to be collagen fragments, especially from the collagen  $\alpha 1$  chain, which probably reflect the physiological turnover of tissue extracellular matrix (41).

#### Technological Aspects of Peptidomics

The discovery of novel biomarkers depends not only on the concentration of the biomarker candidate in the sample and the complexity of the matrix but also on the analytical sensitivity of the detection method and

the sample-preparation steps. The design of study strategies and analyses of bioinformatics data is crucial for reproducible and unbiased results (50). Any peptidomic analysis requires a robust and comprehensive procedure.

#### SAMPLE PREPARATION

To enrich the low molecular weight components in a sample for peptidomics analyses requires sample-preparation steps different from those required for proteomic analyses (17, 51). The preanalytical phase is the most challenging. A wide range of variables, both exogenous and endogenous, can affect the results (52, 53). The considerable stability of the proteome's composition and concentrations in urine allows samples to be stored for 6 h at room temperature with little change and for years at  $-20^{\circ}\text{C}$  (39, 54). Fiedler et al. investigated the influence of many variables on final peptidomics results (55). Significant differences were observed not only between first and second morning urine samples but also between first-stream and mid-stream urine samples. Bacteriuria and hematuria had a great effect, even at low concentrations, on the peptide profile. Freeze-thaw cycles can influence the final results when assessing exogenous variables; thus, reproducibility is improved with once-frozen urine samples.

To minimize such potentially confounding factors and preanalytical variations requires that samples be collected and handled in a standardized manner. The Human Kidney and Urine Proteome Project (<http://www.hkupp.org>) is an international initiative of the Human Proteome Organization to establish collection and manipulation procedures for proteomics. In Europe, the European Kidney and Urine Proteomics organization (<http://www.eurokup.org>) promotes interactions between scientists in the field, with the goal of improving the understanding and assessment of kidney disease through urine proteomics. Both associations have proposed recommendations for standardized urine-processing steps (with special emphasis on sample collection, centrifugation, and thawing), which should minimize biases among studies.

#### USE OF MASS SPECTROMETRY IN PEPTIDOMICS

Traditionally, hypotheses for biomarker discovery have been derived from an understanding of disease biology (56). Over the past few decades, however, many researchers have turned to mass spectrometry to discover candidate molecules that could serve as biomarkers. The advantages of mass spectrometry for identifying and quantifying peptides in complex biological samples have facilitated the development of novel biochemical approaches for diagnosis, not only of cancer but of other diseases as well. Studies of proteins and peptides have used different methodologies.

Two-dimensional gel electrophoresis has been used extensively, but it is a time-consuming technique with poor interassay reproducibility, especially at low molecular weights because it cannot separate and thus distinguish molecules of  $<10$  kDa. Capillary electrophoresis-mass spectrometry yields robust and highly reproducible analyses of low molecular weight peptides and is compatible with many volatile buffers and analytes (19, 24); however, the long processing times make this technique challenging to use for large-scale studies. One of the most suitable platforms for urine peptide profiling is SELDI and MALDI followed by mass spectrometry identification with a TOF detector. This approach focuses on peptides in the range of 1–20 kDa. Immobilization, the key step in the entire process, reduces sample complexity, but at the expense of a great loss of information (52). Finally, liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) is capable of providing large amounts of information with high reproducibility. Only capillary electrophoresis and liquid chromatography are able to interface directly with tandem mass spectrometry instruments for peptidomics studies with the required depth of analysis, dynamic range, and enhanced accuracy of quantification (53). In addition, analytical methodologies that increase analytical sensitivity have been developed. One example is selected reaction monitoring, which uses a nonscanning mode of operation on an LC-MS/MS instrument (57). It increases the detection capability by 2 to 3 orders of magnitude compared with conventional scanning modes.

Mass spectrometry facilitates both biomarker discovery and verification/validation. Mass spectrometers help in characterizing proteins and peptides and their modifications. One of the clearest advantages over other platforms is its capacity to qualitatively screen and analyze thousands of molecules without previous knowledge of their existence or relevance to particular pathophysiological conditions. Quantification of previously discovered candidates is essential for evaluating their diagnostic capabilities.

As with proteomics, both absolute and relative quantification of peptides generally require the use of stable isotope-labeled molecules as internal standards. Their use can overcome the problems of matrix effects, variation in sample preparation, and instrument fluctuations. As outlined elsewhere, the isotopic label should be introduced into the work flow as early as possible to increase the number of steps being controlled and thereby decrease imprecision (58). This technique is fairly time-consuming and expensive, however. Label-free strategies for relative quantification are based on comparing signal intensities produced by identical peptides in different analyses and rely on the accuracy of the hypothesis that identical

peptides will behave similarly across different experiments and therefore permit direct comparisons (59).

With urine samples, even absolute quantification is usually uninformative, so analyte concentration is commonly corrected for creatinine or protein excretion, or it is based on a 24-h urine collection, thus reducing the dietary and exercise effects on variation in results. In contrast to transcriptomics and proteomics, no “housekeeping” peptides have been successfully identified to date (53).

#### DATA PROCESSING AND BIOINFORMATICS

Peptidomics and proteomics require considerable computing power to obtain statistically significant and reproducible data. Peptide identification is one of the most challenging aspects (60). Online databases contain peptide sequences for a variety of body fluids and a myriad of disease conditions, and they serve as universal platforms for aiding in defining and verifying candidate biomarkers (42). A substantial proportion of the human urinary proteome database is derived from studies that assessed transplantation or renal disease, whereas the data derived from studies of prostate, renal, and bladder cancers, as well as pheochromocytomas, are relatively few.

The most demanding issue with peptidomics is related to the nonspecificity of the peptide ends. As Hölttä et al. have stated (26), no restrictions regarding enzyme-cleavage specificity can be applied during analyses of bioinformatics data. The consequence is a huge increase (up to 1000-fold) in the number of sequences to consider. This situation contrasts with that of proteomics, in which protease digestion (usually with trypsin) ensures specific endings for each peptide molecule. For this reason, peptidomics suffers from higher false-positive rates and less accurate results.

#### Urine Peptidomics for Disease Diagnostics

Most of the literature on urine peptidomics addresses impairment of kidney function and outcomes of kidney transplantation (43, 61). The few studies that have searched for cancer biomarkers have focused on bladder, ovarian, and prostate cancers. Genitourinary malignancies are responsible for 1 of every 6 cancer deaths in men and 1 of every 10 in women (1).

#### OVARIAN CANCER

Ovarian cancer is the deadliest gynecologic malignancy. Current diagnostic strategies are based on measuring carbohydrate antigen 125 (CA125) in serum (62) in combination with vaginal ultrasonography. Measurement of CA125 lacks diagnostic sensitivity and specificity for early diagnosis, however, and many efforts have focused on finding protein and peptide mol-

ecules that could be useful as diagnostic biomarkers. Some proteins, such as human epididymal secretory protein 4 (63) and osteopontin (64), have shown utility, although none has surpassed CA125. Using serum, one of the first peptidomics-based studies combined peaks of unknown identity, presumably representing low molecular weight polypeptides, and claimed to distinguish between individuals with no malignancy and patients with ovarian cancer (stages I–IV) with 100% sensitivity and 95% specificity (65). The results described in this report have now been invalidated because of preanalytical, analytical, and bioinformatics artifacts (66).

#### PROSTATE CANCER

Prostate cancer, the most prevalent malignancy in men, ranks second in lethality (1). Novel noninvasive markers with higher diagnostic sensitivity and specificity are needed. Although PSA-derived forms and the ribonucleic acid marker PCA3 (prostate cancer antigen 3) seem to add some degree of diagnostic specificity, they have not met expectations (67). Other protein candidates that have been suggested require large-scale validation. The first comparison of the urine proteome used 2-dimensional gel electrophoresis followed by MALDI-TOF mass spectrometry fingerprinting of voided urine samples after prostatic massage to evaluate age-matched men with benign prostatic hyperplasia (68). Calgranulin B/MRP-8 was highlighted for verification and validation. Subsequent research with urine samples has yielded additional candidate molecules, including the matrix metalloproteinases (69) and engrailed-2 (70), although none have yet been validated with large cohorts. Hypothesizing that first-void urine contains prostatic fluid, Theodorescu et al. (71) used a filter with a 20-kDa cutoff followed by capillary electrophoresis–mass spectrometry analysis and obtained a biomarker panel of 12 urinary peptides based on the results. They proposed that this peptide panel, used in combination with age, free PSA, and total PSA, could improve current diagnosis by increasing the area under the ROC curve from 0.77 (based on the free-PSA percentage and patient age) up to 0.82. Nevertheless, this peptide panel also remains to be validated.

#### BLADDER CANCER

Bladder cancer is the fifth most common cancer in Western societies. Current diagnostic strategies are based on cystoscopy and urine cytology, but these methods have high interobserver imprecision and low reproducibility. Given that the bladder is in intimate contact with urine after its production in the kidney, this body fluid has been mined heavily for both protein and peptide biomarkers that might help, not only in detecting bladder cancer, but also in distinguishing muscle-invasive from noninvasive malignancy (72, 73).

A large number of peptides with different concentrations in urine samples from patients with invasive bladder cancer, compared with patients with noninvasive cancer and with controls, have been found, but most of these peptides appear to be fragments of abundant proteins. In fact, Theodorescu et al. (54) proposed a proteomic pattern of 22 polypeptides with high diagnostic sensitivity and specificity for urothelial cancer and highlighted fibrinopeptide A as a potential diagnostic biomolecule. Bryan et al. (72) identified 8 peptides with significantly different concentrations in patients with and without muscle-invasive urothelial carcinoma. Such peptides were identified as derived from albumin, fibrinogen, hemoglobin, and prealbumin—all high-abundance proteins.

#### OTHER CANCERS

Anatomically distant sites can influence urine composition. Studies of the urine peptidome have used this rationale to pursue possible markers of lung cancer (74) and gastrointestinal cancer (75). Using SELDI, Husi et al. (76) found that a nonnegligible number of the candidate proteins belonged to the family of small calcium-binding proteins, S100, which have been related to the growth of tumors of the upper gastrointestinal tract. None of the identified candidates fulfilled the requirements for a single marker, so a protein-peptide pattern served for screening and prediction of outcome. A diagnostic sensitivity of up to 98% was reported, but most of the candidates had also been described for other malignancies, compromising the pattern's specificity.

If one steps back and considers these results as a whole, one sees that most of the peptide panels have not been validated properly. This lack of validation studies represents one of the major shortcomings of peptidomics for reaching the clinical setting and places the usefulness of peptidomics for cancer diagnostics under a critical eye.

#### Translation to the Clinic

Despite intensive efforts, no molecule described in any proteomics or peptidomics study has entered the clinic. For retrospective and prospective validation studies of candidate molecules and to avoid artifacts and methodology-related false-positive results, other methodologies (e.g., immunoassays) are preferred (3, 77). Sometimes initial studies based on small populations show a statistical significance that, because of bias in patient selection or other confounders, becomes lost in subsequent studies. The lessons from this experience could help in improving the planning of future strategies. Large-scale population studies are rare and carry a large financial burden. Finally, reaching statistical significance is not sufficient for candidate biomarkers. As

with novel drugs, biomarkers have to show some clinical improvement over those currently in use; otherwise, they will not be adopted.

Given the complexity of any biological process, a single biomarker has been widely viewed to be unlikely to discriminate a pathologic process with sufficient sensitivity and specificity. Therefore, the incorporation of combinations of multiple, independent biomarkers into a diagnostic or predictive panel may be more likely to be useful. Nevertheless, each of the individual biomarkers used in any panel must be independently verified and validated to ensure clinical utility. This requirement makes the design of large-scale validation studies even more difficult.

Recently, a new perspective that transcends classic proteomics and peptidomics suggests that the study of individual or global protease activities might also yield indicators or predictors of disease (78–80). This new approach has been termed “functional peptidomics.” It relies on the fact that tumor progression and invasiveness may lead to the differential production and secretion of exoproteases; thus, the study of their functions might not only reflect the true biological/pathologic state of an organism but also overcome reproducibility problems related to preanalytical variables. This approach is still in its beginning stages, however, and conclusions about its applicability cannot yet be drawn.

#### Future Challenges

The impressive growth in high-throughput biology has dominated science during the last decade, mainly owing to the leap in the development of new technologies. Substantial efforts in proteomics have focused on the discovery and validation of sensitive and specific diagnostic biomarkers for many human pathologies. Deep biochemical and pathophysiological knowledge is critical for solving clinical questions, and every step in the procedure must be planned and executed meticulously. Standardized handling procedures are expected to aid tremendously in the generation of clinically useful and reproducible data.

Peptidomics is a relatively new field, and few studies of explorations and characterization of the peptidome have yet been published. There is no strong evidence that peptidomics will yield better results than proteomics, but biological and chemical reasoning supports work in that direction. Proteomics is undoubtedly the dominant technology in the postgenomics era, and peptidomics represents a largely unexplored step forward.

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