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Prostate Cancer Diagnosis and Characterization with Mass Spectrometry Imaging

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Abstract

Background—Prostate cancer (PCa), the most common cancer and second leading cause of cancer death in American men, presents the clinical challenge of distinguishing between indolent and aggressive tumors for proper treatment. PCa presents significant alterations in metabolic pathways that can potentially be measured using techniques like mass spectrometry (MS) or mass spectrometry imaging (MSI) and used to characterize PCa aggressiveness. MS quantifies metabolomic, proteomic, and lipidomic profiles of biological systems that can be further visualized for their spatial distributions through MSI.

Methods—PubMed was queried for all publications relating to MS and MSI in human prostate cancer from April 2007 to April 2017. With the goal of reviewing the utility of MSI in diagnosis and prognostication of human PCa, MSI articles that reported investigations of PCa-specific metabolites or metabolites indicating PCa aggressiveness were selected for inclusion. Articles were included that covered MS and MSI principles, limitations, and applications in PCa.

Results—We identified nine key studies on MSI in intact human prostate tissue specimens that determined metabolites which could either differentiate between benign and malignant prostate

Conflict of interest

NYA is co-founder of BayesianDx and scientific advisor to inviCRO. The other authors declare no conflicts of interest.

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tissue or indicate prostate cancer aggressiveness. These MSI-detected biomarkers show promise in reliably identifying PCa and determining disease aggressiveness.

Conclusions—MSI represents an innovative technique with the ability to interrogate cancer biomarkers in relation to tissue pathologies and investigate tumor aggressiveness. We propose MSI as a powerful adjuvant histopathology imaging tool for prostate tissue evaluations, where clinical translation of this ex vivo technique could make possible the use of MSI for personalized medicine in diagnosis and prognosis of prostate cancer. Moreover, the knowledge provided from this technique can majorly contribute to the understanding of molecular pathogenesis of PCa and other malignant diseases.

Introduction

The American Cancer Society declares prostate cancer (PCa) as the most common cancer in males, representing 19% of all diagnosed cancer cases in American men, with an estimated number of 26,730 deaths in the United States alone in 2017, PCa the second leading cause of cancer death¹.

While the ability of serum prostate-specific antigen (PSA) level to detect early stage disease is evident, its introduction for annual testing in the 1980's also led to the discovery of many predominantly slow growing prostate tumors that would not have become life-threatening during a patient's life^{2–5}. However, a considerable percentage of patients harbor aggressive disease that requires early and appropriate treatment consisting of surgery, radiation, chemotherapy, or hormonal therapy. Unfortunately, at present, the clinical ability to identify these patients with aggressive disease and differentiating them from patients harboring indolent tumors is often limited. Thus, the urge to treat cancer is often challenged by the concern of avoiding over-treatment in the PCa clinic^{6–8}.

At present, histopathological examination can reliably distinguish benign from malignant lesions using the Gleason Score^{9,10} or Prognostic Grade Group system¹¹, but its ability to distinguish indolent PCa from aggressive is still limited. The development and progression of PCa inevitably alters tissue biochemistry, and inspired by the developments in oncological genomics, proteomics, and lipidomics, mass spectrometry (MS)-based metabolic biomarkers have been investigated for their ability to detect and characterize PCa for better prognostication and individual therapy planning. MS investigations have been further enhanced by the innovation of mass spectrometry imaging (MSI), by which metabolic maps of intact tissues are generated^{12–18}.

Here, while we focus our review on the current literature reports of MSI studies of PCa and the promise of this innovative molecular imaging method to improve PCa diagnosis and characterization, we will start our address with brief reviews in the MS and MSI techniques, and in the major achievements in MS evaluations of PCa.

Methods

For this review, PubMed was queried for all publications relating to MS and MSI in human prostate cancer from April 2007 to April 2017. Titles and abstracts of studies found in the

search were reviewed, and relevant studies were advanced to full-text review. With the goal of reviewing the utility of MSI in diagnosis and prognostication of human PCa, MSI investigations were included that described identification of PCa-specific metabolites and metabolites indicating PCa aggressiveness. Additionally, MS articles were included which highlighted principles, applications in PCa, or limitations of the technique, and articles that described MSI principles in overcoming these limitations were selected. In a final step, the reference lists of included papers were screened, and new titles were reviewed for potential inclusion as previously described. Relevant data from the selected studies were summarized in the text and tabulated in Table 1.

Results

Mass spectrometry principles

Mass spectrometry is a chemical analysis technique that measures the mass of molecules by ionizing, separating, and detecting the resulting ions as peaks according to their mass-to-charge ratio. The resulting spectrum plots the relative abundance of each molecular fragment as a peak against its mass-to-charge ratio (m/z), and produces a unique profile for every sample. Similar peak patterns, however, can describe a similar family of molecules. The values of data signatures, or fingerprints, for the analyzed molecules are dependent on the procedures of sample preparation, the selection of the ionization method, and the configuration of the mass analyzer.

Mass spectrometry ionization and analysis methods

Two of the most frequently used MS ionization techniques are electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI).

Initially, the use of MS was been restricted to gases, volatiles, and thermally stable molecules due to the limitations of early ionization techniques. However, the development of electrospray ionization (ESI) in 1984 by John B. Fenn and colleagues¹⁹, and the proposal of MALDI in 1985²⁰ with demonstration by Koichi Tanaka and colleagues in 1987²¹, made it possible to analyze heavy and non-volatile molecules such as nucleic acids, proteins, and metabolites. For their respective contributions to the field, Fenn and Tanaka shared the Nobel Prize in Chemistry in 2002 with Kurt Wüthrich for developments in NMR that enabled direct analysis of biomolecules. Today, ESI and MALDI have become increasingly important techniques in the laboratories for structural analyses or quantitative measurements of metabolites in biological samples.

ESI produces ions using an electrospray in which a high voltage is applied to a liquid, creating a fine aerosol. The process of transferring ionic species from solution into the gas phase involves three steps: dispersal of a fine spray of charged droplets, solvent evaporation, and ion ejection from the highly charged droplets. With the aid of elevated temperature or nitrogen drying gas, the charged droplets are reduced in size by evaporation of the solvent, leading to increased charge density and decreased droplet radius. When the electric field strength within the charged droplet reaches a critical point, ions at the surface of the droplets

are ejected into the gaseous phase. Finally, the ions are accelerated down a pressure and electric potential gradient toward the mass analyzer.

In MALDI, the sample is mixed with a UV-absorbing crystalline matrix material and spotted onto a target plate. The plate is placed in a vacuum and hit with a UV laser, and the matrix absorbs the irradiation – simultaneously volatilizing and ionizing the sample.

The sensitivity of a mass spectrometer relies on the structure of its mass analyzer; both timeof-flight (TOF)²² and quadrupole mass filters $(Q)^{23}$ are commonly used mass analyzer structures. Furthermore, they can be combined to produce a high-resolution mass spectrometer $(Q-TOF)^{24}$. Achieving high mass resolution and accuracy can also be accomplished by means of Fourier transform mass spectrometers, such as Fourier Transform Ion Cyclotron Resonance (FTICR)²⁵.

Mass spectrometry applications in prostate cancer studies

Metabolomic studies of prostate cancer have been accomplished by MS to examine prostate cell lines^{26–28}, animal prostate models^{29–31} and biological fluids including urine, prostatic fluid, semen and blood^{16,32–36}. As this review focuses on prostate cancer diagnosis and prognosis in prostate tissue samples, three major MS studies investigating metabolomics in prostate tissues are noted.

In 2009, Chinnaiyan and colleagues reported a study of 42 prostate samples of adjacent benign (16) and malignant (12 localized and 14 metastatic PCa) tissues, along with 110 matched specimens of urine and plasma from biopsy-positive PCa patients (59) and biopsy-negative subjects (51)³⁷. A total of 1,126 metabolites were quantified using high-throughput gas chromatography (GC)- and liquid chromatography (LC)-MS³⁷. Among the 626 tissue-specific metabolites, 60 metabolites were exclusively found in malignant samples. Another 50 metabolites were increased and 37 were decreased in localized prostate cancer compared to benign prostate tissue samples. A total of 124 metabolites were increased and 107 metabolites were decreased in all biospecimen samples from patients with metastatic compared to patients with localized disease. In general, an upregulation in amino acid metabolism and nitrogen breakdown pathways during tumor metastasis was revealed. Particularly, the study identified sarcosine, an N-methyl derivative of glycine, as a significant metabolite that increased with PCa progression and metastasis and, most importantly, could be detected both in tissue and, non-invasively, in urine samples of PCa patients.

Recently, combined metabolomic, transcriptomic, and immunohistochemistry studies have been reported on malignant and matched adjacent non-malignant tissue samples from 106 patients with PCa³⁸. Metabolomic analyses were performed by GC-MS and LC-MS and identified a set of 254 metabolites. Among them, 173 showed a capacity to differentiate malignant from non-malignant samples. Furthermore, analysis of a subset of metabolites indicated that twelve were up-regulated and four down-regulated in comparison to increased Gleason score. Specifically, positive correlations with pantothenic acid, and negative correlations with maltose, fructose-6-phosphate, gluconic acid and cholesterol, were reported. The study further observed metabolite changes in transmembrane protease serine 2 (TMPRSS2)-ERG translocation-positive PCa, with 55 metabolites increased and 17

decreased when compared to ERG-negative carcinomas. TMPRSS2-ERG gene fusion represents the predominant molecular subtype in PCa, and various studies have shown that TMPRSS2-ERG positivity is often associated with higher disease-related mortality^{39,40}. In accordance with this report, the study reported a negative correlation between ERG positivity and maltotriose and gluconic acid quantified from MS, with the absence of these metabolites correlated with a greater chance of PCa recurrence¹⁷. Although previously correlated with PCa, for the first time the study showed the polyamines spermine and putrescine to be negatively correlated with presence of the poor-prognosis TMPRSS2-ERG fusion gene. In contrast, fatty acid levels (e.g. cerebronic acid, 2-hydroxybehenic acid and tricosanoic acid) were increased in ERG-positive PCa samples. These results clearly demonstrate the dysregulation of fatty acid, sphingolipid, and polyamine metabolism in PCa.

In a recently published study, mass spectrometry-based metabolomic analysis was performed in frozen and matched formalin-fixed paraffin embedded (FFPE) human prostate cancer tissue. A total of 352 metabolites were profiled, and physical-chemical characteristics of metabolites measured were related to their preservation or loss following fixation and embedding. Although FFPE tissue sections showed a decrease of metabolites with functional groups (e.g. carboxamide), global metabolomic profiles obtained from FFPE collections still proved useful for the prediction of biological states and discovery of biomarkers in tissue specimens, which offers great opportunity concerning retrospective tissue analysis⁴¹.

Recent technology developments have also enabled metabolomic MS evaluation of PCa metabolites and histopathology from the same specimen, especially important given the heterogeneity of PCa pathologies. A new preparation method uses methanol to simultaneously extract metabolites for MS measurement and fix the tissue for histopathological evaluation⁴². Pathology analyses, including H&E and immunohistochemistry, as well as metabolomic analyses, were successfully carried out with MS in this fashion⁴². In 96 samples, a total of 260 metabolites in the alcohol extracts were detected, and among them 82 metabolites were increased in PCa containing biopsies compared to histologically benign prostate biopsies.

However, mass spectrometry itself cannot directly attribute the measured MS values to each individual pathology, but the development of mass spectrometry imaging (MSI) has overcome this barrier.

Mass spectrometry imaging principles

As an extension of MS, MSI enables molecular information to be spatially mapped onto the solid biospecimen from which it originated (Figure 1). Histopathology analysis of an adjacent piece of tissue allows metabolites to be localized onto their tissue pathologies of origin. MALDI-MSI, which opened up the histopathology-based MSI field, still remains a first line approach in MSI-based diagnostics. As described above, MALDI occurs in a vacuum and requires sample preparation, in that a tissue section needs to be covered with a UV-absorbing crystalline matrix material. It offers the widest mass range allowing the imaging of small molecules, metabolites, lipids, and proteins, unlike immunohistochemistry, while also remaining the only approach capable of imaging proteins with molecular weights

up to 300 kDa^{43–48}. Moreover, MALDI MSI now offers spatial resolution as high as 5 μ m, and can routinely image at 100 μ m^{49–51}.

Usually separate pieces of tissue are used for MSI and histopathology analysis, but a histology-compatible tissue preparation method was developed by Agar and colleagues in 2007⁵². The matrix solution fixation (MSF) method allows simultaneous tissue fixation and matrix deposition by incorporating MALDI matrix into solvents that preserve tissue integrity. Solvent fixation treatment of frozen tissue sections usually causes solubilization and displacement of proteins, lipids, and cellular membrane structures. MSF, however, preserves tissue integrity through maintenance of cellular structure and the general ensemble of biomolecules, causing subcellular disruption of only 50 to 300 nm. This has been achieved by a combination of cold solvent tissue fixation protocols with matrix deposition. The matrix can be removed after imaging, enabling histology and MSI of the same tissue section. The method is compatible with common MALDI matrixes and solutions commonly used for histology fixation, and it exhibits spectral quality and spatial resolution similar to matrix deposition techniques used in the current literature^{17,29}. Furthermore, this method is shown to afford flexibility in optimizing crystallization parameters, so that a variety of biomolecules can be imaged⁵².

Many technical approaches for surface-based ambient ionization processes have been described in the literature in the past years, and a few have been commercialized, including desorption electrospray ionization (DESI) MSI. This technique involves directing a focused beam of charged droplets at a sample, such as thin-layered tissue on a glass slide. As the solvent pools, it forms a thin film on the surface that extracts molecular components from the sample below. The extracted liquid splashes up into the mass analyzer as subsequent solvent impacts the surface. Ambient interface methods can be used to take a snapshot of the sample's molecular profile or to collect spectra at various points across a sample⁵³. This ambient ionization method enables analysis with basically no preparation procedures. However, this advantage comes at the expense of its lower sensitivity, due to the lack of sample preparation and enrichment procedures; thus, molecules of interest can be lost amidst the signal produced by more abundant but possibly irrelevant compounds. DESI is limited by lower spatial resolution than can be achieved by MALDI (around 200 µm), although values of 40 µm have been reported on non-biological tissues^{54,55}. While it is also limited to the analysis of a smaller mass range, its simple, fast application makes it suitable for clinical translation and able to complement other ionization approaches.

The above mentioned protocols may facilitate clinical acceptance of MS, as verification of tissue histopathology in conjunction with MS analysis is the only way to ensure the accurate interpretation of MS metabolomics. Additionally, the technical advancements of MALDI and DESI MSI, including the development of effective sample preparation procedures, faster and more accurate analysis, and high resolution, encourage mass spectrometry imaging towards further research use and clinical implementation^{56–62}.

MSI identification of prostate cancer-specific metabolites

MSI investigation of tissue specimens represents an innovative field in PCa research. The following studies have focused on either the diagnosis or prognosis of PCa by considering

either single metabolites or metabolomic profiles quantified from MALDI or DESI MSI and have reported differences in expression profiles between benign and malignant human prostate tissue^{63–70}. A number of metabolites have been identified as potential diagnostic markers for PCa by means of MSI, such as mitogen-activated protein kinase/extracellular signal-regulated kinase, cholesterol sulfate, phosphatidylinositols, and biliverdin reductase B^{63–65,67}. An overview of the most recent studies of MSI on human PCa whole tissue specimens is presented in Table 1.

MALDI-MSI was used to evaluate protein expression in PCa tissue versus benign from 75 prostatectomy cases, and six metabolites were increased in PCa containing-tissues. The overexpression of one of these metabolites – identified as a fragment of mitogen-activated protein kinase/extracellular signal-regulated kinase (MEKK2) – in PCa tissues allowed for a reliable discrimination between malignant and benign tissue specimens with a sensitivity of 90.3% and a specificity of 86.4% (AUC = $0.96)^{63}$. MEKK2 is a member of the serine/ threonine protein kinase family and is known for its crucial role in relaying cell surface signals through various downstream mitogen-activated protein kinase (MAPK) signaling pathways. The MAPK signaling pathway regulates growth factor-stimulated cell proliferation, differentiation, survival, and death. Dysregulation of this pathway is implicated in diverse diseases and cancers, and compounds inhibiting steps in MAPK signaling pathway are considered as potential drugs for cancer treatment^{66,71–74}. Thus, the presentation of the overexpression of MEKK2 in PCa tissue may not only be useful for disease diagnosis but also for the design of individualized therapy, as well as for monitoring the effect of therapies.

MALDI-MSI has also been applied to malignant and benign epithelium as well as stromal areas of prostatectomy specimens to identify characteristic molecules for each pathological component. A cross-validation analysis, with 13 prostatectomy cases in the testing set and 10 in the validation set, presented high discriminatory ability for two peaks. One such peak, biliverdin reductase B (BVR), is a cytoprotective and growth promoting protein that is overexpressed in PCa, suggesting this enzyme could serve as a potential biomarker for human PCa⁶⁷. It catalyzes the reaction to bilirubin, which is a potent antioxidant in human cells. Furthermore, BVR activates the expression of genes involved in cell growth, differentiation, and survival through the MAPK- and phosphatidylinositol 3-kinase (PI3K) pathway. This growth-promoting function of BVR as well as its increased expression in tumor cells classifies this enzyme as a tumor promoter⁷⁵. Recent studies have indicated that many of biliverdin reductase's growth promoting functions can effectively be suppressed by inhibitors of BVR activity, which could offer a novel approach in PCa treatment⁷⁶.

To consider an entire cancer metabolomic profile, another MALDI-MSI study created a biomarker algorithm consisting of 22 different metabolites based on protein expression profiles in benign (n=11) and malignant (n=11) human prostate tissues. An overall cross-validation (88.00%), sensitivity (85.21%) and specificity (90.74%) was achieved for the distinction between benign and malignant prostate tissue⁶⁹. Such remarkable sensitivity and specificity in distinguishing between benign tissue and PCa has also been shown in a high-resolution (HR)-MALDI-MSI study of 14 samples that identified increases of 26 metabolites in malignant compared to benign tissue (including phosphatidylinositols, three

Using DESI-MSI, cholesterol sulfate was identified as a biomarker for PCa diagnosis, as it was almost exclusively found in cancerous and precancerous lesions⁶⁴. Therefore, cholesterol sulfate might not only be useful to differentiate benign and cancerous tissue but also for the detection of precancerous lesions within histologically normal tissue (Figure 2). Cholesterol sulfate is known for its stabilizing and regulatory role as a cell membrane component and has been reported as a potential tumor marker in various types of cancers^{77,78}. Since the biological process responsible for the expression of cholesterol sulfate in human PCa is unknown, future studies will have to address this issue.

In a recently published work, endogenous compounds of cancerous and non-cancerous regions in three human prostate cancer tissue specimens were detected and imaged using matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry imaging (MALDI FTICR MSI)⁷⁰. The authors combined two different MALDI matrices (quercetin and 9-aminoacridine) and used the newly developed technique of matrix coating assisted by an electric field (MCAEF)^{79,80}. A total of 1091 metabolites were identified, of which 250 and 217 were exclusively found in either the cancerous or the non-cancerous regions, respectively. Of these, 152 metabolites showed differentiating distributions between these two regions of tissue, such as an increased energy charge and a lower expression of neutral acyl glycerides in the cancerous regions. Sixty-two acyl-glycerides were detectable only in non-cancerous regions, which can be interpreted as a result of known low glycolysis in prostate cancer cells and compensatory increased fatty acid beta-oxidation leading to a depletion of these acyl-glycerides in cancerous tissue regions^{70,81}.

MSI prognostication of prostate cancer aggressiveness

Considering the limitations of histology to reliably determine PCa aggressiveness, the ability of MSI to predict and characterize prostate cancer aggressiveness in terms of biochemical recurrence was evaluated. The relationship between the decrease in the expression of lysophosphatidylcholine and the increase in biochemical recurrence potential of PCa after prostatectomy was evaluated with a study of 31 PCa patients⁸². Reduced expression of lysophosphatidylcholine was an individual predictor and potential prognostic marker of biochemical recurrence (Kaplan-Meier, p=0.027)⁸².

MALDI-MSI measurement of tissue microarrays of 729 human PCa specimens identified four signals associated with a low Gleason score, early disease stage, and low proliferation marker Ki-67, one signal associated with high Ki-67, and one signal associated with a prolonged time to PSA recurrence⁸³. While these signals might serve as biomarkers for the differential diagnosis of PCa, two additional signals were associated with the overexpression of erythroblast transformation-specific related gene (ERG) and five signals associated with ERG negativity⁸³. This discovery is of particular importance, since ERG can fuse with TMPRSS2 to form an oncogenic fusion gene especially common in hormone-refractory

PCa. Therefore, MSI as an imaging tool may identify TMPRSS2-ERG-positive PCa to ensure that affected patients receive individually targeted therapy.

Conclusion and Future Directions

This review highlights the potential significance of MSI in enabling diagnosis and further prognosis of human PCa thus far seen in literature. The potential biomarkers detected during MSI analyses provide a metabolic overview of disease, and at the same time, affirm the potential of metabolomics to improve disease characterization when compared with a single metabolite. Following the development and improvement of various MSI methodologies, applications of the techniques have successfully distinguished PCa from histologically benign prostate tissue, and aggressive from indolent PCa. MSI also offers the valuable ability to map biomarkers onto prostate tissue pathologies, therefore complementing histology, immunohistopathology, and molecular pathology for disease diagnosis and patient prognostication. While the physical principles governing MSI make it an ex vivo technique, improvements in speed, precision, and sensitivity of the technique make feasible MSI clinical use to provide patients with individualized PCa treatment.

Continuing progress in MSI will lead to a higher accuracy in identifying PCa and determining PCa aggressiveness. We consider the next step in the development of MSI to be of three-dimensionality. Working towards this aim, an atmospheric-pressure ion source for MS has recently been developed⁸⁴, with laser ablation electrospray ionization (LAESI)-MS as an innovative approach for depth profiling. In combination with lateral imaging, this new technology will enable three-dimensional molecular imaging with MS⁸⁴.

Another huge advance for the field would be the combination of MSI analysis of PCacontaining tissue samples with traditional histopathological examination to develop a computer-based program that can identify different types and aggressiveness of PCa. This knowledge could also be applied to tissues from all over the body to evaluate other diseases, and it has already been done in breast and brain tissue^{85–88}. Thus, MSI can contribute majorly to understanding of molecular pathogenesis of not just PCa and but to other malignant diseases as well.

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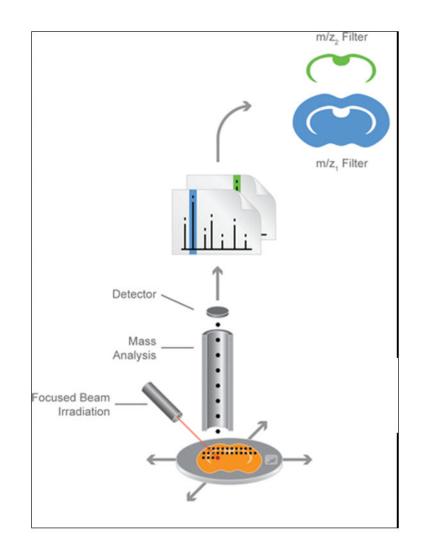


Figure 1.

Schematic of mass spectrometry imaging technique, where focused beam irradiation generates a spectral read-out for each micron-scale area of the biospecimen⁸⁹.

Cholesterol Sulfate (m/z 465.4) DESI Image	H&E Stained Digital Image	
А мно202-37	Tumor PIN	
В мно212-04	Tumor PIN	
С UH0003-32	Tumor PIN	
D UH0003-12	PIN Normal	
E UH0002-29	PIN	

Figure 2.

DESI-MS ion image of m/z 465.4, cholesterol sulfate, in the negative ion mode and H&E stained image of prostate samples (A) MH0202-37, cancer and adjacent normal tissue with PIN, (B) MH0212-04, cancer and normal tissue with PIN, (C) UH003-32, cancer and normal tissue with PIN, (D) UH0003-12, PIN and normal tissue, and (E) UH0002-29, PIN detected within normal regions of tissue⁶⁴.

Table 1

Key studies on mass spectrometry imaging in intact human prostate tissue.

Year	Торіс	Main Findings	Ref
2016	MALDI-FTICR-MSI for PCa diagnosis	Distinction between cancerous and non-cancerous regions in prostate tissue through the expression of neutral acyl glycerides	70
2015	MALDI-MSI for PCa prognosis	Correlation between decreased expression of lysophosphatidylcholine and biochemical recurrence of PCa after surgical treatment	82
2014	MALDI-MSI for PCa diagnosis	Phosphatidylinositols represent potential biomarkers for PCa diagnosis	65
2013	MALDI-MSI for PCa diagnosis	N-glycan profiling provides a new opportunity to evaluate disease status in PCa patients	88
2013	MALDI-MSI for PCa diagnosis	Biliverdin reductase B serves as a potential biomarker for PCa diagnosis	67
2013	MALDI-MSI for PCa diagnosis and prognosis	Identification of potential biomarkers for PCa diagnosis and prognosis (signals associated with Gleason score, disease stage, Ki-67 and PSA recurrence)	83
2010	DESI-MSI for PCa diagnosis	Cholesterol sulfate represents a potential biomarker for PCa diagnosis	64
2009	MALDI-MSI for PCa diagnosis	The overexpression of MEKK2 can be used to discriminate malignant from benign prostate tissue	63
2007	MALDI-MSI for PCa diagnosis	Differentiation of cancerous and non-cancerous prostate tissue with an overall cross-validation of 88.00%, sensitivity 85.21% and specificity 90.74%	69