*Current Drug Metabolism: solicited review*

**Genitourinary Tumors: *Update on molecular biomarkers for diagnosis, prognosis and prediction of response to therapy***

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**Short title**: Biomarkers in genitourinary tumors

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**Abstract: Background:** Research of biomarkers in genitourinary tumors goes along with development of complex emerging techniques ranging from next generation sequencing platforms, applied to archival pathology specimens, cytological samples, liquid biopsies, and to patient-derived tumor models.

**Method:** This contribution is an update on molecular biomarkers for diagnosis, prognosis and prediction of response to therapy in genitourinary tumors. The following major topics are dealt with: Immunological biomarkers, including the microbiome, and their potential role and caveats in renal cell carcinoma, bladder and prostate cancers and testicular germ cell tumors; Tissue biomarkers for imaging and therapy, with emphasis on Prostate-specific membrane antigen in prostate cancer; Liquid biomarkers in prostate cancer, including circulating tumor cell isolation and characterization in renal cell carcinoma, bladder cancer with emphasis on biomarkers detectable in the urine and testicular germ cell tumors; and Biomarkers and economic sustainability.

**Conclusion:** The identification of effective biomarkers has become a major focus in cancer research, mainly due to the necessity of selecting potentially responsive patients in order to improve their outcomes, as well as to reduce the toxicity and costs related to ineffective treatments.

**Key words**:Genitourinary tumors; microbiome; renal cell carcinoma; prostate cancer; bladder cancer; liquid biopsy; PSMA; immunotherapy

1. **INTRODUCTION**

The identification of molecular biomarkers in genitourinary (GU) tumors is of great interest nowadays. This is mainly due to the need to reduce invasive diagnostic procedures and, at the same time, to improve the selection of potentially responsive patients, to avoid toxicity as well as to reduce costs related to ineffective treatments. The research goes along with the development of new and emerging molecular analysis techniques, including next generation sequencing (NGS) platforms, applied to archival tissue specimens, cytological samples and liquid biopsies, as well as patient-derived animal tumor models. All this is aiming at a personalized approach to diagnosis, prognosis and prediction of response to therapy [1].

The scope of this review is give readers an update on immunological biomarkers, including the microbiome, and their potential role and caveats; tissue biomarkers for imaging and therapy, with emphasis on Prostate-Specific Membrane Antigen (PSMA); liquid biomarkers; and biomarkers and economic sustainability.

1. **IMMUNOLOGICAL BIOMARKERS: potential role and caveats**

Recently Santoni et al. [1] gave an exhaustive overview on the role of immune cells in the pathogenesis, growth and metastatization of GU tumors. The immune system has been shown to play a pivotal role in the modulation of response to targeted therapeutic agents. This has led to a variety of immunotherapeutic strategies and to the identification of promising immunotargets, such as Cytotoxic T-Lymphocyte Antigen 4 (CTLA4), programmed death 1 (PD-1) and PD ligand 1 (PD-L1). These immunotargets are currently evaluated for their therapeutic, predictive and prognostic roles in patients with Renal Cell Carcinoma (RCC), Bladder Cancer (BC), Prostate Cancer (PCa), and Testicular Germ Cell tumors (GCTs).

**2.1. Renal Cell Carcinoma**

Immunotherapy is a key factor in the therapeutic algorithm for patients with locally advanced or metastatic RCC. In particular, the approval by the FDA and EMA of several agents, such as anti/PD-1/PD-L1 drugs, have revolutionized the scenario of therapeutic management of metastatic RCC, reaching important clinical end points with extended patients’ survival[2]. Robust and reliable biomarkers are crucial for patient’s selection for treatments with immunomodulatory drugs.

Each approved PD-1/PD-L1 drug is paired with a PD-L1 immunohistochemistry (IHC) based assay. The use of a single biomarker for patient selection may not be feasible, because the immune responses are dynamic and evolve over time. In addition, the predictive value of PD-L1 IHC expression is controversial, as showed in both the Checkmate 025 [3] and 214 [4] trials. For instance, the efficacy of nivolumab in the Checkmate 025 was not related to PD-L1 tissue expression. Patients expressing PD-L1 more than 1% showed a worse overall survival (OS) in the treatment arm, suggesting a prognostic role rather than a predictive value. An exploratory analysis in the Checkmate 214 trial in patients with previously untreated advanced or metastatic RCC showed a progression-free survival (PFS) benefit in patients expressing PD-L1 (1% or greater). However, patients with higher PD-L1 expression showed greater benefit with the immune-combination of Nivolumab with Ipilimumab vs. Sunitinib alone. Such results give support to the concept that PD-L1 IHC expression is not an absolute predictor of response in patients with untreated advanced or metastatic RCC receiving immune checkpoint inhibitors (ICI) [5–9].

There are some additional issues and questions that need to be taken into consideration. One is the intratumoral heterogeneity of PD-L1 expression. Another is that PD-L1 is a dynamic biomarker because a prior exposure to VEGF and mTOR inhibitors modulates its expression [10,11]. Primary resistance to ICIs is linked to several factors, such as poor intrinsic antigenicity of tumor cells, low mutational tumor burden [12-13], lack of priming related to potentially immunogenic pretreatment with radio- or/and chemotherapy [14], poor antigen presentation, immunosuppression by extracellular metabolites, and functional exhaustion of lymphocytes in the tumor [15,16].

**2.2. Bladder and prostate cancers**

In the last few decades BC treatment, namely systemic therapy, has been characterized by the lack of newer therapeutic options other than chemotherapy. Chemotherapy, both in first and second-line, has shown a modest clinical advantage with non-negligible toxicity. Targeted therapies are rather ineffective in BC. The new generation of immunotherapeutic agents represents the most promising path for systemic treatment of BC. Checkpoint inhibition, that is, the PD1/PD-L1 pathway inhibition, paired with a PD-L1 immunohistochemistry (IHC) based assay, has shown important results in many other types of tumors. It is expected to become a major player in the treatment of BC (Figure 1). Other immunotherapy-based strategies, such as fusion proteins, should represent a future and promising option.

Innate and adaptive immunity is involved in PCa carcinogenesis and progression. Based on this, several immunotherapeutic approaches have been developed and investigated in patients with PCa. ICIs, such as anti-CTLA-4, anti-PD-1 and anti-PD-L1 agents, represent promising drugs for the treatment of PCa patients, used either alone or in combined strategies, potentially together with oncolytic viruses and vaccines.

**2.3. Testicular germ cell tumors**

# The ability to predict prognosis and treatment response in GCTs has not improved for several years. Trials with novel targeting agents, conducted in patients with refractory GCTs, have not achieved better outcomes. Various molecular biomarkers have been investigated in order to refine the prognosis and treatment of these malignancies.

# Low expression of PD-L1 of GCT tissue by IHC is associated with a significantly better PFS and OS, whereas high expression of PD-L1 in tumor infiltrating lymphocytes (TIL) is highly predictive of better prognostic outcomes compared to low PD-L1 expression in TIL[17]. However, the abundant expression of PD-L1 in tumor cells is not predictive of response to treatment with ICIs [18]. The poor response to ICIs in GCTs may be partly explained by the very low mutational load and insufficient neoantigen signal [19]*.* A phase II study with the anti-PD-L1 agent avelumab is ongoing. It is hoped that it will shed more light on single agent immunotherapy in refractory GCTs (NCT03403777).

**2.4. The Microbiome**

A close biological relationship has emerged among the microbiome of the gut, the metabolism of the body, as well as the immune system in cancer development and treatment. With the advent of high-throughput NGS techniques, the analysis and understanding of the mechanisms of the complex microbial network that colonizes our body areas have been explored. It has been shown that the microbiome might be a novel and pivotal diagnostic, preventive, prognostic and therapeutic tool. In particular, in RCC patients gut microbiome composition could influence primary resistance to PD-1 blockade. Antibiotic therapy inhibits the clinical benefit of immunotherapy in patients with cancer in an advanced stage and represents a predictor of resistance to PD-1 blockade [20].

Studies on the microbiome of patients with BC and PCa have shown significant differences in terms of abundance in bacterial composition compared to healthy controls. Moreover, the identification of microbiome-derived metabolites and related pathways are significantly different between PCa samples compared to controls [21,22]. This information has been used to develop a microbiome profile to predict PCa risk groups. Even though at its beginning, research on microbiome represents an important step forward in the understanding of the mechanisms underlying oncogenesis, progression and resistance to therapy of tumors (Figure 2).

1. **TISSUE BIOLECULAR BIOMARKERS FOR IMAGING AND THERAPY, WITH EMPHASIS ON PROSTATE CANCER**

The rising detection rate of the genitourinary cancers, in particular of PCa, has increased the demand for improved diagnostic, imaging and therapeutic approaches. Prostate-specific membrane antigen (PSMA), a tissue marker highly expressed in high grade PCa, represents an attractive target for cancer detection and treatment.

**3.1 PSMA and PCa aggressiveness**

PSMA IHC expression increases steadily from focal within the normal prostate and to a diffuse pattern in secondary deposits. This was shown in a study based on a radical prostatectomy specimen with a lymph node in the adjacent periprostatic tissue [23]. In another investigation, a sharp increase in PSMA tissue expression by IHC, in comparison with the previous naïve PCa, was seen in the bone metastasis in the castration-resistant phase [24-25].

**3.2 PSMA, cell location in PCa, and internalization function**

Concerning PSMA location at the cell level and its changes or pattern expression in relation to therapeutic procedures, little attention has been paid by surgical pathologists. In particular, “a kind of bias is accepted by them based on the name of the molecule: prostate-specific membrane antigen, an antigen broadly or basically located in the cell membrane”[26]. This is not exactly what can be revealed by IHC. Its location can change. PSMA can be seen on the surface of the cells, at the cell membrane level, and/or in the cytoplasm (Figure 3). These three patterns should correspond to the extracellular domain of the molecule, its transmembrane region, and its cytoplasmic domain. A predominant cell location on the surface of the cell was seen following androgen ablation therapy, whereas a preferential cytoplasmic location was seen following radiation therapy. When considering the function of the PSMA molecule, the three IHC cell patterns should “statically” represent the different stages of its internalization mechanism [27].

**3.3 PSMA and PCa imaging and treatment**

An accumulation on the cell surface, enhanced by androgen ablation, could improve cancer visualization with imaging techniques. “On the contrary, a predominantly cytoplasmic location could not be easily accessible to molecules used for imaging purposes”[26]. All this could help to explain the findings in the study by Rauscher et al. [28]. Probably, the same could be said when PSMA molecules are targeted for therapeutic purposes or potentially for imaging and therapy at the same time [29].

1. **LIQUID MOLECULAR BIOMARKERS**

The selection of cancer patients more likely to benefit from a specific therapeutic approach as well as the early identification of patients with aggressive disease, that could benefit from a more aggressive treatment strategy, are key issues in the oncology field. Circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA), including circulating cell-free DNA (cfDNA), DNA methylation and mutations, circulating tumor cells, miRNA, IncRNA and mRNAs, cell-free proteins and peptides and exosomes, are promising and attractive approaches.

The liquid biopsy of CTCs, which belongs to the larger family of circulating rare cells (CRC), has been validated and approved by the FDA as a useful prognostic tool in a variety of cancer types [30-32]. This is based not only on the ability of CTCs to mirror tumor heterogeneity but also on the possibility to combine the genetic and transcriptomic status of single CTCs with epigenome analyses. Although CTCs have received great attention based on their potential in evaluating the status of localized and metastatic diseases, their clinical implementation is not yet widespread.

* 1. **Prostate cancer**

To date, the CellSearch system assay is the only FDA approved method for CTCs. Detection of CTCs in PCa patients is seen in the 57% of patients [33]. A CTCs baseline levels lower than < 5 CTCs per 7.5 mL of blood is significantly associated with a better overall survival (21.7 *vs*. 11.5 months) compared to the unfavorable group with a CTC number of 5 or greater [33]. In addition, a decrease in CTC number during or following treatment is associated with improved survival [34]. NGS analysis of CTCs and tumor sample of a single patient with advanced PCa showed a concordance in the order of 86% between the mutations in CTCs and genomic anomalies identified in primary or metastatic tumors [35-36]. However, no validated or prospective studies have been carried out yet.

ctDNA is detectable in the peripheral blood and detection of known driver aberrations can be obtained in more than 97% of cases. Moreover, modifications in ctDNA genomic mutations is evaluable by repeated analyses of ctDNA with high degree of concordance with genomic assessment of primary tumors or metastases [37,38]. Patients with metastatic castration resistant PCa with AR point mutations detected in ctDNA before the first administration of abiraterone have shown a significant worse OS [39]. Detection of mutations in DNA repair genes in ctDNA is gaining increasing interest due to the possible benefit with a PARP targeted treatment.

**4.1.1 PSMA of liquid biopsy in PCa patients: would it be feasible?**

The question is how information on PSMA expression and cell location can be obtained in the absence of tissue from the primary tumor or its secondary deposits. An answer could come from a recent study “Heterogeneous PSMA expression on circulating tumor cells: a potential basis for stratification and monitoring of PSMA-directed therapies in prostate cancer” [40]. The authors found that 12 out of 20 CTC-positive patients showed PSMA-positive CTCs. Further studies on PSMA expression, including its cell location, on circulating tumor cells could be a potential basis for stratification and monitoring of PSMA-directed therapies in PCa.

* 1. **CTC isolation and characterization in RCC**

RCC may benefit from the development of non-invasive biomarkers, allowing early and personalized treatment changes. The introduction of CTC analysis within daily clinical practice for patients with RCC is in the early phases. However, the advances in the last 5 years in isolating and analyzing CTCs bring optimism about the future therapeutic landscape in patients with RCC.

The collection, identification, enrichment and analysis of CTC require the use of different methods. The first method consists in the detection of CTCs through epithelial markers, such as the Epithelial cell adhesion molecule (EpCAM). EpCAM is a [transmembrane](https://en.wikipedia.org/wiki/Transmembrane) [glycoprotein](https://en.wikipedia.org/wiki/Glycoprotein) involved in cell signaling, migration, proliferation, and differentiation [41]. The number of CTCs that can be isolated by EpCAM is usually low [42]. This is based on the biological behavior of clear cell RCC, which often transdifferentiate through a process named “Epithelial-to-mesenchymal transition (EMT)”, a morphological transformation that is phenotypic of RCC cells [43,44] and that leads to the acquisition of mesenchymal features. Detecting EMT markers in CTCs provides fundamental information on the status of the disease, considering the straight association between EMT and the prognosis of RCC patients, as well as its role in the acquisition of resistance to anti-VEGF TKIs [43].

More recently, antibodies directed against membrane Carbonic Anhydrase 9 (CAIX) and CD147 (a widely expressed membrane glycoprotein involved in matrix metalloproteinase induction, cell adhesion and T cell activation) [45] have been developed to increase the number of CTCs in RCC patients. Indeed, Liu *et al.* reported that while EpCAM was found only in about the 18% of clear cell RCC tumors, CAIX and CD147 were present more than 97% of samples [46].

The second method is based on a RT-PCR-based approach. The three main targets of this technique are *CAIX*, *VHL* and *Cadherin-6 (CDH-6)*. *VHL* gene alterations detected in tumor samples have reported concordance with those identified on peripheral blood in about the 75% of cases [47]. On the other hand, *CDH-6* gene expression by RT-PCR has been observed in 45% of clear cell RCC blood samples [48].

Technical advances should aim to isolate a greater number of CTCs in metastatic patients than in patients with localized disease and to find the same mutations present in the corresponding histologic samples, either from primary or metastatic tissues.

* 1. **Bladder cancer** **with emphasis on biomarkers detectable in the urine**

BC is one of the most common cancer world-wide. It is classified in non-muscle invasive (NMIBC) and muscle invasive (MIBC) BC. NMIBC recurs and progresses to MIBC with a reduced survival rate. The detection and diagnosis of BC require cystoscopy and bladder biopsy, which are costly procedures. Thus, there is an urgent need to develop novel diagnostic methods less invasive and less expensive, for early detection and surveillance, both in MIBCs and NMIBCs.

Multiple urine-based tests are commercially available. Currently NMP22, NMP22 BladderChek, and UroVysion have the FDA approval for BC detection and surveillance, while uCyt+, BTA TRAK/STAT only for surveillance. However, at present, sensitivity, specificity and diagnostic accuracy of these urine-based assays are still suboptimal and, in an attempt to improve them, novel molecular markers as well as multiple-assays must to be translated into clinic.

At present there are studies exploring the use of liquid biopsy to identify biomarkers in urologic malignancy. DNA- and RNA-based markers in body fluids, such as urine and blood, are promising potential markers in terms of diagnosis, prognosis, prediction and monitoring urological malignancies[49]. For these reasons, circulating cell-free DNA, DNA methylation and mutations, circulating tumor cells, miRNA, IncRNA and mRNAs, cell-free proteins and peptides and exosomes have been assessed in urine specimens [50-52]. However, proteomic and genomic tests must be validated in well-designed multicenter clinical studies, before they can be employed in clinical practice.

The classical approach, both in the liquid biopsies and in the tissues in patients with cancer, including BC, is based on the processing of data considering only the expression of each single gene, regardless of the expression of other genes [53-55]. These complex gene interaction networks can be revealed by a recently developed systems biology approach called Weighted Gene Co-Expression Network Analysis (WGCNA) [56-57]. It takes into account the expression of all genes assessed in an experiment in order to reveal the clusters of co-expressed genes (modules) that, very probably, are also co-regulated.

**4.4. Testicular germ cell tumors**

# Liquid biopsy has been applied in GCT patients [58] to find a valuable alternative to the commonly used serum tumor markers, such as alpha fetoprotein, human chorionic gonadotropin and lactate dehydrogenase. The Targeted serum miRNA (TSmiR) test, based on the serum levels of MicroRNA miR-371a-3p, appears to be an informative biomarker for the follow-up of testicular germ cell cancer patients [59-61].

**5. MOLECULAR BIOMARKERS AND ECONOMIC SUSTAINABILITY**

The introduction of new targeted therapies and immunotherapies has led to important advances regarding life expectancy of cancer patients. However, the increase in the number of new cases, the introduction of new drugs as well as the utilization of new and additional biomarkers have all “boosted” the costs of treatments.

All this places more emphasis on the need to ensure the future economic sustainability of these methods and treatments in order to guarantee their applicability in daily clinical practice and the access for all cancer patients.

In this context, it has been estimated that the cost for a biopsy varies according to tumor type and location, generally reaching thousands of dollars (i.e. $ 14,000 for a single lung biopsy). The cost of a liquid biopsy dependents on the selected methods. It has been estimated at around $ 500 per test, with a market that has been valued at more than $ 10 billion over the next three years [62].

In consideration of the increasing use of these new diagnostic methods in clinical practice, it is absolutely necessary to evaluate the effectiveness of these techniques, their cost and, above all, their cost-benefit in terms of impact on patients’ survival and Quality of Life [63]. This is essential in order to optimize the use of these tools and ensure their economic sustainability in future years.

**CONCLUSIONS**

The identification of effective biomarkers in GU tumors has become a major focus in cancer research, mainly due to the need for selecting potentially responsive patients to improve their outcomes, as well as to reduce the toxicity and costs related to ineffective treatments. All this is leading towards a personalized approach to diagnosis, prognosis and prediction of response to therapy.

**CONSENT FOR PUBLICATION**

Not applicable.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest, financial or otherwise.

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**Figure Legends**

Figure 1. PD-L1 immunohistochemical expression in bladder cancer. Immunostaining is present both in the epithelial component and in the stromal chronic inflammatory infiltrate.

Figure 2. Role of gut microbiota in modulating the response to immunocheckpoint inhibitors in renal and lung cancer.

Figure 3. PSMA immunohistochemical expression in metastatic prostate cancer.

Figure 1

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Figure 2

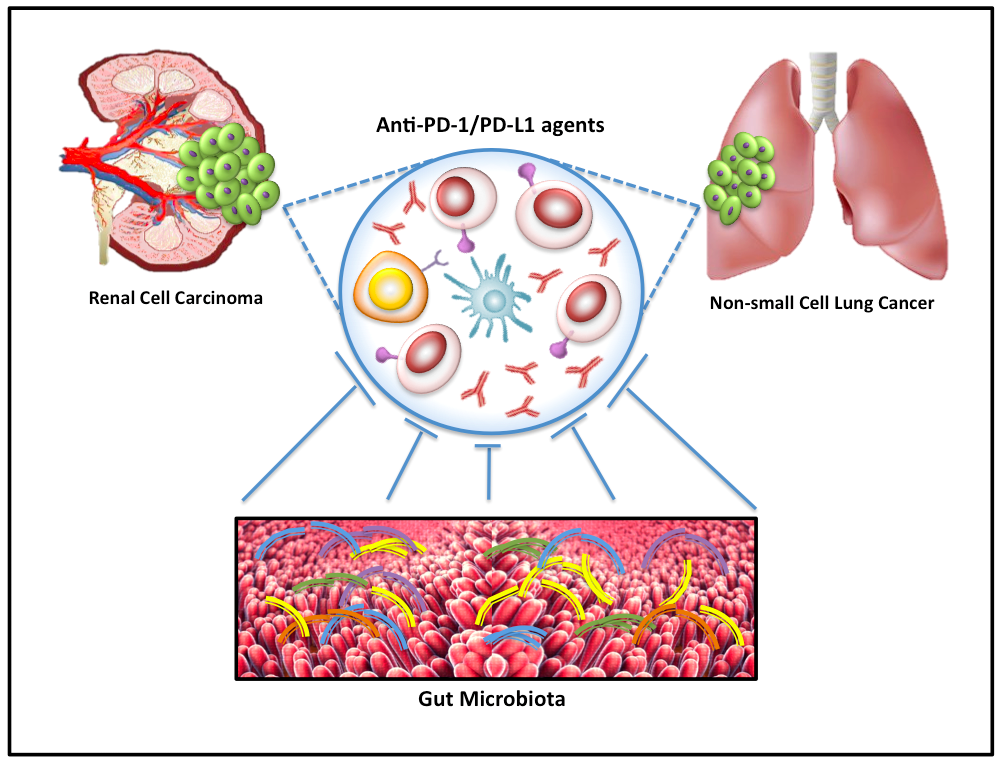


Figure 3

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