Chapter 4

Bladder Cancer Markers and Recent Innovations

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Abstract

Bladder cancer (urothelial carcinoma) is the most common tumor of the urinary tract. It occurs more frequently among men about 65 years old on average. Two forms of the tumor are known: a non–muscle-invasive one and a muscle-invasive one. The latter turns out to be very aggressive with a survival of 5 years average. The non–muscle-invasive form frequently recurs (60–70%) and in 15% of cases, it progresses into the invasive form. The diagnosis is made mainly by cystoscopy and urine cytology. A high number of researches were dedicated in order to find a simple test using voided urine to frequently monitor possible tumor recurrence. During the last 10 years, many tests were proposed concerning either special proteins of which the most common are the bladder tumor antigen (BTA) and the nuclear matrix protein 22 (NMP22) or the presence of genetic mutations [most frequently, fibroblasts growth factor receptor 3 (FGFR3) and TP53], alteration of DNA methylation, chromatin structure and, more recently, the presence of specific micro-RNA. Recently the analysis of lipids present in voided urine showed a difference in fatty acids between healthy individuals and those affected by non-invasive forms. These markers appear to have a high specificity and sensitivity: a deepening of these results could lead to the development of a test that avoids invasive treatment and the cost of cystoscopy.

Keywords: bladder cancer, bladder cancer screening, urinary markers, DNA mutations, lipid metabolism

1. Introduction

1.1. Epidemiology

Urothelial carcinoma of the bladder (BC) is one of the most common urogenital cancers, and according with the American Cancer Society in 2016, it accounts for about 5% of all

new cancers in the USA [1]. Chances for men to suffer from BC are three to four times higher than for women. This trend does not seem to be simply due to differences in lifestyle between the sexes (smoke, occupation). Perhaps, women are more protected from this disease than men because estrogen does not stimulate or (maybe) encourage the inhibition of oncogenesis [2]. This fact is supported by both *in vivo* experiments and data concerning mortality incidence [3]. Usually the first diagnosis is performed between 65 and 70 years, but it may occur earlier [2].

1.2. Etiology

Tobacco smoking is the major risk factor for bladder cancer; in fact, smokers develop bladder cancer four times more than non-smokers [4]. Long time smokers have a higher risk of getting sick, and this trend remains even in former smokers up to 20 years [2, 4]. The process that leads to carcinogenesis has not yet been described in detail nor has there been a clear definition as to which of the 60 carcinogens found in tobacco smoke are directly involved with cancer development [5]. However, some free radicals seem to have clear links with tumor development [5]. A linear relationship was found between smoking status and 4-ABPDNA adduct formation and an increase in alterations in p53 tumor suppressor gene (TSG) expression [6]. Metabolism of arylamines are also involved in bladder carcinogenesis. In fact, N-hydroxy metabolites are filtered into the bladder lumen and hit the epithelial tissue, thereby triggering a process of tumorigenesis. The processes of detoxification, catalyzed by *N*-acetyltransferase, are under genetic control. The risk of developing bladder cancer is stronger in individuals with *N*-acetyltransferase-2 slow acetylators. There is a strict relationship between environmental exposure and genetic risk factor [7, 8]. Therefore, the link between smoking and suffering from bladder cancer seems to be weaker than the one between smoking and developing respiratory tract tumors. In fact, there are populations with high smoking rates but low bladder cancer rates [2]. Certain occupational groups are at increased risk of developing bladder cancer because of their chronic exposure to chemical agents. Also, in this case, the probability of developing a bladder tumor depends on an individual susceptibility to this disease [9]. However, some workers mainly employed in textile and tire industries are at increased risk. Several substances, such as naphthylamine, 4-aminobiphenyl (ABP), and benzidine, were unambiguously associated with bladder cancer and are now banned. Other substances such as ortho-toluidine, polychlorinated biphenyls, formaldehyde, asbestos, and solvents such as benzene, dioxane, and methylene chloride are considered strong candidates for bladder carcinogens. Other risk factors for the development of a bladder cancer are chronic infection or irritation of the bladder, which causes an involuntary contraction of the bladder muscles that results in an urgent, uncontrollable need to urinate. A higher possibility of developing genetic mutations is also favored by an increased cell proliferation due to infections and inflammatory processes that favor the formation of nitrite and nitrosamines [2]. The intake of analgesics that contain phenacetin or cyclophosphamide are also correlated with an increased risk of developing urothelial carcinoma. Travis et al. found that the risk of developing bladder cancer following therapy with cyclophosphamide increased by 4.5-fold and was dependent upon a cumulative dose [10]. A study concerning the risk of bladder cancer in women found that radiotherapy in the pelvic area for treatment of ovarian cancer is a potential risk factor [11].

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Infection (in the Middle East) with parasites such as *Bilharzia* and *Schistosoma haematobium* is also a known risk factor for bladder cancer [12, 13]. Rare cases of hereditary bladder cancer were found to be related to a mutation in the retinoblastoma tumor suppressor gene [2, 14].

1.3. Pathology

The most common bladder tumors in Western countries are urothelial transitional cell carcinoma (that counts over 90% of total cases), but there are other less common forms of tumor: squamous cell carcinoma, adenocarcinoma and neuroendocrine tumors [4]. Schistosomiasis (*Schistosoma haematobium*), which is endemic in Africa and in the Middle East, causes chronic granulomatous cystitis. This condition favors the development of squamous metaplasia of the transitional epithelium, which can cause squamous cell carcinoma [15]. Adenocarcinoma begins in glandular cells that are found in the lining of the bladder and is a very rare type of bladder cancer [4, 16].

1.4. Staging

Cancer that is in the lining of the bladder is known as non–muscle-invasive bladder cancer (NMIBC), and about 75% of patients show this degree of severity at first diagnosis [4]. These comprise papillary tumors designated Ta (urothelial) and T1 (extending into the lamina propria) or carcinoma *in situ* (CIS), a flat erythematous lesion with a propensity to progress to muscle-invasive disease (MIBC). This is a very aggressive cancer with a life expectancy of around 5 years. In fact, during stages T2–T4, the cancer spreads to the muscle layer of the bladder, invades the muscle wall of the bladder, and then disseminates to nearby organs and lymph nodes [4, 16].

Since 2002, a universal staging system can be used, namely, the TNM classification (tumor, lymph nodes, and metastasis). It is very important to be able to define a classification system that is easy to use and reproducible, because it can be crucial to adopt the most appropriate therapy and to predict prognosis [2].

1.5. Signs and symptoms

In most cases, lower urinary tract symptoms and hematuria are the most common early symptoms (85% of patients). Other symptoms include changes in bladder habits, increase in urinary frequency, bladder irritability, and dysuria. Symptoms of advanced bladder cancer are weight loss, abdominal pain, and renal impairment secondary to ureteric obstruction [2].

1.6. Bladder cancer diagnosis and surveillance

Cystoscopy is a diagnostic procedure to examine directly the bladder and is considered the current gold standard for diagnosis and surveillance [4]. This type of examination allows for a possible biopsy that can provide important information about tumors regarding the different developmental stages [17]. It has the disadvantage of expense and is rather invasive. Nevertheless, bladder cancer has a very high cost per patient because NMIBCs have a low

progression rate, but are characterized by a high recurrence rate (50–70% within 5 years). For this reason, patients need frequent and expensive surveillance protocols [18, 19]. Despite a correct treatment, some patients may develop muscle-invasive disease that has a high malignant potential and is associated with considerable progression and cancer death rates.

A chemical dipstick for hematuria offers another diagnostic test. Unfortunately, hematuria from bladder cancer may be intermittent, and it occurs also in people who do not have bladder cancer. Furthermore, the detection of the disease may be uncertain and may require repetitive screening [2]. Intravenous urography (IVU) is a diagnostic test in patients with hematuria. It is very specific for upper tract surveillance and diagnosis while the early detection of small urothelial tumors may be particularly difficult to visualize.

Computed tomography (CT) scanning is useful to identify lesions within the ureter, renal pelvis, and renal parenchyma, but is not specific.

Urine-based cytology is more useful for patients who have a history of urinary tract cancer. This test may be ordered to screen patients who are at high risk for bladder cancer and as an adjunct in long-term surveillance protocols [2, 17].

1.7. Urine cytology

Urine cytology is the most commonly used diagnostic technique in clinical practice. Recently, more research on other urine-based markers has been done [20].

Urinary cytopathology is a test used to detect bladder cancer and inflammatory diseases of the urinary tract. It is based on the interpretation of morphological changes in disaggregated cells. Even in the presence of a negative cystoscopic examination, morphological changes such as increased size, increased nuclear-to-cytoplasmic ratio, nuclear pleomorphism, coarse and irregular chromatin ,and frequent mitotic figures are features associated with a higher risk for bladder cancer [18, 21]. This kind of analysis requires a professionally trained and experienced cytopathologist. Incorrect sample collection and processing can adversely affect the accuracy of the results [2]. The possibility of obtaining false positive is very high with intravesical therapy, radiation treatment, and infection, which are able to alter the results [18].

This method is not suitable for screening patients because it is not very sensitive in the cases of low-grade tumors. In fact, it is not easy to identify tumor cells in urine specimens of patients with noninvasive low-grade carcinoma. These cells have particular characteristics that are not always similar to the malignant tumor cells. The early stage, dysplasia, is characterized by the formation of flat, fibrovascular stalks that cannot be identified as neoplastic formations in histological sections.

Urinary cytology is mainly indicated for the detection of high-grade and high-stage disease that shows more recognizable features (nuclear pleomorphism, coarsely clumped chromatin, and large nucleoli) [2]. In fact, urinary cytological analysis can detect carcinoma *in situ* (CIS) with a sensitivity of 80–90% and a specificity of 98–100% because it consists of cells that are significantly abnormal. Urinary cytology can be used in long-term surveillance programs, but it is an expensive technique [18].

1.8. Biomarkers in bladder cancer

The correct treatment of patients with bladder cancer requires an early and accurate diagnosis, which is followed by long-term surveillance. Currently, the most effective tests that could diagnose and monitor the progression of the disease are cystoscopic examination, cytology, and histology. These techniques have promoted a dramatic decrease in the mortality associated with the disease, but they are inadequate for several reasons. They do not allow us to understand the molecular mechanisms underlying the tumor in question; the possibility of human error is quite high; they are expensive and, in the case of cystoscopy, invasive [17].

In recent years, due to improved molecular biology techniques, new classes of diagnostic and prognostic biomarkers have been identified. They have taken into account not only individual proteins but also the interactions between molecules in pathways known to be tumorigenic [22].

Tumorigenesis occurs often with a clear involvement of distinct pathways that can lead to the development of NMIBC or of MIBC. This feature can be used to find out new markers which are able to highlight the pathway involved and, hence, the type of tumor. The study of gene expression in bladder cancer is another type of investigation that is developing [17]. Identifying the ideal marker is not a simple task. Indeed, it needs to feature many requirements together: high sensitivity and specificity, the ability to predict the degree of tumor malignancy, lack of susceptibility to human errors, user-friendliness, noninvasiveness, and price convenience. It should not be influenced by the presence concurrently of other diseases and by the presence of hematuria or irritative symptoms that often occur in patients with bladder cancer [20]. An ideal biomarker should be also able to identify, in a clear way, the disease before its clinical manifestation and, at the same time, to provide accurate prognostic information [17, 23]. By developing non-invasive molecular assays which are able to analyze body fluids, it would be easier to perform a large-scale screening of individuals most at risk [20, 22]. It would be important to be able to develop a test that can express a very strict diagnosis, which allows patient treatment in a personalized way. This ideal marker could relieve the inconvenience inherent in the use of invasive procedures to strictly necessary cases. In the last few years, the aging of the population has caused a dramatic rise in diseases related to age. The incidence of bladder cancer has increased by 36%. This is another reason why it would be useful to have an effective screening method, as is the case for the screening of prostate and colon cancer [20].

The following discussion illustrates the current diagnostic assays for bladder cancer and discusses some of the emerging biomarkers. Some of these studies are at a preliminary stage, but they appear to deserve further investigation and a mention in this text.

2. Current diagnostic assays

2.1. Urine-based assay

2.1.1. Bladder tumor antigen (BTA) assay

The original bladder tumor antigen (BTA) test is an agglutination assay that measures the level of a membrane protein that is released into the urine when the cancer invades the bladder wall.

The BTA test can be used for the detection of low-grade disease and shows a higher sensitivity than cytology. Unfortunately, it cannot be used in patients affected by other disorders such as urinary infections, stones, or benign prostatic hypertrophy (BPH). In such cases, the presence of concomitant diseases can lead to false-positive results [20]. To overcome this lack of precision, two other BTA tests were developed: BTA STAT and BTA TRAK. Both are immunoassays that can detect factor H–related protein (cFH), which is released into the urine by tumor cells [2, 18, 20].

BTA STAT is a qualitative dipstick test [24]. This assay has several advantages: it is fast, is inexpensive, and works with only a few drops of urine. The sensitivity is higher than either the original version of the test or cytology. The results are more accurate in cases of high-grade bladder cancers. Unfortunately, this test has the same problems as the original version. False-positive results are quite common with rates of 2–5%. This assay cannot be used in patients with dysuria, incontinence, and hematuria [20]. Also in the case of bacillus Calmette-Guérrin (BCG) instillation in patients has led to a sharp drop in its accuracy, even if the treatment was made 2 years before. For this reason, BTA STAT cannot be used for the monitoring of the disease in patients who have undergone immunotherapy [20, 22]. The reasons for its low specificity yet its high sensitivity are still not clear. The use of BTA STAT was approved by the U.S. Food and Drug Administration (FDA). However, a constant monitoring of patients is recommended also through cystoscopy. Hence, this technique is not definitive for diagnosis, but it is only indicative [20, 24].

BTA TRAK is a quantitative assay: an ELISA test that detects levels of cFH [20, 22]. The cFH protein can be detected also in case of bladder bleeding [22]. Furthermore, other studies argue that cFH is definitely produced and secreted by the Kupffer cells, hepatocytes, vascular endothelial cells, and platelets, but it has not been proven that bladder cancer cells secrete cFH [22, 25]. The BTA TRAK test shows a higher sensitivity than does either the BTA STAT test or cytology also for the detection of low-grade tumors. However, this test presents problems of low specificity for the same reasons as the BTA test. For this reason, also this test cannot replace urine cytology or cystoscopy. An increase of the test values is often related to the possibility of the development of a recurrence. BTA TRAK may be useful for quantifying the correct time interval between the cystoscopic analyses individually in a patient with a recurrence, but this assumption needs further work [20].

2.1.2. Nuclear matrix protein 22 (NMP22)

Nuclear matrix proteins (NMPs) have a structural role in cellular nuclei and are involved in DNA replication, transcription, RNA processing, and gene expression. In particular, NMP22 is a specific protein of the nuclear matrix that is involved in the correct distribution of the genetic material to daughter cells during mitosis [20]. Two different texts to detect this protein have been developed: NMP22 test, which is a quantitative immunoassay and NMP22 BladderChek, which is a qualitative point-of-care test. Apoptosis causes the release of this protein into the urine that can be detected using monoclonal antibodies. Urine of patients with bladder cancer contains a greater amount of this protein compared to healthy controls [2, 18, 20]. Thus, this test provides a measure of cell turnover. The sensitivity is about 51–85% and

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increases with the support of cytology [18]. Also other bladder diseases affect the reliability of this test negatively and the rate of false positive is quite high [20, 24]. NMP22 is not suitable to be used for screening exams; however, some studies have found positive correlations between NMP22 levels and the aggressiveness of the cancer. This correlation can be exploited to make assessments on patient prognosis [20, 26, 27]. The evaluation of NMP22 levels may also have predictive value for the risk of recurrence. In fact, Soloway et al. found that patients who exceed a threshold value of NMP22 level (20 U/ml) have a greater probability to develop a recurrence [28].

2.1.3. BLCA-1 and BLCA-4

BladderCancer-1/BladderCancer-4 (BLCA-1 and BLCA-4) are members of six bladder-specific nuclear matrix proteins (NMPs) discovered in 1996 by Getzenberg et al. [29]. These proteins are involved in important cellular functions such as DNA replication and RNA synthesis and in nuclear morphology [30]. Many NMPs are identified as specific markers for several cancers [31]. BLCA-1 and BLCA-4 are considered specific urinary markers of bladder cancer. In particular, they are associated with tumor cell proliferation, survival, and angiogenesis [29–31]. The expression of these factors is important to detect bladder cancer at an early stage, since they are expressed early in carcinogenesis [18]. BLCA-1 was originally identified from bladder tumor tissue, while BLCA-4 is expressed in both tumor and adjacent benign areas of the bladder, but not in bladders without malignancy [29, 30]. Probably they play different roles in the regulation of the gene expression in bladder cancer [32]. Although BCL-1 has high sensitivity and specificity while its role is not yet clear in the development of bladder cancer. BCL-4 has sensitivity and specificity higher than other urinary tumor markers. The detection BCL-4 expression is not affected by the presence benign bladder disorders but may increase in patients with spinal cord injuries. Both BCL-1 and BLCA-4 assays still need further refinement and validation if they are to be included into clinical practice [30].

2.1.4. Urinary Bladder Cancer test (UBC)

Cytokeratins (CKs) are intracellular proteins in the intracytoplasmic cytoskeleton of epithelial cells. They are overexpressed in bladder cancer and are released into the urine as soluble fragments after proteolytic degradation following cell death [18, 33]. Because of such features, various assays measuring the concentration of soluble cytokeratins, such as TPS for cytokeratin 18 and TPA for cytokeratin 8, 18, and 19, have been used to detect bladder tumors [34]. Immunological assays UBC-Rapid and UBC-ELISA tests are used to detect the concentration of a combination of cytokeratin 8 and 18 fragments [18]. The **UBC-Rapid test** is a qualitative point-of-care assay that shows a high variability of sensitivities and specificities that probably is due to the histological and clinical characteristics of bladder cancer [18, 33]. This assay requires no special knowledge as the case of BTA STAT and is more sensitive and specific. However, it has a low sensitivity in the detection of bladder cancer recurrence and cannot replace, but only lower, the number of cystoscopies heeded during the patient's follow-up [35–37]. **UBC-ELISA** is a quantitative assay. It is limited by a strong possibility of human error and it is characterized by a high variability of sensitivity and specificity, a high false-positive

rates and the inability to detect low-grade tumors. Further studies are needed to assess their potential diagnostic role and to increase the utility of these tests for bladder tumor surveillance [18].

2.1.5. CYFRA 21-1

Cytokeratin Fragments (CYFRA 21-1) is also an ELISA-based assay that detects the concentration of soluble fragments of cytokeratin using two mouse monoclonal antibodies. Various studies on the sensitivity and specificity of the assay have reported different results. Possible reasons include the different tumor grade of the patient populations and the differences in the method of urine collection and storage [34]. The centrifugation step is very important to remove cells and cell debris that contains a large amount of CYFRA 21-1, though the accuracy of the assay is not improved yet. In fact, after centrifugation an equivalent decrease in the number of true positive and false positive can be observed [38]. CYFRA 21-1 is found at high concentrations in the urine of patients with benign diseases of the bladder. Also intravesical immunotherapy with BCG increased concentrations of urinary CYFRA 21-1, even after years of treatment, when there was no evidence of a bladder tumor. This assay has a high sensitivity for the detection of high-grade and CIS tumors, but it cannot be used for early detection. This assay shows greater accuracy for the detection of primary tumors than for the recurrence. Currently, it cannot be used as a substitute for cystoscopy and has achieved only an arguable and marginal role in daily clinical practice [34].

2.1.6. Survivin

Survivin is a member of the inhibitors of apoptosis proteins (IAP) gene family that is involved in apoptosis inhibition [39]. Survivin is expressed during embryonic and fetal development but not in normal adult tissues. It is also abundantly expressed in transformed cells and in many tumors. Survivin promotes an abnormal elongation of cell life that leads to an accumulation of genetic mutations and can promote resistance to immune-surveillance. Researchers are interested in exploiting the information on survivin for diagnostic and clinical purposes. For this reason, they are studying its role as a promoter of carcinogenesis but also the pathways in which it is involved. The detection of survivin in the urine is obtained by a BioDot microfiltration detection system. Dots of the urine samples are blotted on nitrocellulose membranes and survivin can be detected using various anti-survivin antibodies and standard dot blot detection reagents [40]. Survivin has a high sensitivity for detecting low-stage and low-grade bladder tumors often underdiagnosed by other diagnostic tests. High level of the urine's survivin is associated with increased risk of bladder cancer, of tumors of higher grade, and of bladder cancer recurrence. This assay is more accurate than cytology tests using urine. Moreover, it is also more accurate than NMP22 tests and avoids false-positive results.

Survivin is present in the urine of 78% of patients with localized disease. Its level could be skewed in patients with either T1 or higher tumor stage than in patients with Ta disease or CIS. Its use in the diagnosis would require further evaluation and a deepening study of the signaling pathways in the bladder cancer [41].

2.2. Cell-based assay

2.2.1. ImmunoCyt/uCyt+ assay

Fradet and Lockhard developed the immunofluorescence-based test ImmunoCyt/uCyt+ in 1997. These are two markers that are expressed in malignant exfoliated urothelial cells and few normal umbrella cells and are not found in the other normal cells [17, 18, 42]. This assay uses a cocktail of three antibodies: the first antibody (19A211) is directed against a glycosylated carcinoembryonic antigen and is labeled red; the other two antibodies, M344 and LDQ10, labeled with fluorescein, are directed against mucins [18, 42]. The mucins are glycoproteins that are normally found on the surface of epithelial cells. In normal cells, these glycoproteins are found in heavily glycosylated form while in malignant cells these glycoproteins are less glycosylated. This test uses LDQ10 and M344 antibodies that can recognize some portions of the protein backbone [42]. The recognition of the results is given a lot to the cythopathologist's skill. In fact, to have a negative response, cytology slide should contain at least 500 cells that are negative for fluorescence while the presence of one fluorescent cell is considered to be positive. This assay is not very influenced by factors such hematuria or inflammatory conditions because it is a cellular assay. ImmunoCyt/uCyt+ has a superior sensitivity to detect early pathological stage than cytology. It can also improve the detection of CIS and increase the sensitivity of the urinary cytology [18]. However, to be valid, this assay must always be accompanied by cytological analysis, and it is only suitable for the surveillance of patients with a history of bladder cancer [18, 42].

A combination of multiple immunological tests is considered useful for diagnosis and prognosis. Recently a combination of p40, GATA3, and uroplakin II (transmembrane protein) antibody has been proposed [43].

2.2.2. UroVysion fluorescence in situ hybridization (FISH)

UroVysion is also a fluorescence-based assay. It was developed in 2000 and is FDA approved as a urine marker for the diagnosis and the surveillance of bladder cancer [24]. Exfoliated urothelial cells are analysed exploiting the technique of fluorescence *in situ* hybridization (FISH) to detect the aneuploidy of chromosomes 3, 7, and 17 and loss of the 9p21 locus [17, 18]. As the case of ImmunoCyt/uCyt+ assay, this assay is quite complex and requires the interpretation of a skilled cytopathologist. In fact, the result is considered to be positive if either at least five cells are found with two extra chromosomes, or ten cells with an extra chromosome, or a homozygous deletion of 9p21 in >20% of the epithelial cells [24]. UroVysion has a higher sensitivity in diagnosis or recurrence of bladder cancer than other types of analysis. A positive result of UroVysion test, in patients who have been previously treated with BCG, can warn doctors about treatment failure. Unfortunately, this assay has the disadvantages of providing many false-positive results and is found to be more sensitive in cases of high-grade tumor than in case of low-grade disease. Thus, it is recommended to use UroVysion after ImmunoCyt/uCyt+ test or cytological analysis as a confirmatory test [18, 44].

2.2.3. DD23

DD23 is an IgG murine monoclonal antibody (MAb) derived from the immunization of a BALB/c mouse that recognizes a 185-kDa tumor-associated antigen. This antigen is not detected in normal urothelium while it is expressed in human bladder cancer cells, both *in vitro* and *in vivo* [45]. However, a study observed that in patients with bladder cancer, DD23 antigen is expressed in cancerous as well as in noncancerous cells. One theory holds that normal urothelial cells in response to external signals from the malignant cells express DD23 [45, 46]. This immune-cytochemical assay is performed using an avidin-biotin alkaline phosphatase, with a single urothelial cell exhibiting intense immunostaining sufficient to make a positive call. However, there are various methods of DD23 detection [47]. For example, Bonner et al. used a highly sensitive automated quantitative fluorescence image analysis system, whereas Gilbert et al. used a commercial clinical reference laboratory-adapted qualitative immunocytochemistry assay with light-based microscopy [45, 46]. The cytological analysis of urine sediment carried out using murine IgG1 monoclonal antibody (MAb) together with Papanicolaou (Pap) staining is difficult both from practical use and from an interpretative point of view. Even this technique has a higher sensitivity for the detection of high-grade tumors than low-grade ones [47–52]. DD23 antigen expression must be supported by cytological analysis. It can enhance the sensitivity of the cytopathology diagnosis of 21%, especially in low-grade cancers and can increase the detection of unclassified TCC cases [45, 53, 54]. This assay is not very suitable for the detection of recurrence in patients who have previously received an intravesical therapy. DD23 can be a support of cystoscopic examination to increase the sensitivity to monitor and diagnosis recurrent bladder cancer. The use of this biomarker needs further clinical studies [45].

These assays are summarized in **Table 1** with their characteristics.

	Sensitivity	**Specificity**	**Limitations**
Urine-based assay			
BTA STAT	53–83%	67–72%	High false-positive rates.
BTA TRAK	66–72%	51–75%	High false-positive rate.
Nuclear matrix protein 22 (NMP22)	51–85%	77–96%	False positive in case of hematuria or inflammatory bladder conditions.
BLCA-1	80%	87%	It needs futher validation.
BLCA-4	89–96%	90–100%	It needs futher validation.
UBC-Rapid test	36–78%	63–97%	High variability of sensitivities and specificities. Hight false-positive rate. Low sensitivity in the detection of low-grade bladder tumors.
UBC-ELISA	40–70%	63–75%	Hight false-positive rate. Low sensitivity in the detection of low-grade bladder tumors.
CYFRA 21-1	61–85%	75–91%	False positive in case of inflammatory bladder conditions.
Survivin	64–83%	88–93%	It needs futher validation.

	Sensitivity	Specificity	Limitations
Cell-based assay			
ImmunoCyt/uCyt+ assay	68%	72%	The test results depend on specimen stability and handling.
UroVysion fluorescence *in situ* hybridization (FISH)	69–75%	82–85%	Low sensitivity in the detection of low-grade bladder tumors. Lack of consensus regarding criteria used to evaluate abnormal cells.
DD23 (*)	70.3%	59.8%	It can be used as an adjunct to cytopathologic evaluation.

All the data derived from Ref. [17], except (*) from Ref. [23].

Table 1. Sensitivity and specificity of urine-based and cell-based assays.

3. Nucleic acids alterations

Circulating DNA has been demonstrated in all body fluids, including urine, and it is the demonstration of many tumor-related alterations, particularly for colon cancer. For this reason, many researches were made on urine and bladder samples in order to analyze free DNA and RNA fragments present. An increase in DNA was found in both the voided urine and plasma of patients with NMIBC with respect to non-affected patients [55]. Aggressive tumors show very high DNA values. Analysis of the DNA may represent a novel diagnostic tool to indicate the presence of residual disease or to discover aggressive forms early in the bladder cancer course. Analysis of the DNA showed 302 mutations of exons, 204 segmental alterations in genomic copy number. and 22 genomic rearrangements for a given sample [56]. Three different groups were identified based on the gene alterations. Group A, classified as focally amplified, is enriched in focal somatic mutations in many genes; group B is characterized by CDKN2A-deficient fibroblast growth factor receptor 3 (FGFR3) mutant defined papillary from the histological aspect; and group C, classified as "tumor protein p53 (TP53) cell-cycle mutant", is enriched with RB1 mutations and amplifications of E2F3and CCNI [56].

It has been suggested that these differences may represent different oncogenic mechanisms. In non-invasive tumors, mutations were found especially in the oncogenes Harvey rat sarcoma (HRAS), fibroblasts growth factor receptor 3 (FGFR3), and phosphatidylinositol-3-kinase (PK3CA).

In invasive tumors, mutations have been found in tumor suppressor genes, especially TP53 and retinoblastoma (RB1) [57]. The presence of the FGFR3 mutation in urine is observed for low-grade tumors and seems to be associated with concomitant or future recurrence [57, 58]. Methylation is also important: five targets were identified, including ventral and anterior homeobox 1 (WAX1) KCNV1, TAL1, PPOX1, and CFTR, which have a sensitivity of 88.68% and a specificity of 87.25%. An increase in methylation is also observed for the tumor suppressor gene RUNX3 gene [59, 60]. In particular, methylation of VAX1 and LMX1A appears to be associated with bladder cancer recurrence. Promoter hypermethylation of some genes

combined with FGFR3 mutations may represent a sensitive diagnostic assay [61]. Methylation of the tumor suppressor gene H-cadherin (CDH13) has been reported in many cancers, and an increase is observed especially in Asian patients affected by bladder cancer [62]. The CDH13 gene, located on 16q24, encodes a protein that belongs to the cadherin family [63]. CDH13, a tumor suppressor gene (TSG), is also called H-cadherin or T-cadherin and plays a pivotal role in cell–cell adhesion [64]. The expression of this gene favors the adhesion between the cells, thus inhibiting metastatic diffusion.

3.1. Microsatellite analysis

Microsatellites (or short tandem repeats) are repeated noncoding DNA sequences consisting of very short repeat units (mostly 2–4 bp each) arranged in a tandem repeat. They can be used as molecular markers of loci and play an essential role in the structure of chromosomes. Two types of microsatellite alteration are involved in many cancers: loss of heterozygosity due to a deletion and genomic instability due to an alteration of microsatellite repeat length [40, 65].

Several studies have been published on the correlation between microsatellite alterations in bladder transitional cell carcinoma (TCC). In particular, they have observed 17 microsatellite loci. The loci on chromosomes 9 and 18 have proven to be the most informative [66–69]. Saidi et al. compared two microsatellite loci (GSN and/or D18S51) of patients with histopathologically confirmed bladder TCC, with normal bladder mucosa and with non-malignant diseases. They found alterations in 46 out of 70 patients with TCC, but none in the tissue samples from the control group [70]. This analysis showed a sensitivity of 65.71%, and specificity of 100% but, according to the literature data, frequency of microsatellite alterations in urinary bladder TCC varies a great deal [67, 70, 71]. Unfortunately, microsatellite analysis is time-consuming, expensive, and requires highly trained personnel. Furthermore, Saidi et al. have not found a close correlation between the pathological stages as recurrence, metastasis, and death, in patients with TCC, followed by at least 2 years [70]. The study of the possible use of microsatellites as a tumor marker is complicated also by other point of view: the correct selection of microsatellite loci, their polymorphic nature and ethnic differences in patient populations are features that affect the results between studies [70, 72].

4. Chromatin alterations

Mutations in chromatin could lead to alterations in genes involved in human carcinomas [73]. In high-grade bladder cancer over 2300 alterations were found as well as in genes involving chromatin modifiers [74]. This implies that epigenetic modulators could have a therapeutic role in urothelial cancers [73]. Varticovski et al. performed comparative bio-informatic analysis on mutations in several previously identified genes associated with bladder cancer. They found mutations in TP53, RAS, and TERT genes and mutations/deletions in several chromatin modifiers KDM6A and MLL2/3. They found also mutations in novel sites distant from promoters showing a reprogramming of regulatory networks. Many of these mutations

occur in characteristic sites unique for each stage of the tumor progression. This feature has potential clinical application providing valuable new information on bladder cancer biology and tumor progression [74]. The coiling of DNA around nucleosome particles is the basis of genome organization with histone modifications being associated with both active and repressed regions of chromatin. Acetylation or phosphorylation may change the chromatin structure by altering the net positive charge of the histone proteins so changing the accessibility of the underlying information sequences. Modifications of histones are reported for many tumors and are correlated to tumor stage and prognosis, but with contradicting results [75]. Histone acetylation is catalyzed by a specific enzyme family, histone acetyltransferases (HATs), and correlates with nucleosome remodelling and transcriptional activation, whereas deacetylation of histone tails is catalyzed by histone deacetylases (HDACs) induces transcriptional repression through chromatin condensation [76]. Altered expression of different HDACs has been reported inr various human cancers [77–83]. Bladder tumor chemotherapies, which act as inhibitor of acetyltransferase, have shown that the decrease in tumor cellular growth *in vitro* is due to the acetylation of histone lysine with a consequent imbalance between histone acetylation and de-acetylation.

4.1. Telomerase

The telomere is the terminal region of eukaryotic chromosome and is composed of highly repetitive DNA sequences (e.g., TTAGGG in humans). It was thought to be a non-coding region while recent discoveries have speculated that it is involved in the regulation of telomerase. Telomerase is a ribonucleoprotein organized to form a complex that includes: an RNA component, human telomerase RNA (hTR), and telomerase reverse transcriptase (hTERT) that is a catalytic protein. In normal cells, DNA polymerase is not able to replicate the chromosome until its termination. In fact, after each DNA replication, there is a progressive shortening of chromosomes with a consequent loss of genetic information, which causes chromosomal instability and cellular senescence. Telomerase maintains telomere length in several cancer cells types [40]. It keeps intact chromosomes adding telomeres at the ends chromosomes, lengthening again the shortened telomeres. In normal cells it is active only during embryonic development while in most normal adult tissues its expression is repressed [84]. The expression of telomerase in tumor cells immortalize them [85]. This mechanism can be considered a crucial step in tumorigenesis [84].

The telomeric repeat amplification protocol (TRAP) assay is used to measure telomerase activity. Telomerase reaction products are detectable by several commercial kits that provide optimized sets of primers and reagents for telomerase detection. Another method allows measurement of hTERT mRNA levels by RT-PCR. The sensitivity of these assays is variable: it depends on sample manipulation but also on the presence of inflammatory conditions that can contaminate benign cells with telomerase activity [86–96]. The specificity of both the TRAP assay and hTERT RT-PCR is also variable. Telomerase assays are not suitable for use in clinical settings. To overcome these problems, it requires more clinical trials [40].

5. RNA messenger

The detection of (*hTERT*), mRNA of human telomerase reverse transcriptase, using RT-PCR was considered as a valid tool for a noninvasive tumor diagnosis test. However, in urine we can found a significant amounts of cell-free RNA and for this reason RNA tumor markers (e.g., *hTERT*, *UPK1A*, *HTATIP2*), cannot be used unconditionally for RT-qPCR–based analysis of whole urine [97]. Another system that has been intensively studied for its role in tumorigenesis is *uPA*: *ETS2*. The *uPA* promoter region contains *ETS2* binding sites, a member of the *ETS* family of transcription factors. *uPA* mRNA content in tissue and urinary protein concentrations of uPA are suitable for bladder cancer diagnostics. In the case of bladder cancer higher *ETS2* RNA concentration it is observed compared with *uPA*. This tumor marker ratio of *ETS2*: *uPA* could be an interesting diagnostic tool [97–101]. Hedegaard et al. prepared a total RNA-sequencing (RNA-seq) libraries for 476 patients with bladder cancer at different stages (Ta, T1, *in situ* [CIS], MIBC). They mapped sequences to the human genome and then explored the heterogeneity in early-stage bladder cancer of 8074 genes [102]. The results were interpreted by statistical analysis and, on the basis of these results; the bladder tumors were sub-grouped into three major classes [102–104]. They found that tumors of high stage and grade were more frequently observed in classes 2 and 3 than in class 1.

Particularly, MIBC and high-risk NMIBC were more frequently observed in the same cluster 2 showing similarities. Differences in gender or smoking status do not influence the results [102]. Class 3 tumors show basal-like characteristics. They are mainly associated with repressed genes and with modifications of histones and/or chromatin. Class 1 tumors are characterized by a good prognosis, while class 2 shows poor prognosis and high expression of late cell-cycle genes, which have previously been associated with aggressiveness in bladder cancer [102, 105, 106]. Class 1 and class 2 tumors display different levels of aggressiveness. This study can be overlapped with other previous studies [105]. This kind of analysis could be a way for optimized surveillance programs and treatment selection [102].

6. Micro RNA

Micro RNA are short non-coding RNAs (18–25 nucleotides) that can change the expression of mRNA. This kind of regulation can take place directly by interaction with mRNA, by translation regulation of the protein product or by mRNA degradation. A single miRNA can regulate multiple genes or more miRNA can be involved in a single target (**Figure 1**) [107].

They can, therefore, regulate the expression of many genes and the analysis of these molecules may be important for understanding many biological processes such as epithelial to mesenchymal transition, which is a relevant mechanism in bladder cancer tumor development. Micro RNAs are present in many body fluids including urine, are resistant to RNase

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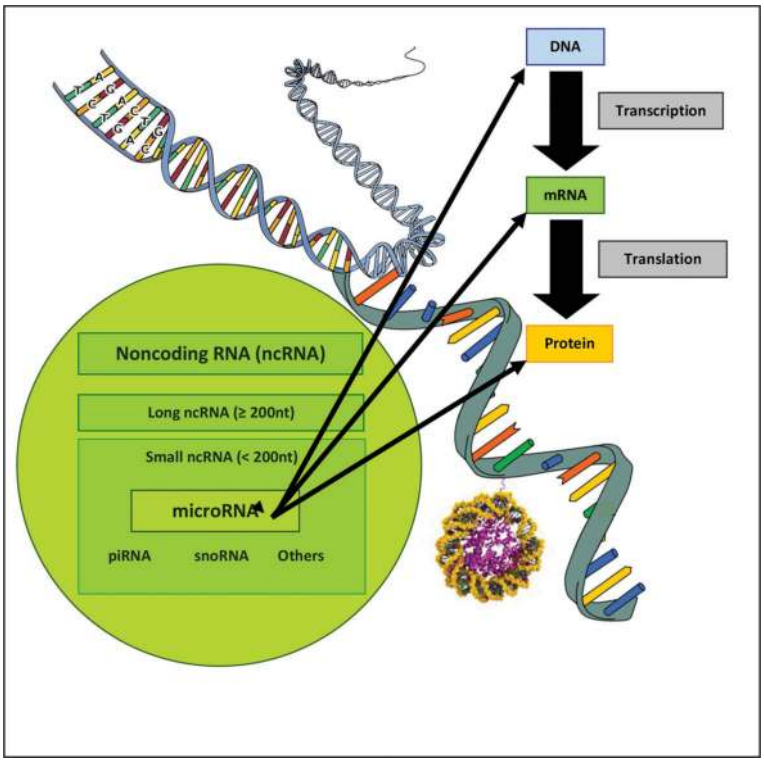

Figure 1. Functions of mi-RNA.

and also can be preserved for long time, so they can be determined using non-invasive methods. Many miRNAs were found in the urine, some of them might be correlated with renal or prostatic diseases, as well as bladder cancer [108].

Recently, a list of miRNAs detected in bladder cancer as well as their target genes has been reported. At the moment, a complete analysis is unavailable, but some results indicate some differences in relation to bladder cancer, particularly in relation to their invasiveness and prognosis.

An increase of miR 21, the cluster miR 183-96-1, miR 210, and miR 205 is observed to influence the epithelial-mesenchymal transition, cell growth and migration. On the other hand, a decrease is observed in many others, like miR 29c, miR 124, miR 409, miRs 23 family, miRs-200 family, miR-214, that favors apoptosis and inhibit cell proliferation. miR-205 discriminates between low-grade papillary urothelial carcinoma and high grade papillary urothelial carcinoma, whereas miR-145 distinguished high-grade papillary urothelial carcinoma from infiltrating carcinoma [109]. The increase of the expression levels of miR-200c and miR-141 in patients with bladder cancer after surgical removal of the tumor appears to return to normal values [110]; miR-126, miR-182, and miR-199a are the most abundant in the urine of bladder cancer patients with respect to the controls [107]. Ratios of miR-12 s6/miR-152

and miR-182/miR-152 are considered as makers capable of distinguishing bladder cancer patients from controls and patients affected by infections. Their sensitivity and specificity are not very high ranging from 55 to 82%. Yamada et al. found that the detection of miR-96 in the urine has a sensitivity of 71.8% and a specificity of 89.2% [111]. Shimizu et al. reported also good specificity and sensitivity by testing a methylation panel of miR9-3, miR124-2, miR124-3, and miR 137 [112]. As regards to a prognostic evaluation, miR2103, miR214, miR152, and miR3187-3p are predicting a recurrence-free survival [113–115].

7. Proteins

Proteomics analysis allows the identification of proteins in the urine of patients. Some of these proteins such as alpha-defensin, apolipoprotein A-1 (APOA1), and alpha-1-antitrypsin are present in increased levels in tumor cells [116]. Metabolomic analysis allowed the identification of some metabolites associated with bladder tumors. However, the correct association of the results with a type of tumor can be difficult to establish due to small molecule renal filtration.

There are several proteins that are worth further study for evaluation as new possible biomarkers. **Fibronectin** is a multifunctional, extracellular matrix glycoprotein. It is produced by a wide variety of cells and is present in many tissues. Some studies have shown that increases in the urine content in case of the bladder cancer [117–119]. **Clusterin** is a heterodimeric disulfide-linked glycoprotein, implicated in a number of biological processes. It is a chaperone protein present in two isoforms (1 and 2) with antagonistic actions exhibiting different cellular locations. Clusterin gene increases its expression in bladder cancer particularly in invasive disease [120]. High clusterin expression is associated with poor prognosis. However, it is expressed in all body fluids and this feature limited its specificity. It would be necessary to detect the individual expression of the two isoform rather than total clusterin levels [121]. Tilki et al. reported **CEACAM1** (Carcinoembryonic antigen-related cell adhesion molecule 1, also known as CD66a) as a novel urinary marker for bladder cancer. Sensitivity was higher for MIBC than NMIBC and thougth patients with diabetes were excluded from the study. Furthermore, CEACAM1 immunostaining disappears in urothelium of NMIBC while appears in adjacent tumor-associated endothelial cells [122]. **Calprotectin** is a protein with antimicrobial properties [123]. It was suggested as a prognostic indicator because it results in upregulation in bladder cancer with 80% sensitivity at 92% specificity [124, 125]. However, it is released also by neutrophils during inflammatory processes and this feature compromises its accuracy [126]. **Stathmin-1** and **CD147** are two urinary proteins studied for their correlation with bladder cancer. In fact, there are reports that overexpression of stathmin-1 and CD147 are associated with aggressive bladder cancer and a poor prognosis [127, 128]. **γ-synuclein** is involved in the pathogenesis of neurodegenerative diseases and is also used as a marker in breast tumors. It shows 87.5% sensitivity and 90.0% specificity. **DJ-1** has been reported to be overexpressed in aggressive high-grade bladder cancer [129]. A pilot study found urinary DJ-1 to be significantly increased only in MIBC [130]. The specificity of **Apolipoprotein A4 for bladder** cancer is not confirmed even if various apolipoproteins increase in the urine

of bladder cancer patients [131]. In Kumar et al probably was overestimated the presence **coronin-1A** (a cytoskeletal protein) in urine of patient with bladder cancer [130, 132]. Orenes-Piñero et al. realized an immunohistochemistry analysis, followed a proteomics analysis, that showed Reg-1 (lithostathine-1-alpha) overexpression in bladder tumors [133]. Urinary levels of matrix metallopeptidase 9 (**MMP9**) are elevated in many cases of invasive bladder cancer. However, it shows a modest sensitivity and specificity in case of low-grade disease [134–141]. The majority of the biomarker studies could not be classified as unequivocal. This conclusion may be due to a loss of information (stage/grade/sensitivity/specificity) or due to a non-representative patient population that overstates the sensitivity and specificity [130].

8. Lipids

There have been very few published analyses of lipids as potential biomarkers, although an increase in the serum of lipids and proteins associated with sialic acid were demonstrated to differentiate patients from healthy control groups, showing a sensitivity of around 80% and a specificity of 70% [142]. A rare variant of urothelial carcinoma has been characterized by large epithelial cells with an eccentric nucleus and vacuolated cytoplasm that resemble lipoblasts, which are also positive for cytokeratin 7, 20, CAM 5.2, and other proteins. Only in one publication on bladder tissue has the composition of fatty acids been taken into consideration; it was shown that there was an increase in the levels of stearic and oleic acid and a decrease in the level of arachidonic acid in pathological tissue with respect to healthy tissue excised from the same patients [143]. In addition, the levels of some polyunsaturated fatty acids were significantly reduced, suggesting an altered lipid metabolism occurring *in vivo* during human bladder tumor-genesis. The only attempt to determine lipid concentrations in urine was made more than 30 years ago in 1985, comparing bladder tumor patients with healthy controls and additionally some patients with bacterial infections. It was shown that there was a marked difference in phospholipid levels compared to fatty acids with 100% specificity and 80% sensitivity. However, macrohematuria or infection can affect the results that subsequently appear similar to the tumor samples [142].

9. Isolated particle

9.1. Exosomes as possible marker

Exosomes are small membrane vesicles particles (30–100 nm) released by the cells into the extracellular environment and play different roles in many physiological situations like the immune response. Exosomes isolated from the urine of patients with muscle-invasive bladder cancer induced epithelial- to mesenchymal transition in urothelial cells [144].

9.2. Analysis of isolated virtosomes from voided urine

Circulating DNA has been isolated using chromatographic separation or by ultracentrifugation. In this way not only cell debris or particles, but also exosomes are eliminated. In the

supernatant obtained after ultracentrifugation are present virtosomes that are comprised of newly synthesized DNA, RNA, proteins, and phospholipids and are released from cells in a regulated fashion [145, 146]. In a research made on human lymphocytes in culture, it has been demonstrated that the cells released virtosomes that differ in composition in relation to the stimulation of proliferation made with phytohaemoagglutinin. The virtosomes present in the medium may also be capable of influencing the non-stimulated lymphocytes and *vice versa* [146].

The isolation of virtosomes from voided urine of non-invasive bladder cancer patients has been attempted. The analysis of the material present in the voided urine and from the cell cytoplasm of the control group and the patients are reported in **Table 2**. The nucleic acid content was lower in the patient group, but the difference was not significant and was not confirmed by the cytoplasmic results.

	Urine		**Cytoplasm**	
	Controls	**Patients**	**Controls**	**Patients**
Proteins %	1.5 ± 1.4	1.39 ± 1	0.223 ± 0.19	0.236 ± 0.259
DNA %	0.39 ± 0.31	0.1 ± 0.03	0.143 ± 0.79	0.17 ± 0.029
RNA %	0.09 ± 0.07	0.022 ± 0.007	0.096 ± 0.033	0.056 ± 0.030
PLs %(*)	98.5 ± 1.81	98.35 ± 0.95	99.4 ± 0.32	99.16 ± 0.23

(*) phospholipid.

Table 2. Composition of protein, DNA, RNA, and phospholipid in the voided urine and cell cytoplasm in the control and patient groups.

The values shown in **Table 2** are very different with respect to those obtained from healthy lymphocytes either in the culture medium or in the cytoplasm using the same procedure (**Table 3**), where a much larger proportion of protein and RNA was detected, and only a small proportion of phospholipid [146].

	Medium	**Cytoplasm**
Proteins %	37.91	41.01
DNA %	4.65	3.45
RNA %	53.41	35.09
PLs %(*)	3.94	19.90

(*) phospholipid.

Table 3. Composition of virtosomes isolated from lymphocytes.

The chromatographic analysis of the phospholipids of the voided urine and cell cytoplasm did not show any significant difference between the control and patient groups, unless a small increase in the amount of sphingomyelin in the patient group is considered. However, the most important result was the presence of a large amount of lipids. Preliminary analysis highlighted some differences in the fatty acids that indicated the need for a more in depth analysis (**Table 4**).

Fatty acids presents in the virtosomes					
	Control	**%**	**Low-grade tumor (Ta) %**		
Dodecanoate C12:0am==	3.3 μM	1.17	7.9 μM	3.24	↑
Myristate C14:0	22.6 μM	8	15.3 μM	6.28	↓
Pentadecanoate C15:0	27 μM	9.57	13.2 μM	5.4	↓
Palmitate C16:0	98.6 μM	34.9	84.7 μM	34.78	
Heptadecanoate C17:0	16 μM	5.67	11.8 μM	4.84	
Linoleate C18:2n6	18.6 μM	6.59	12 μM	4.92	
Oleate C18:1n9c	20.1 μM	7.12	21.2 μM	8.70	↑
18-methyl nonadecanoate C19:0	1.9 μM	0.67	1 μM	0.41	
Tricosanoic acid C23:0	0.9 μM	0.32	0.3 μM	0.12	↓
Tetracosanoic acid C24:0	2.9 μM	1.02	0.8 μM	0.32	↓
Stearate C18:0	70.2 μM	24.88	75.6 μM	31.04	↑

Table 4. Preliminary analysis that highlighted differences in the fatty acids.

An analysis was also made using chromatographic and mass spectrometric separation by extracting lipids from 20 patients with non-invasive bladder cancer and 20 controls of similar ages (**Figure 2**).

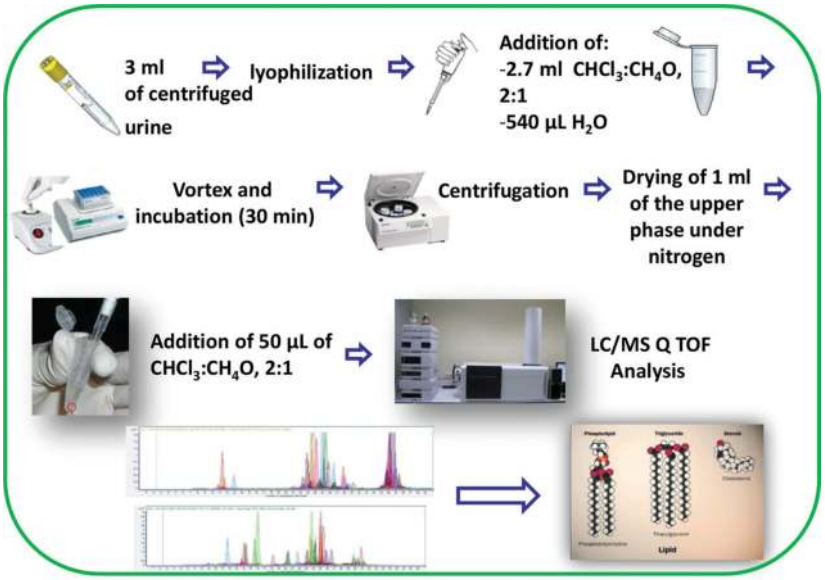

Figure 2. Lipid extraction from urine. Chromatoghraphy and mass-spectrometry of lipids extract from urine [148].

Two hundred and fifty lipids were analyzed and a significant difference was observed, in particular for 25 lipids that appear to be characteristic for the tumor patient. Their identification may offer the possibility of a marker that appears to have sensitivity and a specificity of around 100% (**Figure 3**) [147].

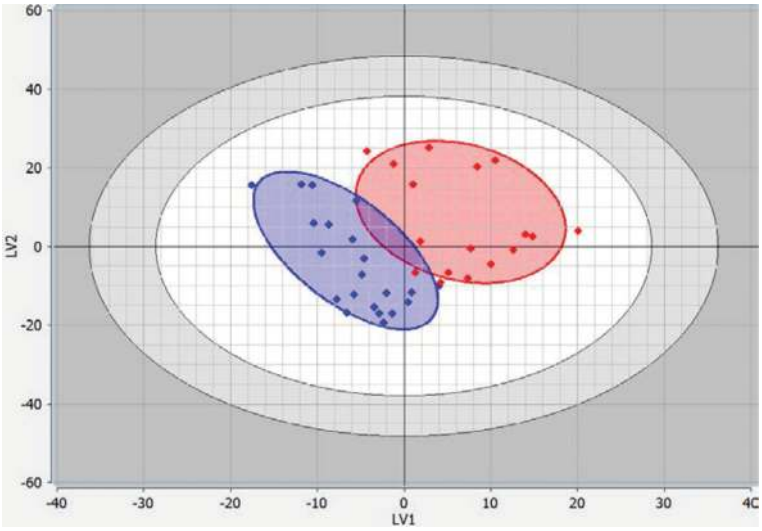

Figure 3. Partial least squares discriminant analysis (PLS-DA). Separation of lipids between patients and heathy controls [146].

10. Conclusions

The numerous publications clearly indicate how important it is to find markers for bladder cancer that may substitute cystoscopy and many efforts were made to find them. The symptoms of the tumor are not always significant and so it is necessary to determine new, specific and sensitive markers for an accurate diagnosis in order to prevent the diffusion of the pathology and to determine the eventual patients at an early stage. This explains the continuous attempts to find good, non-invasive markers, possibly present in the urine. Ideally, they must be cheap so as to allow frequent repeated tests per patient and not technically complex for their use in all laboratories.

The existing markers (such as BTA or NMP22) are not considered sufficiently specific and sensitive for a correct diagnosis and need to be supported by other tests, principally cystoscopy and cytology in which the interpretation depends much on the experience of the analyzer. Therefore, numerous attempts have been made to find other kinds of markers such as DNA mutations, DNA methylation, chromatin modifications, and specific proteins and, more recently, microRNAs present in the urine. To date, all results have been unsatisfactory given the complexity of the analysis and the limited specificity and/or sensitivity. Recently the composition of lipids in

the voided urine of patient with non-invasive form and healthy control subjects showed some significant differences thus offering new markers with high sensitivity and specificity. If these preliminary data can be confirmed, a simple and inexpensive test may be produced that is useful for both screening and prognosis.

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