International Journal of Biochemistry and Cell Biology 99 (2018) 236–256

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Review article

Urinary extracellular vesicle biomarkers in urological cancers: From discovery towards clinical implementation

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A R T I C L E I N F O

Keywords:
Biomarkers
Omics
Extracellular vesicles
Exosomes
Urine
Cancer

A B S T R A C T

Urine contains cellular elements, biochemicals, and proteins derived from glomerular filtration of plasma, renal tubule excretion, and urogenital tract secretions that reflect an individual's metabolic and pathophysiologic state. Despite intensive research into the discovery of urinary biomarkers to facilitate early diagnosis, accurate prognosis and prediction of therapy response in urological cancers, none of these markers has reached widespread use. Their implementation into daily clinical practice is hampered by a substantial degree of heterogeneity in performance characteristics and uncertainty about reliability, clinical utility and cost-effectiveness, in addition to several technical limitations. Extracellular vesicles (EV) have raised interest as a potential source of biomarker discovery because of their role in intercellular communication and the resemblance of their molecular content to that of the releasing cells. We review currently used urinary biomarkers in the clinic and attempts that have been made to identify EV-derived biomarkers for urological cancers. In addition, we discuss technical and methodological considerations towards their clinical implementation.

1. Urine: composition and liquid biopsy potential

Urine provides an alternative to blood plasma as a potential source of disease biomarkers. It is available in large quantities using non-invasive collection procedures. As a proximal fluid and resulting from the glomerular filtration of the blood, it is interesting not only from the renal or urogenital perspective, but also for a broad variety of disorders which may translate into urine metabolite or protein content alterations. Despite these advantages, to date, no biomarker or biomarker combination, has achieved widespread clinical application as a diagnostic assay.

Pre-analytical confounders are important considerations for urinalysis and may hamper clinical breakthrough (Delanghe and Speeckaert, 2016). The confounders are situated at 4 levels: sampling method (first-voided vs mid-stream specimens; first vs second morning; on spot specimens; 24-h collection), transport, preservation and sample dilution of urine. Urine samples can be normalized by analysis of osmolality (measures the osmoles of solute per liter of solution), specific gravity (compares the density of urine to the density of water), conductivity (conduction of electricity determined by the electrolyte concentration) and urinary creatinine (creatinine, the breakdown product of creatine phosphate during muscle metabolism and filtered out of the blood into the urine by the kidney). These parameters allow to correct for dilution effects due to hydration and to interpret urinalysis findings regardless of the sample dilution. Quality control studies that take into consideration these pre-analytical factors will further lead to improved guidelines for the standardization of urinalysis.

In general, urine is considered as a much simpler biofluid than plasma. Urine samples are divided into sediments and supernatant. Sediments are microscopically investigated for crystals (oxalate, carbonate, phosphate, urate and cystine), casts (hyaline and cellular), bacteria/yeast and cells that will give a clue to what is happening upstream. The supernatant is examined chemically for proteins and low-molecular weight molecules such as albumin, hemoglobin and metabolites. A first indication of the higher complexity of urine was shown in 1995 (Kanno et al., 1995). The full length, transmembrane water channel protein, aquaporin-2 (AQP2), was detected in urine supernatants. Sediments obtained by ultracentrifugation of urine supernatants contained abundant amounts of AQP2. Immunoelectron microscopical analysis of these sediments showed vesicular structures

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https://doi.org/10.1016/j.biocel.2018.04.009
Received 30 December 2017; Received in revised form 5 April 2018; Accepted 8 April 2018
Available online 11 April 2018
1357-2725/ © 2018 Published by Elsevier Ltd.

Table 1
Current urinary biomarkers for diagnosis and surveillance of genitourinary cancers implemented into clinical practice, including performance characteristics and limitations.

Cancer Type	Diagnostic Test	Urine Type	Biomarker	Biomarker Type	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC	SOE (GRADE)	Limitations	Reference (s)
Prostate	**Progensa PCA3***	Post-DRE	PCA3:PSA	Diagnosis (Re-biopsy) Transcriptomic	**54–82**	**66–89**	**48–75**	**74–90**	**0.66–0.87**	**Low**	1 Conflicting evidence on value in predicting clinical/pathological features of PC and performance to alter diagnosis or management decisions 2 Clinical utility and cost-effectiveness have not been confirmed	Auprich et al. (2011), Bradley et al. (2013), Luo et al. (2014), Marks et al. (2007), Vlaeminck-Guillem et al. (2010)
Prostate	**Mi-Prostate score (MiPS)**	Post-DRE Spot urine First catch	PCA3 and TMPRSS2-ERG *(+ serum PSA)*	Diagnosis (Biopsy) Transcriptomic	**80–93**	**33–90**	**33**	**93**	**0.73–0.88**	**Low**	1 Threshold values are not validated in an independent study 2 Reliability, clinical utility and cost-effectiveness have not been confirmed	Cornu et al. (2013), Salami et al. (2013), Sanda et al. (2017), Tomlins et al. (2016)
Prostate	**Select MDx**	Post-DRE Spot urine First catch	HOXC6 and HLX1 *(+ serum PSA, clinical factors)*	Diagnosis (Biopsy) Transcriptomic	**NS**	**NS**	**NS**	**98**	**0.86–0.90**	**Low**	1 Reliability, clinical utility and cost-effectiveness have not been confirmed and need independent validation	Van Neste et al. (2016)
Prostate	**ExoDx Prostate IntelliScore**	Non-DRE Spot urine First catch	EV-derived PCA3 and ERG; SPDEF (normalization) *(+ serum PSA, clinical factors)*	Diagnosis (Biopsy) Transcriptomic	**92–97**	**28–34**	**36–37**	**91–96**	**0.73–0.77**	**Low**	1 Reliability, clinical utility and cost-effectiveness have not been confirmed and need independent validation	McKiernan et al. (2016)
Bladder	**NMP-22**											
	1. BC test kit*	Spot urine	NMP-22	Surveillance Proteomic	**26–100**	**41–92**	**27–89**	**40–99**	**0.68–0.74**	**Moderate**	1 False positive in: infection, inflammation, hematuria, urolithiasis, bowel interposition, other genitourinary cancer, instrumentation 2 Reliability and clinical utility are uncertain 3 No clearly defined threshold value 4 Substantial degree of heterogeneity in diagnostic performance 5 Requires trained laboratory technician	Behrens et al. (2014), Chahal et al. (2001), Chou et al. (2015), Friedrich et al. (2002), Giannopoulos et al. (2001), Gutiérrez Baños et al. (2001), Horstmann et al. (2009), Lotan et al. (2017), Miyake et al., (2012a), O'Sullivan et al. (2012), Paoluzzi et al. (1999), Ponsky et al. (2001), Saad et al. (2002), Sánchez-Carbayo et al. (2001a); Sawczuk et al. (2000); Serretta et al. (2000); Shariat et al., 2006; Sharma et al. (1999); Toma et al. (2004); Wiener et al. (1998); Witjes et al. (1998)

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Table 1 (*continued*)

Cancer Type	Diagnostic Test	Urine Type	Biomarker	Biomarker Type	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC	SOE (GRADE)	Limitations	Reference (s)
	2. BladderChek* **POC**	Spot urine	NMP-22	Diagnosis Surveillance Proteomic	**11–86**	**78–96**	**20-71**	**71-97**	**0.56-0.76**	**Low**	1 False positive in: infection, inflammation, hematuria, urolithiasis, bowel interposition, other genitourinary cancer, instrumentation 2 Reliability and clinical utility are uncertain 3 No clearly defined threshold value 4 Substantial degree of heterogeneity in diagnostic performance	Barbieri et al. (2012); Behrens et al. (2014), Chou et al., 2015, Grossman et al. (2006, 2005), Gupta et al. (2009), Lotan et al., (2017, 2009), Miyake et al., (2012a,b), Ritter et al. (2014)
Bladder	**BTA**											
	1. BTA stat* **POC**	Spot urine or Catheterized	Complement factor H-related protein and complement factor H	Surveillance *(adjunct to cystoscopy)* Proteomic	**32–100**	**63–92**	**16–87**	**70–95**	**0.75**	**Moderate**	1 False positive in: infection, inflammation, hematuria, urolithiasis, BPH, bowel interposition, other genitourinary cancers, history of BCG instillations, presence of foreign bodies in the urinary tract 2 Reliability and clinical utility are uncertain 3 Substantial degree of heterogeneity in diagnostic performance	Babjuk et al. (2002), Chou et al. (2015), Friedrich et al. (2002), Giannopoulos et al. (2001), Guo et al. (2014), Gutiérrez Baños et al. (2001), Mian et al. (2000a), Miyake et al. (2012b), Nasuti et al. (1999), Raitanen (2008), Saad et al. (2002), Serretta et al. (2000), Sharma et al. (1999), Toma et al. (2004), Wiener et al. (1998)
	2. BTA TRAK*	Spot urine or Catheterized	Complement factor H-related protein and complement factor H	Surveillance *(adjunct to cystoscopy)* Proteomic	**52–78**	**63–86**	**45–70**	**78–88**	**NA**	**Low**	1 False positive in: infection, inflammation, hematuria, urolithiasis, BPH, bowel interposition, other genitourinary cancers, history of BCG instillations, presence of foreign bodies in the urinary tract 2 Reliability and clinical utility are uncertain 3 Substantial degree of heterogeneity in diagnostic performance 4 Requires trained laboratory technician	Babjuk et al. (2002), Chou et al. (2015), Mahnert et al. (2003), Miyake et al. (2012b), Serretta et al. (2000)

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Table 1 (*continued*)

Cancer Type	Diagnostic Test	Urine Type	Biomarker	Biomarker Type	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC	SOE (GRADE)	Limitations	Reference (s)
Bladder	**Urovysion***	Spot urine	Chromosomal abnormalities (aneuploidy of chromosomes 3, 7, 17, and loss of 9p21 locus)	Diagnosis *(adjunct to standard procedures)* Surveillance Genomic	**33–100**	**63–100**	**33–88**	**71–92**	**0.63–0.87**	**Moderate**	1 False positive in: inflammation, hematuria, urolithiasis, BPH, other genitourinary tumors 2 Reliability, clinical utility and cost-effectiveness are uncertain 3 No clearly defined criteria exist for a positive assay 4 Substantial degree of heterogeneity in diagnostic performance 5 Accuracy is dependent on technical aspects: Experience with performing FISH; Sample quality with a sufficient number of tumor cells 6 Requires trained cytopathologist	Bonberg et al. (2013), Chou et al. (2015), Gudjónsson et al. (2008), Hajdinjak (2008), Horstmann et al. (2009), Lotan et al. (2017), May et al. (2007); Placer et al. (2002), Sarosdy et al. (2002), Song et al. (2010), Sullivan et al. (2009), Toma et al. (2004)
Bladder	**ImmunoCyt/ uCyt+***	Spot urine	Carcinoembryonic Antigen HMW form (M334) and mucoproteins (LDQ10/19A11)	Surveillance *(adjunct to cystoscopy)* Proteomic	**32–100**	**62–91**	**26–82**	**74–99**	**0.54–0.85**	**Moderate**	1 False positive in: infection, inflammation, hematuria, urolithiasis, BPH 2 Reliability, clinical utility and cost-effectiveness are uncertain 3 Substantial degree of heterogeneity in diagnostic performance 4 Poor sensitivity in T2 bladder cancer (antigens are not found in muscle-invasive BC) 5 Accuracy is dependent on technical aspects: Experience in interpretation of the staining; Sample quality with need for a large number of exfoliated cells (more than 500 per slide); Requirements concerning laboratory equipment; Steep learning curve; Substantial interobserver variability; Need for constant quality control 6 Requires trained cytopathologist	Beiche et al. (2002), Cha et al. (2012), Chou et al. (2015), Comploj et al. (2013), Greene et al. (2006), Horstmann et al. (2009), Lodde et al. (2003), Messing et al. (2005), Mian et al. (1999), Olsson and Zackrisson (2001), Schmitz-Dräger et al. (2007), Sullivan et al. (2009), Têtu et al. (2005), Toma et al. (2004), Vriesema et al. (2001)
Bladder	**UBC**											

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Table 1 (*continued*)

Cancer Type	Diagnostic Test	Urine Type	Biomarker	Biomarker Type	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC	SOE (GRADE)	Limitations	Reference (s)
	1. UBC-ELISA	Spot urine	Cytokeratin 8 and 18	Diagnosis Surveillance Proteomic	**21–84**	**71–98**	**29–93**	**28–94**	**0.51–0.72**	**Low**	1 False positive in: infection, inflammation, urolithiasis, BPH, other genitourinary malignancies 2 Reliability and clinical utility are uncertain 3 Substantial degree of heterogeneity in diagnostic performance 4 Requires trained laboratory technician	Babjuk et al. (2002), Boman et al. (2002), Giannopoulos et al. (2001), Hakenberg et al. (2004), Heicappell et al. (2000), May et al. (2007), Mian et al. (2000b), Mungan et al. (2000), Ritter et al. (2014), Sánchez-Carbayo et al. (2001b,a, 1999a),
	2. UBC-Rapid **POC**	Spot urine	Cytokeratin 8 and 18	Diagnosis Surveillance Proteomic	**36–79**	**57–92**	**41–88**	**30–87**	**0.62–0.75**	**Low**	1 False positive in: infection, inflammation, urolithiasis, BPH, other genitourinary malignancies 2 Reliability and clinical utility are uncertain 3 Substantial degree of heterogeneity in diagnostic performance	Babjuk et al. (2002), Ecke et al. (2017, 2015), Hakenberg et al. (2004), Mian et al. (2000a), Pichler et al. (2017), Ritter et al. (2014), Schroeder et al. (2004), Styrke et al. (2017)
Bladder	**CxBladder**	Spot urine Mid-stream Second urine of the day	IGFBP5, HOXA13, MDK, CDK1; CXCR2 *(+ clinical factors)*	Diagnosis Surveillance Transcriptomic	**73–95**	**77–95**	**NS**	**96-98**	**0.73–0.87**	**Low**	1 False positive in: hematuria, urolithiasis 2 Reliability and clinical utility are uncertain and need independent validation 3 Requires trained laboratory technician	Breen et al. (2015), Kavalieris et al. (2017,2015), Lotan et al. (2017), O'Sullivan et al. (2012)

Abbreviations: AUC: area under the curve; BC: bladder cancer; BCG: Bacille Calmette-Guérin; BPH: benign prostatic hyperplasia; DRE: digital rectal examination; PC: prostate cancer; EV: extracellular vesicles; HMW: high molecular weight; NPV: negative predictive value; PPV: positive predictive value; Se: sensitivity; Sp: specificity; SOE (GRADE): strength of evidence (Grading of Recommendations, Assessment, Development and Evaluations Working Group).
*FDA approved; POC: point-of-care test.

with membrane-associated AQP2. Tandem mass spectrometric profiling of proteins present in ultracentrifuged sediments from normal human subjects identified hundreds of distinct proteins implicated in the genesis of multivesicular bodies and endosomes suggesting the presence of extracellular vesicles (EV) (Street et al., 2012). Specifically, these EV isolates contain proteins characteristic of every cell type in the urinary tract, including podocytes, renal tubular epithelial cells and urothelium from the urinary collecting system. In the case of prostate-specific diseases, excretion of prostate-specific factors into the urine can be stimulated by prostate massage during digital rectal examination (DRE). Prostate massage increases prostate-derived EV release into the urethra and subsequently in the collected urine fraction (Nilsson et al., 2009). Circulating blood-derived EV cannot pass through the glomerular filtration membrane in physiological conditions, since EV are larger than the effective pore size of the glomerular wall (< 10 nm).

The term "liquid biopsy" has attracted considerable interest in oncology (Siravegna et al., 2017). It describes the possibility of probing the molecular landscape of cancer via a blood draw by characterizing circulating cell-free tumor DNA (ctDNA), tumor-derived RNA (such as miRNAs) and/or circulating tumor cells (CTC). In addition to blood, urine has been shown to contain tumor-derived genetic material (Birkenkamp-Demtröder et al., 2018; Christensen et al., 2017). ctDNA detected in urine of patients with urogenital cancers originates primarily from shedding of tumor cells or their breakdown products directly into the urinary tract (Casadio et al., 2013). However, renal clearance of ctDNA from the blood results in transrenal DNA (tr-DNA) and, for example, allows to identify KRAS and EGFR mutation in respectively urine from colon cancer patients and lung cancer patients (Botezatu et al., 2000; Reckamp et al., 2016). In urological tumors, ctDNA can be shed into both the blood and urine (Birkenkamp-Demtröder et al., 2016). Some studies demonstrated concordance between plasma and urinary ctDNA alterations (Utting et al., 2002), while others showed they could provide complementary information (Birkenkamp-Demtröder et al., 2016). Additional concordance studies on matched urinary and plasma ctDNA samples will be necessary to investigate to what extent they give additional information.

Despite the high concentration of RNA-hydrolysing enzymes in urine, mRNA and small and long non-coding RNAs are preserved in urine. This is most likely because of binding to the argonaute-2 protein or their presence within EV (Berrondo et al., 2016; Hendriks et al., 2016; Motamedinia et al., 2016; Nilsson et al., 2009; Sole et al., 2015). Liquid biopsies of urine in disease surveillance could be a preferable choice compared to other body fluids as this approach is truly non-invasive and can be performed at home by the patients themselves (Siravegna et al., 2017).

2. Biomarkers in urological oncology

The ideal biomarker improves clinical decision making in conjunction with clinicopathological parameters. It has a highly reproducible and robust detection method that is both sensitive and specific and has high positive and negative predictive values (PPV, NPV). It should be obtained in a non-invasive way from an easily accessible body fluid (Atkinson et al., 2001). Diagnostic biomarkers detect or confirm the presence of a medical condition, while prognostic biomarkers are used to identify the likelihood of a clinical event, disease recurrence or progression in patients with the condition of interest. Prognostic biomarkers are distinguished from predictive biomarkers, which are used to identify patients who experience a favorable or unfavorable response to a particular treatment (FDA-NIH Biomarker Working Group, 2016).

Urine is exposed to cells from the renal system and urothelium lining the renal pelvis, ureters, bladder, and urethra. In addition, prostatic secretions are expelled into the urine through the prostatic ducts. It also contains cells that have been shed. Urine can thus contain a variety of molecular markers for malignancy. Intensive research over the past couple of years has identified many promising urological cancer biomarkers. Nonetheless, only a few commercially available tests have reached clinical practice and several limitations hinder their widespread use (Table 1).

2.1. Prostate cancer

Prostate cancer (PC) is a major health issue in men. In developed countries, it remains the most commonly diagnosed male malignancy and the third leading cause of cancer-related mortality (Ferlay et al., 2015; Siegel et al., 2017). An important clinical problem in PC is the inability of current diagnostic tests to discriminate between indolent and aggressive cancers (Welch and Black, 2010). Measuring serum prostate-specific antigen (PSA) has limitations, including low PC specificity (Thompson et al., 2004). This leads to overdiagnosis and overtreatment of PC (Carter, 2004). Therefore, the search for better diagnostic and prognostic biomarkers is an important research objective in PC.

2.1.1. PCA3

Prostate cancer antigen 3 (PCA3) is a prostate-specific long noncoding (lnc)RNA. PCA3 is strongly overexpressed in PC compared to normal prostate tissue and benign prostatic hyperplasia (BPH) (Bussemakers et al., 1999). The *Progensa PCA3 test (Hologic, Marlborough, MA, USA)* measures urinary PCA3 lncRNA, which is normalized with urinary PSA mRNA (PCA3 score). In contrast to serum PSA, the PCA3 score is independent of prostate volume (Deras et al., 2008), has a higher specificity and has a better PPV and NPV, although its sensitivity is lower. The sensitivity of PCA3 varies according to the PCA3 score cutoff value (Luo et al., 2014; Vlaeminck-Guillem et al., 2010). The PCA3 test has been U.S. Food & Drug Administration (FDA)-approved for men 50 years of age or older with a previous negative biopsy to assist in decision-making regarding repeat biopsies (Haese et al., 2008), but its clinical utility and cost-effectiveness for this purpose have not been confirmed (Calonge, 2014; Nicholson et al., 2015).

2.1.2. Mi-Prostate score (MiPS)

The *TMPRSS2-ERG* fusion gene comprises the androgen-responsive genes *TMPRSS2* (transmembrane protease, serine 2) and *ERG* (ETS-related gene) and is observed in 40–80% of prostate cancers (Tomlins et al., 2005). *TMPRSS2-ERG* transcripts are detected in urine of clinically localized PC patients after digital rectal examination (DRE) (Laxman et al., 2006), which is associated with a high specificity and PPV, but a low sensitivity for detection of PC (Hessels and Schalken, 2013). The *TMPRSS2-ERG* score (urine TMPRSS2-ERG mRNA normalized to urine PSA mRNA) is not yet approved as a PC biomarker to assist in clinical decision making.

Considering PC heterogeneity, combining markers into a biomarker panel might provide additional diagnostic and prognostic value. The commercially available *Mi-Prostate score (MiPS) (University of Michigan MLabs, Ann Arbor, MI, USA)* combines urinary levels of PCA3 and TMPRSS2-ERG with serum PSA levels to generate a PC risk assessment score (Leyten et al., 2014; Sanda et al., 2017). In two validation cohorts, urinary *TMPRSS2-ERG* and *PCA3* scores improved the performance of serum PSA for predicting (high-grade) PC, allowing for risk stratification and avoiding unnecessary biopsies (Sanda et al., 2017; Tomlins et al., 2016).

2.1.3. SelectMDx

Gene expression profiling on urinary sediments identified a three-gene panel (*HOXC6*, *TDRD1*, and *DLX1*) showing higher accuracy to predict high-grade PC (Gleason score ≥ 7) compared with PCA3 or serum PSA (Leyten et al., 2015). Two of these urinary sediment biomarkers (HOXC6 and DLX1) were incorporated into a multimodal model with traditional clinical risk factors (age, serum PSA, PSA density, family history, DRE, history of negative prostate biopsies) to identify patients with high-grade PC. The risk model, named *SelectMDx*

(*MDxHealth, Irvine, CA, USA*), predicted Gleason score ≥7 PC with a higher accuracy than the Prostate Cancer Prevention Trial risk calculator and PCA3. Therefore, *SelectMDx* might reduce the number of unnecessary prostate biopsies (Van Neste et al., 2016). In addition, a retrospective study showed promising results regarding the correlation between the *SelectMDx* risk score and multiparametric MRI (mpMRI) outcomes (Hendriks et al., 2017).

2.1.4. ExoDx Prostate IntelliScore

ExoDx Prostate IntelliScore uses the combination of EV-derived PCA3 lncRNA and ERG mRNA in first-catch urine samples for detection of high-grade PC. It reached the market as the first commercially available EV-derived urinary biomarker test (McKiernan et al., 2016) and will be discussed more extensively in the part of the review devoted to EV-derived biomarkers.

2.2. Bladder cancer

Bladder cancer (BC) is the ninth most frequent type of cancer and its incidence among urological cancers is second to that of PC (Ferlay et al., 2015; Siegel et al., 2017). Early diagnosis and treatment of non-muscle-invasive BC improves prognosis, but because of a high recurrence rate, continuous surveillance of these patients is required. Positive voided urinary cytology can indicate urothelial cancer anywhere in the urinary tract, but detection of BC ultimately depends on cystoscopic evaluation of the bladder and histological examination of sampled tissue. Driven by the low sensitivity of cytology for low-grade tumors and the fact that cystoscopy remains an invasive examination, much research is focused on finding better urine-based assays to assist in diagnosis and surveillance of BC. Despite the high clinical need, none of these markers have been accepted for diagnosis or follow-up in routine practice or clinical guidelines (Babjuk et al., 2017).

2.2.1. NMP-22

Nuclear matrix proteins (NMP) support nuclear shape and DNA organization, and facilitate chromatin distribution to daughter cells during mitosis (Berezney and Coffey, 1974; Pardoll et al., 1980). NMP-22 is overexpressed in urothelial cancers and released into the urine following tumor cell apoptosis (Keesee et al., 1996). Patients with BC have an elevated concentration of urinary NMP-22 compared to healthy persons (Carpinito et al., 1996). The *NMP*-22 BC *test* kit (*Matritech, Newton, MA*) is a quantitative ELISA test indicating the precise concentration of antigen in a urine sample. BC detection is based on the appropriate diagnostic cut-off value. The NMP-22 *BladderChek* (*Alere, Waltham, MA, USA*) is a qualitative point of care (POC) test performed at the time and place of patient care, providing a simple positive or negative result. Both are FDA-approved for monitoring disease recurrence following treatment, while the *NMP-22 BladderChek* Test is also approved for BC diagnosis in symptomatic or at risk patients.

Of concern with the *NMP-22* tests is the inconsistency of their performance characteristics in detecting BC (Chou et al., 2015). Since NMP-22 is released from apoptotic cells, many benign urological conditions can cause false-positive test results (Table 1).

2.2.2. BTA

The bladder tumor antigen (BTA) test (*Polymedco, Cortlandt Manor, NY, USA*) detects human complement factor H-related protein (hCFHrp) in voided urine specimens. This protein is produced by BC cells and may confer a selective growth advantage to cancer cells by decreasing complement activity, thus preventing lysis by immune surveillance (Kinders et al., 1998). *BTA stat*, a qualitative POC test, and *BTA TRAK*, a quantitative ELISA test, have both been FDA approved as adjunct to cystoscopy.

Sensitivity of BTA is superior to that of urinary cytology, but specificity and AUC (area under the curve) are lower (Guo et al., 2014). Because hCFHrp is present in blood, *BTA* testing will be positive when hematuria is present, regardless of the presence of BC (Lüdecke et al., 2012) (Table 1).

2.2.3. Urovysion

UroVysion (*Abbott Molecular, Des Plaines, IL, USA*) is a multicolor FISH (fluorescence in situ hybridization) assay that identifies common BC–associated chromosomal alterations in exfoliated cells. (Halling et al., 2000; Sandberg and Berger, 1994). It is FDA-approved for BC detection in hematuria patients (as adjunct to standard procedures) and surveillance.

UroVysion has a higher sensitivity compared to urinary cytology, but different urological conditions and technical aspects might confound the results leading to a lower specificity (Table 1).

2.2.4. ImmunoCyt/uCyt+

The *ImmunoCyt/uCyt+* test (*Scimedex, Denville, NJ, USA*) is an immunocytological assay identifying a high-molecular weight form of carcinoembryonic antigen and two mucoproteins in exfoliated urothelial cells in urine (Fradet and Lockhard, 1997; Mian et al., 1999). The test is FDA-approved for BC surveillance, as an adjunct to cystoscopy.

Immunocyt/uCyt+ improves sensitivity over urinary cytology, especially in low-grade BC, while specificity is lower. Sensitivity improves through combination of *Immunocyt/uCyt+* and cytology without a significant decrease in specificity (Mian et al., 1999; Pfister et al., 2003; Têtu et al., 2005). In a large meta-analysis, *Immunocyt/uCyt+* showed the highest sensitivity in BC detection and surveillance compared to other urinary biomarkers (Chou et al., 2015).

False positives are common in benign conditions of the urinary tract and the test is hampered by interobserver variability and technical limitations (Beiche et al., 2002) (Table 1).

2.2.5. UBC test

The expression pattern of cytokeratins (CK) is largely specific to particular tissue epithelia and overexpression of certain CK is associated with BC (Moll et al., 1982). The *UBC-ELISA* and *UBC-Rapid* tests (*IDL Biotech, Bromma, SWE*) detect CK 8 and 18 fragments in urine. *UBC-Rapid* is a qualitative POC assay (Sánchez-Carbayo et al., 1999a,b), which can also be quantitatively analyzed using a photometric reader (Ritter et al., 2014).

UBC generally has a low sensitivity and comparisons with other biomarkers are not in favor of *UBC* testing (Babjuk et al., 2002; Schroeder et al., 2004). False positives are observed in patients with benign urinary tract disorders and other urological malignancies (Sánchez-Carbayo et al., 1999a,b).

2.2.6. CxBladder

Cxbladder (*PacificEdge, Dunedin, NZL*) quantifies 4 mRNAs with overexpression in BC compared with normal urothelium and low expression in blood and inflammatory cells: cyclin-dependent kinase 1 (CDK1), homeobox A13 (HOXA13), midkine (MDK), and insulin-like growth factor-binding protein 5 (IGFBP5). Another mRNA – chemokine receptor 2 (CXCR2) – is highly expressed in neutrophils and is included in the test to reduce false-positive results due to nonmalignant acute and chronic inflammatory conditions (Holyoake et al., 2008; O'Sullivan et al., 2012).

In the detection setting, *Cxbladder* has a higher sensitivity compared to that of cytology and *NMP-22* (O'Sullivan et al., 2012). In patients undergoing surveillance for BC, sensitivity and NPV are superior compared to cytology, *NMP-22* and *Urovysion* (Lotan et al., 2017). These results have yet to undergo independent validation in larger studies.

2.3. Renal cell carcinoma

Renal cell carcinoma (RCC) is the most common type of kidney cancer in adults (Siegel et al., 2017). Early diagnosis of small renal

tumors leads to better treatment outcomes, but the absence of symptoms characterizing early stages of RCC makes its detection a challenging problem (Ljungberg et al., 2015). RCC comprises a broad spectrum of histopathological entities differing in prognosis and subsequent response to therapy (Moch et al., 2016). Imaging studies may not always accurately distinguish benign kidney tumors from malignant ones (Choudhary et al., 2009; Hindman et al., 2012), whereas kidney biopsy is an invasive investigation associated with certain complications (Marconi et al., 2016). Reliable biomarkers facilitating early detection of kidney tumors and differential diagnosis of their subtypes are therefore warranted. So far, no commercial urinary RCC biomarker has reached the market.

3. EV-derived biomarkers in urological cancers

Extracellular vesicles (EV) encompass a heterogeneous group of bilayer membrane vesicles that are released by all human cell types (Yáñez-Mó et al., 2015). Two biologically distinct classes of EV are actively shed by living cells: exosomes that derive from fusion of multivesicular bodies with the plasma membrane and have a size of 50–200 nanometer (nm), and microvesicles/ectosomes that bud directly from the plasma membrane and have sizes of 50–1000 nm. An elaborate discussion of the biogenesis and characteristics of EV subtypes will not be included here as this has been published previously (Raposo and Stoorvogel, 2013).

EV play a role in intercellular communication by shuttling functional proteins and nucleic acids between cells (Dhondt et al., 2016; Tkach and Théry, 2016). Several aspects make them appealing from a biomarker perspective. EV can be found in all body fluids, including blood, urine, saliva and sweat, which makes them compatible with non- or minimally invasive liquid biopsies (Minciacchi et al., 2017; Perakis and Speicher, 2017). Their cargo is a spatiotemporal fingerprint of the cell of origin, reflecting pathophysiological processes within the source tissue. Moreover, not only protein, but also nucleic acid, lipid and metabolite composition of EV can be used to discriminate normal from diseased state, offering multiple options for biomarker detection within the same entity (Table 2).

Despite intensive research, no urinary biomarkers with sufficient clinical utility are yet available to guide decision-making in urological oncology. EV-derived biomarkers could potentially improve on current limitations (Sheridan, 2016). Differential protein, RNA and lipid expression are represented in urinary EV of prostate, bladder and renal cancer patients (Table 2).

3.1. Prostate cancer

Urine and prostatic fluids are enriched in prostate-derived EV and provide a potential source for biomarker discovery in PC. Prostatic fluids are collected in first void urine after DRE and prostate massage (Drake and Kislinger, 2014; Principe et al., 2013). EV-associated transmembrane proteins CD9 and CD63 are enriched in urine from PC patients, collected post-DRE and after correction for urinary PSA (Duijvesz et al., 2015).

Several studies have investigated the proteomic cargo of urinary PC-derived EV. δ-Catenin, which is significantly overexpressed in PC, was identified in EV isolated from urine of PC patients (Lu et al., 2009). The presence of prostate markers PSA and PSMA, and the cancer-associated antigen 5T4, which is a non-soluble molecule within biological fluids, has also been demonstrated in urinary EV (Mitchell et al., 2009). Integrin α3 and integrin β1, adhesion proteins involved in cellular invasion, were found at increased levels in urinary EV of patients with metastatic PC, compared to patients with BPH or localized PC (Bijnsdorp et al., 2013). Several proteins and protein-combination panels were identified as urinary EV-derived biomarkers by mass spectrometry proteome analysis (Fujita et al., 2017; Øverbye et al., 2015), while other studies validated previously identified EV biomarkers using targeted proteomics and immuno-assays (Sequeiros et al., 2017; Wang et al., 2017) (Table 2). This approach demonstrated differential enrichment of protein biomarker candidates in urinary EV compared to plasma-derived EV (Welton et al., 2016). Glycan profiling of urinary EV derived from prostatic secretions indicated that changes in glycosylation of N-linked glycoproteins, such as an increase in larger tetra-antennary glycans, might reflect the clinical status of PC, but no conclusions could be drawn for these limited pilot analyses on pooled samples (Nyalwidhe et al., 2013).

The majority of transcriptomic changes in urinary EV encompass the differential expression of known PC-markers (TMPRSS2-)ERG and PCA3, with varying clinical usefulness and diagnostic value (Dijkstra et al., 2014; Donovan et al., 2015; Hendriks et al., 2016; Motamedinia et al., 2016; Nilsson et al., 2009; Pellegrini et al., 2017). EV-derived PCA3 lncRNA and ERG mRNA levels in non-DRE urine samples demonstrated good clinical performance in predicting biopsy result for high-grade PC (Donovan et al., 2015). Based on these findings, the *ExoDx Prostate Intelliscore* (Exosome Diagnostics, Cambridge, MA, USA), a risk score to estimate initial prostate biopsy outcome, was developed. It combines urinary EV-derived PCA3 lncRNA and ERG mRNA levels, normalized to SPDEF (SAM pointed domain-containing ETS transcription factor) mRNA, with clinical risk factors (serum PSA, age, race, and family history). In a large validation study, it was associated with improved discrimination between high-grade and low-grade and benign disease compared with the standard of care (McKiernan et al., 2016).

CDH3 (P-Cadherin), which possibly exerts a tumor suppressive function, showed reduced levels in PC urinary EV. In coherence with this observation, CDH3 mRNA expression was significantly decreased in tissue from patients with PC compared to BPH (Royo et al., 2016b). Splice variant transcripts of the AGR2 gene were also identified as potential diagnostic biomarkers (Neeb et al., 2014).

Other studies have evaluated urinary EV-derived non-coding RNA in PC. Levels of long intergenic non-coding (linc)RNA-p21, a suppressor of p53 signaling, in urinary EV may help to distinguish PC from benign disease (Işin et al., 2015). Several EV-derived miRNAs and miRNA-panels demonstrated potential diagnostic value as urinary biomarkers in PC (Bryzgunova et al., 2016; Rodríguez et al., 2017; Samsonov et al., 2016; Wani et al., 2017). In addition, urinary EV are enriched in different miRNA biomarker candidates compared to serum-derived EV (Xu et al., 2017) and urinary pellets (Foj et al., 2017). Next generation sequencing also revealed the possible utility of miRNA isoforms (isomiRs) as PC biomarkers (Koppers-Lalic et al., 2016).

Analysis of the lipid composition of EV shows potential for biomarker development in PC. Several lipid classes, such as diacylglycerol (DAG) and triacylglycerol (TAG) species, are differentially enriched in urinary EV from PC patients and healthy controls, demonstrating possible diagnostic utility (Skotland et al., 2017; J.S. Yang et al., 2017).

Analysis of small molecule metabolites can potentially reveal dynamic changes in the metabolism downstream of genetic and proteomic regulation. Metabolomic profiling of urinary EV has proven to be feasible and might identify disease profiles not revealed by conventional analysis of the original urine samples. For example, levels of adenosine, glucuronate, isobutyryl-L-carnitine and D-ribose 5-phosphate were significantly lower in pre-prostatectomy urine samples as compared to post-prostatectomy and control samples (Puhka et al., 2017).

3.2. Bladder cancer

Urine is an excellent biological fluid for biomarker discovery in BC, since it has been in direct contact with tumor cells lining the bladder wall. Consequently, BC-derived EV are released directly into the urine. The concentration of CD63-positive urinary EV is significantly elevated in BC patients compared to healthy controls, demonstrating the concept of using the EV concentration as a biomarker for disease (L.G. Liang et al., 2017).

Various studies have characterized the proteomic profile of urinary

Table 2
EV-derived urinary biomarker candidates in genitourinary cancers.

Cancer type	Urine type	Biomarker candidate	Biomarker type	Isolation method(s)	EV Normalization	EV-METRIC (%)	Reference
Prostate	Spot urine sample	δ-catenin	Diagnosis Proteomic	Differential (ultra)centrifugation	NP	11	Lu et al. (2009)
Prostate	Morning urine sample	PSA, PSMA, 5T4	Diagnosis Monitoring Proteomic	Differential (ultra)centrifugation + Sucrose cushion	Protein amount	11–22	Mitchell et al. (2009)
Prostate	NS	Integrin α3, Integrin β1	Diagnosis Proteomic	Differential (ultra)centrifugation	PDCD6IP (ALIX)	11	Bijnsdorp et al. (2013)
Prostate	Whole urine sample pre- or post-DRE	CD63 + EV signal intensity	Diagnosis Proteomic	Immunocapture (Time-resolved fluorescence immunoassay)	Urinary PSA/Creatinine	0	Duijvesz et al. (2015)
Prostate	Morning urine sample (P); First morning urine sample (HC)	TM256, LAMTOR1, ADIRF	Diagnosis Proteomic	Differential (ultra)centrifugation + filtration	Protein amount	33	Øverbye et al. (2015)
Prostate	Morning urine sample (P); First morning urine sample (HC)	TMEM256, flotillin 2, Rab3B, PARK7, LAMTOR1	Diagnosis Proteomic	Differential (ultra)centrifugation + filtration	Protein amount	44	Wang et al. (2017)
Prostate	Spot urine sample (first morning urine excluded)	Afamin, cardiotrophin-1, CDON, endoplasmatic reticulum aminopeptidase 1, FGF19, IL17RC, NAMPT, IL1RAPL2, CD226, IGFBP2, CCL16, TNFSF18, IGFBP5; Aromatic-L-amino-acid decarboxylase	Prognostic Proteomic	Differential (ultra) centrifugation + Filtration + Size-exclusion chromatography	NP	29–63	Welton et al. (2016)
Prostate	First catch urine sample post-DRE	FABP5	Diagnosis Prognosis Proteomic	Differential (ultra)centrifugation	CD9	33	Fujita et al. (2017)
Prostate	First catch urine sample post-DRE	TGM4 + ADSV; PPAP + PSA + CD63 + SPHM + GLPK5	Diagnosis Prognosis Proteomic	Differential (ultra)centrifugation + Filtration	NP	33	Sequeiros et al. (2017)
Prostate	First catch urine sample post-DRE	Vesicle-associated PSA extraction ratio	Diagnosis Proteomic	Centrifugation + n-butanol extraction	NP	0	Vermassen et al. (2017)
Prostate	Urine sample post-DRE	N-linked glycans	Diagnosis Prognosis Glycomic	Differential (ultra)centrifugation	NP	0	Nyalwidhe et al. (2013)
Prostate	Urine sample pre- and post-DRE	PCA3, TMPRSS2-ERG	Diagnosis Transcriptomic	Differential (ultra)centrifugation + Filtration	NP	12.5	Nilsson et al. (2009)
Prostate	First catch urine sample pre- and post-DRE	PCA3	Diagnosis Transcriptomic	Centrifugation + Filtration + Ultrafiltration	NP	0	Dijkstra et al. (2014)
Prostate	First catch urine sample	AGR2 SV-G, AGR2 SV-H	Diagnosis Transcriptomic	Differential (ultra)centrifugation + Filtration	NP	44	Neeb et al. (2014)
Prostate	First catch urine sample	EXO106 score: PCA3 + ERG (normalized to SPDEF)	Diagnosis Prognosis Transcriptomic	Filtration + Exosome Diagnostics Urine Clinical Sample Concentrator Kit (Ultrafiltration)	NP	0	Donovan et al. (2015)
Prostate	First catch urine sample pre-and post-DRE	PCA3, ERG	Diagnosis Transcriptomic	Centrifugation + Filtration + Ultrafiltration	NP	0	Hendriks et al. (2016)
Prostate	First catch urine sample	ExoDx Prostate IntelliScore: ERG + PCA3 (normalized to SPDEF and combined with serum PSA and clinical risk factors)	Diagnosis Prognosis Transcriptomic	Filtration + Exosome Diagnostics Urine Clinical Sample Concentrator Kit (Ultrafiltration)	NP	0	McKiernan et al. (2016)
Prostate	Spot urine sample	TMPRSS2:ERG, BIRC5, PCA3, ERG, TMPRSS2	Diagnosis Transcriptomic	Ultrafiltration	NP	0	Motamedinia et al. (2016)
Prostate	Morning urine sample	CDH3	Diagnosis Transcriptomic	Differential (ultra)centrifugation + filtration	NP	44	Royo et al. (2016b)
Prostate	First catch urine sample post-DRE	PCA3, ERG	Diagnosis Transcriptomic	Centrifugation + Filtration + Ultrafiltration	NP	0	Pellegrini et al. (2017)

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Table 2 (*continued*)

Cancer type	Urine type	Biomarker candidate	Biomarker type	Isolation method(s)	EV Normalization	EV-METRIC (%)	Reference
Prostate	Urine sample post-DRE	lincRNA-p21	Diagnosis Transcriptomic	Differential centrifugation + Urine Exosome RNA Isolation Kit (Norgen)	NP	0	Işin et al. (2015)
Prostate	Spot urine sample	miR-19b	Diagnosis Transcriptomic	Differential (ultra)centrifugation + filtration	NP	25	Bryzgunova et al. (2016)
Prostate	Morning urine sample	miR-574-3p, miR-141-5p, and miR-21-5p	Diagnosis Transcriptomic	Differential centrifugation + Lectin-induced precipitation	NP	0–22	Samsonov et al. (2016)
Prostate	Spot urine sample	miRNA-21, miR-375, let-7c	Diagnosis Prognosis Transcriptomic	Differential (ultra)centrifugation	NP	14	Foj et al. (2017)
Prostate	Morning urine sample (P); First morning urine sample (HC)	miR-196a-5p, miR-501-3p	Diagnosis Transcriptomic	Differential (ultra)centrifugation + Filtration	NP	33	Rodríguez et al. (2017)
Prostate	NS	miR-2909	Diagnosis Prognosis Transcriptomic	Exiqon miRCURY™ exosome isolation kit	NP	0	Wani et al. (2017)
Prostate	First morning urine sample	miR-145	Diagnosis Prognosis Transcriptomic	Differential (ultra)centrifugation or Hydrostatic filtration dialysis	NP	0–25	Xu et al. (2017)
Prostate	First catch urine sample post-DRE	Isoforms of miR-204-5p, miR-21-5p and miR-375	Diagnosis Transcriptomic	Differential (ultra)centrifugation (+ Sucrose density gradient as validation)	NP	0–12	Koppers-Lalic et al. (2016)
Prostate	Morning urine sample (P); First morning urine sample (HC)	Lactosylceramide d18:1/16:0, phosphatidylserine 18:1/18:1, phosphatydylserine 18:0-18:2	Diagnosis Lipidomic	Differential (ultra)centrifugation + filtration	Protein amount	33	Skotland et al. (2017)
Prostate	NS (P); Morning urine sample (HC)	22:6/22:6-phosphatidylglycerol, (16:0, 16:0)-diacylglycerol, (16:1, 18:1)-diacylglycerol, high abundant triacylglycerol species	Diagnosis Lipidomic	Differential (ultra)centrifugation + Flow Field-Flow Fractionation	NP	0–22	J.S. Yang et al. (2017)
Prostate	Midstream spot urine sample	Adenosine, glucuronate, isobutyryl-L-carnitine, D-ribose 5-phosphate	Diagnosis Metabolomic	Differential (ultra)centrifugation + Filtration	EV volume, EV number, CD9 optical density, metabolites, urine volume or urine creatinine	77	Puhka et al. (2017)
Bladder	Spot urine sample	Resistin, GTPase NRas, EPS8L, EPS8L2, Mucin 4, Retinoic acid-induced protein 3, Alpha subunit of GsGTP binding protein, EH-domain-containing protein 4 (cancer EV); Galectin-3-binding protein (non-cancer EV)	Diagnosis Proteomic	Differential (ultra) centrifugation	NP	22	Smalley et al. (2008)
Bladder	Spot urine sample	CD36, CD44, 5T4, basigin, CD73	Diagnosis Proteomic	Differential centrifugation + Sucrose cushion	NP	14	Welton et al. (2010)
Bladder	NS	EDIL-3	Prognosis Proteomic	Differential (ultra) centrifugation + Filtration + Sucrose cushion	NP	11	Beckham et al. (2014)
Bladder	First morning urine sample	TACSTD2	Diagnosis Proteomic	Differential (ultra)centrifugation	NP	22	Chen et al. (2012)
Bladder	First morning urine sample	Alpha-1 antitrypsine, histon H2B1K	Diagnosis Prognosis Proteomic	Differential (ultra)centrifugation	NP	22–33	Lin et al. (2016)
Bladder	Perioperative urine sample (MIBC), Postoperative urine sample (NMIBC), NS (HC)	Periostin	Prognosis Proteomic	Differential (ultra) centrifugation	CD9	22	Silvers et al. (2016)
Bladder	NS	CD63 + EV signal intensity	Diagnosis (POC) Proteomic	Centrifugation + Filtration + Integrated double-filtration microfluidic device	NP	12.5	L.G. Liang et al. (2017)
Bladder	Perioperative urine sample (MIBC), NS (HC)	HEXB, S100A4, SND1	Diagnosis Prognosis Proteomic	Differential (ultra)centrifugation	ALIX	22	Silvers et al. (2017)

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Table 2 (*continued*)

Cancer type	Urine type	Biomarker candidate	Biomarker type	Isolation method(s)	EV Normalization	EV-METRIC (%)	Reference
Bladder	Spot urine sample	GALNT1, LASS2 (cancer EV), ARHGEF39, FOXO3 (non-cancer EV)	Diagnosis Transcriptomic	Differential (ultra)centrifugation + Filtration	NP	14	Perez et al. (2014)
Bladder	Spot urine sample (P), NS (HC)	HOTAIR, HOX-AS-2, MALAT1, SOX2, OCT4, HYMA1, LINC00477, LOC 100506688, OTX2-AS1	Diagnosis Prognosis Transcriptomic	Differential (ultra) centrifugation	NP	11	Berrondo et al. (2016)
Bladder	NS	miR-4454, miR-21, miR-720	Diagnosis Transcriptomic	Centrifugation + Urine Exosome RNA Isolation Kit (Norgen)	NP	0	Armstrong et al. (2015)
Bladder	NS	miR-940	Diagnosis Transcriptomic	Differential (ultra)centrifugation	NP	11	De Long et al. (2015)
Bladder	First morning urine sample	miR-375, miR-146a, apoB	Diagnosis Prognosis Transcriptomic/ Proteomic	Differential (ultra)centrifugation + Filtration	NP (Transcriptomic) CD9 and ERM (Proteomic)	33–44	Andreu et al. (2017)
Bladder	NS	miR-141-3p, miR-200a-3p, miR-205-5p	Prognosis Transcriptomic	Differential centrifugation + Total Exosome Isolation Kit (Life Technologies)	RNA amount	0	Baumgart et al. (2017)
Bladder	Spot urine sample	miR-21-5p	Diagnosis Transcriptomic	Differential (ultra)centrifugation	Urinary creatinine	11	Matsuzaki et al. (2017)
Renal Cell Carcinoma	Second morning urine sample	CP, MMP9, CAIX, PODXL, DKK4; CD10, DPEP1, EMMPRIN, Syntenin1, AQP1	Diagnosis Proteomic	Differential (ultra)centrifugation (+ Iodixanol density gradient as validation)	Urinary creatinine	44	Raimondo et al. (2013)
Renal Cell Carcinoma	Second morning midstream urine sample	GSTA1, CEBPA, PCBD1	Diagnosis Transcriptomic	Differential (ultra)centrifugation + Filtration	NP	0	De Palma et al. (2016)
Renal Cell Carcinoma	Spot urine sample	miR-126-3p, miR-449a, miR-34b-5p, miR-486-5p	Diagnosis Transcriptomic	Centrifugation + Urine Exosome RNA Isolation Kit (Norgen)	NP	0	Butz et al. (2016)
Renal Cell Carcinoma	NS	Mono-charged lyso-phosphoatidilethanolammine (LysoPE)	Diagnosis Lipidomic	Differential (ultra)centrifugation	Urinary creatinine	0	Del Boccio et al. (2012)

Abbreviations: DRE: digital rectal examination; EV: extracellular vesicles; HC: healthy controls; NP: not performed; NS: not specified; P: patients; POC: point-of-care.

EV of BC patients and identified possible biomarkers for BC. Of interest is that the proportion of identified proteins common to each study is limited (Chen et al., 2012; Smalley et al., 2008; Welton et al., 2010). Based on proteomic analysis of urinary EV, a strong association between levels of tumor-associated calcium-signal transducer 2 (TACSTD2) and BC was shown (Chen et al., 2012). Another study identified urinary EV-derived alpha 1-antitrypsin and histone H2B1K as diagnostic and prognostic biomarkers for BC. Verification by immunohistochemistry revealed significantly higher expression of these markers in BC tissues than in normal tissues (Lin et al., 2016). EV secreted by bladder cancer facilitate tumor progression by enhancing endothelial and urothelial cell angiogenesis and migration. This might be mediated through delivery of EGF-like repeat and discoidin I-like domain-containing protein-3 (EDIL-3), an angiogenic and cancer-associated integrin ligand, which was found at elevated levels in urinary EV from patients with BC compared to those from healthy controls (Beckham et al., 2014). Muscle-invasive BC might transfer periostin in an EV-mediated paracrine manner to promote disease progression. Treating low grade BC cells with periostin-rich EV increases cell aggressiveness, while periostin suppression reverses these effects. Positive immunohistochemical staining of muscle-invasive BC is also correlated with worse prognosis. BC patient urinary EV were found to have markedly higher levels of periostin than controls. Following tumor resection, urinary EV periostin levels became indistinguishable from healthy controls (Silvers et al., 2016). Additionally, several proteins that have been identified in EV derived from the MIBC cell line TCCSUP are enriched in urinary EV from (N)MIBC patients compared to healthy controls (Silvers et al., 2017).

Using a whole transcriptome array, followed by PCR validation, differential gene expression in urinary EV from patients with BC and healthy subjects was analyzed. LASS2 and GALNT1, encoding proteins involved in BC progression, were found exclusively in urinary EV from BC patients, whereas ARHGEF39 and FOXO3, associated with tumor suppression, were only present in controls (Perez et al., 2014). Different studies have focused on diagnostic and prognostic miRNA biomarker discovery in BC (Andreu et al., 2017; Baumgart et al., 2017; Matsuzaki et al., 2017). Interestingly, microRNA profiling from matched samples in BC patients demonstrated that a significant number of upregulated microRNAs in BC tissue are identifiable in urinary EV of the same patient, but not in plasma EV (Armstrong et al., 2015). Some extracellular miRNAs with significantly higher levels in urine from BC patients compared to healthy controls, are enriched within urinary EVs, while others are enriched in EV-depleted urine (De Long et al., 2015). Urinary EV may also contain lncRNA for biomarker discovery. HOTAIR, shown to facilitate tumor initiation and progression, and other tumor-associated lncRNAs are enriched in aggressive BC cancer cell line EV and urinary EV from patients with high-grade muscle-invasive BC (Berrondo et al., 2016).

3.3. Kidney cancer

Few studies have investigated the role of urinary EV as biomarkers in RCC. Proteomic analysis of urinary EV from RCC patients and healthy controls demonstrated a reproducibly different protein enrichment between both. An RCC-specific signature of 10 up- or downregulated proteins, derived from these differential proteomic profiles, was validated using immunoblotting (Raimondo et al., 2013). Transcriptome analysis of urinary EV demonstrated a significant difference in mRNA content in clear cell RCC (ccRCC) patients compared to healthy controls and patients with other types of RCC. Decreased levels of EV-derived GSTA1, CEBPA and PCBD1 mRNA levels were specific for ccRCC and one month after treatment these levels returned back to the normal level (De Palma et al., 2016).

MicroRNA expression screening showed that EV-derived miRNA combinations could differentiate ccRCC patients from healthy controls and patients with benign lesions (Butz et al., 2016). Characterization of the lipidome of urinary EV demonstrated a differential lipid composition between RCC patients and healthy subjects, suggesting a relationship between lipid composition of urinary EV and RCC (Del Boccio et al., 2012).

4. EV-derived biomarkers in clinical practice

Urinary EV represent a valuable material for medical diagnostics. Conventional methods for EV purification, such as differential centrifugation, ultrafiltration and precipitation, provide material of highly variable quality (Alvarez et al., 2012; Lozano-Ramos et al., 2015; Paolini et al., 2016; Van Deun et al., 2014; Vergauwen et al., 2017). Consequently, there is a clear interest for high-throughput or automatable EV purification methods for easy and reproducible EV isolation in a clinical setting.

Promising technologies include biophysical separation such as size-based exclusion chromatography and nanofiltration, and immune affinity adsorption using protein-based antigens or lectin immunoassays. Automated sample preparation in different steps of the isolation procedure must be considered to increase reproducibility. Some approaches already validated with other biological samples could be easily adapted (reviewed in Popović and de Marco, 2017), while other specific applications have been developed starting from urine samples. EV purity has often been sacrificed to achieve affordable sample analysis in real clinical setting. For instance, nanomembrane concentrators have been evaluated positively for clinical diagnostics because they enabled rapid EV isolation and the detection of the podocalyxin biomarker in minimal volume urine samples from patients with chronic kidney diseases and abundant proteinuria (Cheruvanky et al., 2007). Collector devices provided with detachable filter tips suitable for being operated by low-speed centrifugation in 96-well microplates have been proposed (Murakami et al., 2014). In another case, size-exclusion chromatography of pre-concentrated urine produced EV fractions with enough material for accurate downstream analysis of the EV content (Lozano-Ramos et al., 2015). Although the available characterization data are not sufficient to evaluate the output quality, the obtained material enables repeatable diagnostic analysis. Affinity purification methodologies aim at separating EV sub-populations by exploiting selective biomarkers with the objective of analyzing their differential content (Popović and de Marco, 2017). There are interesting applications in the case of urinary EV. Podocyte-specific EV have been immune-captured by using beads coated with anti-CR1 antibodies (Prunotto et al., 2013), EV containing aquaporin-2 were selectively recovered using anti-CD9 antibodies (Salih et al., 2016), $CD133^{+}$ EV were isolated by magnetic cell sorting (Dimuccio et al., 2014), dipeptidyl peptidase-IV-positive EV were captured by means of the monoclonal antibody AD-1 (Sun et al., 2012), and preliminary data show the possibility to use lectins and lateral flow immunoassay to recover highly homogeneous EV fractions (Echevarria et al., 2014; Oliveira-Rodríguez et al., 2016). The identification of proteins from glomerular, tubular, prostate, and bladder cells in urinary EV (Salih et al., 2014) suggests that it will be possible to identify specific biomarkers for selective immunopurification of EV subpopulations originating from different cell types.

Microfluidics may revolutionize the field for higher-throughput EV recovery and analysis. Current technologies require prior EV purification procedures followed by subsequent EV quantification and molecular analysis. This is impractical for clinical use, since relatively large sample volumes are required and conventional methods are labor-intensive, time consuming and low-throughput. Microfluidic approaches based on immunoaffinity (Chen et al., 2010) and immunomagnetic bead based separation (Zhao et al., 2016), membrane based filtration (Cho et al., 2016; Rho et al., 2013), nanowire trapping (Wang et al., 2013; Yasui et al., 2017), acoustic nanofiltration (Lee et al., 2015), deterministic lateral displacement (Wunsch et al., 2016) and viscoelastic flow sorting (Liu et al., 2017) have successfully demonstrated the

Table 3
Microfluidic platforms for integrated EV isolation, detection and analysis from biological fluids in a clinical setting.

Device	EV isolation method	EV detection/analysis method	BiologicalFluid	Target	Minimal Sample Volume	LOD/Sensitivity	Assay time	Throughput	Disease	Reference
μNMR	*Off-chip EV isolation*	EV Labeling with target-specific MNP and NMR detection	Plasma	CD63, EGFR, EGFRvIII, IDH1, R132H, PDPN	1 μL EV	~10^4 EV	< 10 min *(excl. EV isolation)*	Low (Single Channel)	Glioblastoma Multiforme	Lee et al. (2008), Shao et al. (2012)
Microfluidic Exosome Profiling Device	Immunomagnetic	EV lysis – Immunomagnetic capture of intra-EV protein targets – Fluorometric ELISA	Plasma	IGF-1R and p-IGF-1R on EpCAM + EV	30 μL	0.281 pg/ml (IGF-1R), 0.383 pg/mL (p-IGF-1R)	< 90 min	Low (Single Channel)	Non-Small Cell Lung Carcinoma	He et al. (2014)
nPLEX	Immunoaffinity on nanohole array	Surface plasmon resonance through periodic nanohole arrays (Multiplexed)	Ascites	EpCAM and CD24 (normalized to CD63)	10 μL / marker	~3000 EV	< 30 min	High (> 1000 measurement sites / 10^5 sensing elements)	Ovarian cancer	Im et al. (2014)
ExoChip	Immunoaffinity	Fluorescent lipophilic dye (DiO) staining – Fluorescence intensity measurement	Serum	CD63 + EV	400 μL	0.5 pM fluorescence	< 90 min	Low to Intermediate (Single- to Multi-Channel)	Pancreatic cancer	Kanwar et al. (2014)
Multiplexed ac-EHD Device	Tunable Nanoshearing and Immunoaffinity	Colorimetric ELISA (Multiplexed)	Serum	HER2 + and CD9 + EV	500 μL	2760 EV/μL	~2 h	Low (3 Channels)	Breast Cancer	Vaidyanathan et al. (2014)
RInSE	Rapid Inertial Solution Exchange and Immunoaffinity	Fluorescence flow cytometry	Blood after RBC lysis	CD81 on EpCAM + EV	NS	NS	~4 h30	Low (Single Channel)	Breast Cancer	Dudani et al. (2015)
iMER	Immunomagnetic	EV lysis – RNA adsorption and elution – RT-qPCR (Multiplexed)	Serum	EPHA2, EGFR, PDPN, MGTM and APNG mRNA in EGFR/ EGFRvIII + EV	100 μL	NS	~2 h	Low (Single Channel – 4 PCR chambers)	Glioblastoma Multiforme	Shao et al. (2015)
iMEX	Immunomagnetic	Electrochemical sensing	Plasma	EpCAM and CD24 on CD63 + EV	10 μL	~10^4 EV	~1 h	Low (8 Channels)	Ovarian cancer	Jeong et al. (2016)
μMED	Immunoaffinity (Neg. and Pos. Enrichment)	Fluorometric ELISA	Serum (mouse)	GluR2 on CD81 + EV	100 μL	10^7 EV/mL	< 1 h	Low (Single Channel)	Concussion	Ko et al. (2016)
SPR biosensor	Immunoaffinity	Surface plasmon resonance	Serum	HER2 on CD9+/ CD63 + EV	NS	2070 EV/μL	< 90 min	Low (Single Channel)	Breast Cancer	Sina et al. (2016)
Nano-IMEX	Immunoaffinity on GO/ PDA interface	Fluorometric ELISA (Multiplexed)	Plasma	EpCAM, CD81 and CD9 on CD81 + EV	2 μL	~50 EV/μL	NS	Low (5 Channels)	Ovarian cancer	Zhang et al. (2016)
ExoSearch	Immunomagnetic	Fluorometric ELISA (Multiplexed)	Plasma	CA-125, EpCAM and CD24 on CD9 + EV	10 μL	750 EV/μL	~40 min	Low to Intermediate (Single- to Multi-Channel)	Ovarian cancer	Zhao et al. (2016)
Immuno-chip	Immunomagnetic	Fluorometric ELISA	Plasma	EpCAM and HER2 on CD63 + EV	50 μL	NS	NS	Low (Single Channel)	Breast Cancer	Fang et al. (2017)
nPES	Immunoaffinity	EV Labeling with target-specific GNP and nano-plasmon enhanced scattering	Plasma	EphA2 and CD9 on CD81 + EV	1 μL	0.23 ng EV protein/μL	~5 h	Intermediate (50 measurement sites)	Pancreatic Cancer	K. Liang et al. (2017)
DFMD	Double filtration	Colorimetric ELISA	Urine	CD63	8 mL	NS	~5 h	Low (Single Channel)	Bladder Cancer	L.G. Liang et al. (2017)
ExoDisk	Double filtration	Colorimetric ELISA	Urine	CD9 and CD81	1 mL	NS	~1 h	Low (2 sample chambers)	Bladder Cancer	Woo et al. (2017)
ACE microarray chip	Dielectrophoretic separation	Fluorometric ELISA	Plasma, Serum, Blood	CD63 and Glypican-1	25 μL	NS	30–90 min	Low (Single Channel)	Pancreatic/Colon cancer	Ibsen et al. (2017), Lewis et al. (2018)

Abbreviations: μNMR: micro nuclear magnetic resonance; ACE: alternating current electrokinetic; ac-EHD: alternating current electrohydrodynamic; DFMD: double filtration microfluidic device; ELISA: enzyme-linked immunosorbent assay; EV: extracellular vesicles; GO/PDA: graphene oxide/polydopamine; GNP: gold nanoparticles; iMER: immunomagnetic exosomal RNA; iMEX: integrated magnetic-electrochemical exosome; mL: milliliters; LOD: limit of detection; MNP: magnetic nanoparticles; nano-IMEX: nano-interfaced microfluidic exosome; ng: nanograms; nPES: nanoplasmon-enhanced scattering; nPLEX: nano-plasmonic exosome sensor; NS: not specified; pg: picograms; pM: picomolar; RBC: red blood cell; RInSE: rapid inertial solution exchange; RT-qPCR: quantitative reverse transcription polymerase chain reaction.

isolation of EV. Microfluidic chip-based technologies, capable of integrated EV isolation and analysis, have significantly lowered the limit of detection, sample consumption and analysis time (Table 3), facilitating the realization of EV-based diagnostics in clinical settings. Optical sensing methods have been efficiently implemented in microfluidic devices for on-chip fluorescence, colorimetry and surface plasmon resonance (SPR) based EV analysis. SPR is the resonant oscillation of conduction electrons at the surface of a metal–dielectric interface when illuminated by polarized light. An SPR sensor monitors binding events of biomolecules at the sensor surface, which cause a change in the local refractive index linear to the number of molecules bound to the sensor surface (Brolo, 2012). For label-free, high-throughput profiling of immune-immobilized EV, the nano-plasmonic exosome (nPLEX) sensor, which uses transmission SPR through periodic nanohole arrays to detect specific EV populations, was developed (Im et al., 2014). The nPLEX sensor has been successfully applied to determine a diagnostic EV protein signature from pancreatic cancer patients (K.S. Yang et al., 2017). Similarly, several other integrated microfluidic devices combining immunoaffinity and SPR-based sensing have been developed for selective screening of tumor-derived EV (K. Liang et al., 2017; Sina et al., 2016). If effectively miniaturized, optical methods with single biomolecule sensitivity, such as surface enhanced Raman spectroscopy (SERS), could have the potential for on chip, high-throughput screening of all major clinically relevant properties of single EV in biological fluids (Stremersch et al., 2016). High-resolution flow cytometry instruments are under development as powerful tools for enumeration, sizing and molecular profiling of single EV (Stoner et al., 2016). In addition, a single EV analysis (SEA) technique capable of multiplexed measurement of protein biomarker expression on individual EV was recently described. EV are immobilized on the surface of a microfluidic chip, immunostained and imaged by microscopy. After imaging, fluorochromes are quenched for subsequent staining and imaging cycles, followed by multi-dimensional data analysis (Lee et al., 2018).

Non-optical sensing methods that have been successfully implemented on chip include miniaturized nuclear magnetic resonance (μNMR), electrochemical detection and RT-qPCR. A microfluidic system incorporating μNMR allowed detection of GBM (glioblastoma multiforme) EV labeled with target-specific magnetic nanoparticles (Shao et al., 2012). The integrated magnetic-electrochemical exosome (iMEX) sensor was used to immunomagnetically capture and profile plasma EV from ovarian cancer patients using chronoamperometry (Jeong et al., 2016). The same technique was used to monitor kidney transplant rejection by detecting EV released by immune cells into urine (Park et al., 2017). Immuno-magnetic exosome RNA (iMER) analysis was developed as a microfluidic platform integrating on chip EV isolation, RNA extraction and transcriptomic analysis by RT-qPCR. The iMER assay demonstrated diagnostic and drug resistance monitoring feasibility in clinical samples from GBM patients (Shao et al., 2015a). Another novel development, enabling more efficient and rapid extraction and analysis of EV-derived RNA, consists of two serial microfluidic chips for EV lysis using surface acoustic waves and RNA detection through an ion-exchange nanomembrane sensor (Taller et al., 2015).

Apart from the microfluidic technologies described above, an amplified luminescent proximity homogeneous assay (ExoScreen) was developed, enabling detection of cancer-derived EV in liquid biopsies from patients without a prior purification step. EV are immunocaptured by two antibodies and detected by photosensitizer-beads (Yoshioka et al., 2014).

5. Technical issues to consider for EV-based biomarker discovery

A consensus on standard operating procedures (SOP) for urine sample selection, collection, preparation, storage and shipping is required to preserve urine composition and to improve biomarker discovery and validation. Existing recommendations on bioanalytical method evaluation might provide guidance for developing such protocols (Biopharmaceutic Coordinating Committee, 2013). In addition, precise coordination of sample processing SOP and clinical data collection in (multisite) clinical biomarker trials is essential, requiring well-trained study nurses, study coordinators and technicians.

The Human Kidney and Urine Proteome Project (HKUPP), associated with the Human Proteome Organisation (HUPO) and Europrot, has produced a tentative standard protocol for urine sample collection and processing (EU COST action, 2012). However, since pre-analytical variables in urine processing procedures – such as centrifugation steps, addition of protease inhibitors or chemical stabilizers, and urine fractionation strategies – significantly impact the content of urine, they should be adjusted based on the analytes of interest and the clinical question being addressed in the study (Harpole et al., 2016). Although the potential impact of various pre-analytical factors during clinical sample processing on EV studies is increasingly recognized, few have been undertaken using urine (Vergauwen et al., 2017; Zhou et al., 2006). A quality control study assessing the impact of multiple pre-analytical factors including membrane filters for concentration, temperature, storage, pre-clearance centrifugation steps taking into account the integrity of EV studied by nanoparticle tracking analysis and electron microscopy amongst other complementary characterization methods would allow for a complete standardization of the pre-analytical steps. These pre-analytical variables, together with clinical and patient data, should be adequately reported to increase transparency, reproducibility and validation of identified EV-associated biomarkers (Van Deun et al., 2017; Van Deun and Hendrix, 2017).

Our recent review of EV-related literature found 34 distinct EV isolation protocols in 131 studies performed on urine, indicating a lack of standardized protocols (Van Deun et al., 2017). EV are most commonly isolated from urine by differential ultracentrifugation for diagnostic biomarker discovery (Table 2). By extension, searching the EV-TRACK knowledgebase (www.evtrack.org) (Van Deun et al., 2017) for all experiments on urine revealed that it is the most frequently reported isolation method to retrieve EV. Differential centrifugation isolates EV based on their size and density by sequentially increasing the centrifugal force to pellet cells and debris ($< 1500g$), large EV (10,000–20,000g), and small EV (100,000–200,000g). Although well established and commonly used, differential ultracentrifugation results in clumping of EV (Linares et al., 2015), co-isolates non-EV components such as protein aggregates and other contaminants (György et al., 2011; Rood et al., 2010; Tauro et al., 2012; Van Deun et al., 2014) and potentially damages EV during the final ultracentrifugation step (Ismail et al., 2013). In addition, this procedure results in a highly variable and relatively low recovery of EV (Lamparski et al., 2002; Lane et al., 2015), and rotor type, g-force and centrifugation times significantly influence EV yield and purity, making study to study comparison difficult (Cvjetkovic et al., 2014). Although efforts have been made to compare the performance of EV isolation methods for urine (Royo et al., 2016a), quality control studies comparing the performance of EV isolation methods in combination with complementary characterization methods such as particle analysis, protein analysis, contamination assessment and electron microscopy are missing. Whenever EV are isolated, adequate quality control experiments should be performed to assess the true biomarker composition of the isolated EV sample. The most commonly analyzed EV-associated proteins in urine studies are TSG101, CD9, ALIX and AQP2 (Van Deun et al., 2017). As for contaminants, the analysis of Tamm-Horsfall protein (THP) should be considered, given that polymers of this high abundance protein are easily co-isolated with EV and can thus confound both proteomic analyses, by masking of low abundance proteins (Hiemstra et al., 2011), and transcriptomic analyses, by pulling down nucleic acids (Wachalska et al., 2016). Besides THP (ca. 10% of studies), other commonly assessed contaminants in urine EV samples are albumin (8%) and cell organelle proteins (7%). Using the EV-TRACK knowledgebase, we assessed the transparency in reporting by analyzing EV-METRICs of articles reviewed in this manuscript (Table 2 and Fig. 1). EV-METRICs are expressed as a percentage

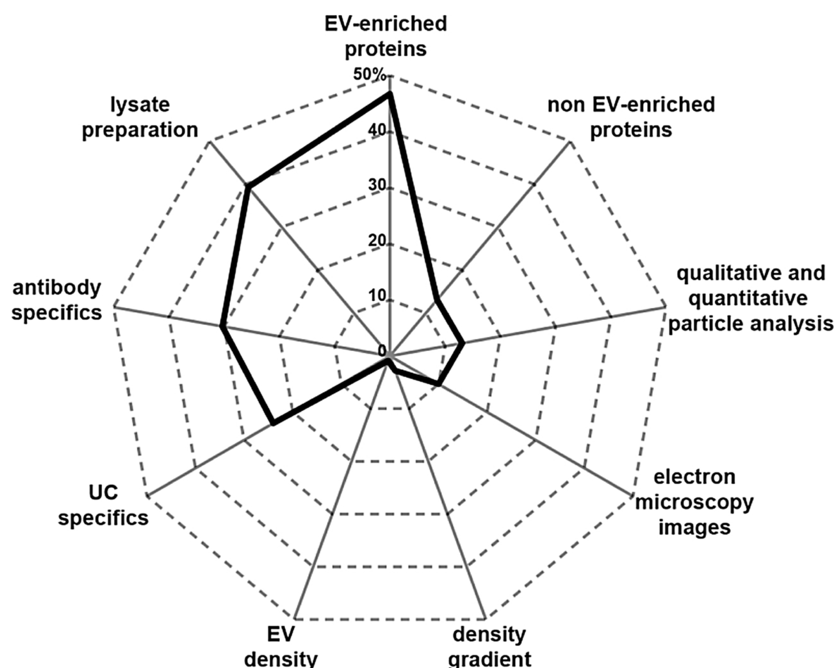

Fig. 1. Quality controls in EV urinary biomarker studies. Spider chart representing the percentage of urinary EV biomarker experiments (listed in Table 2) that adhere to each of the respective EV-METRIC parameters.

of fulfilled components from a list of nine, which were argued by the EV-TRACK consortium to be indispensable for unambiguous interpretation and independent replication of EV experiments (Van Deun et al., 2017). Almost one third (30%) of studies performed no characterization at all of EV samples isolated from urine for biomarker discovery (i.e. EV-METRIC of 0%). The implementation of a density gradient to validate EV isolation from urine is barely reported (2/50 studies) and EV samples are poorly characterized by electron microscopy (5/50 studies provide both wide-field and close-up EM image).

Finally, normalized analysis of extracellular vesicles is a prerequisite to allow patient-to-patient comparison of samples. Whether the intrinsic inter- and intrasubject variability of urine (pH, osmolality, bladder residency time, etc.) is also reflected in urinary EV is not clear. It has been suggested that EV-associated protein in first and second morning urine is largely similar (Zhou et al., 2006). One single-center study found that the presence of urinary EV is higher in females than males and decreases with age (Turco et al., 2016). Further in depth studies of the variability of urinary EV in healthy subjects are scarce. Indeed, rigorous analysis of the presence and composition of urinary EV is hampered by variable physicochemical parameters, such as viscosity and protein content that can affect EV isolation results (Momen-Heravi et al., 2012; Royo et al., 2016a; Witwer et al., 2013). There are several methods of normalization, including urine flow rate, urine volume, urinary creatinine and protein concentration, particle numbers and EV-enriched protein (CD9, ALIX) signal (Table 2) (Zhou et al., 2006). Although some groups have established normalization strategies for protein and metabolite analysis in urine, studies searching for optimal normalization strategies of EV in urine are largely missing. Importantly, only 20% of studies reviewed in this manuscript reported on the normalization strategy (Table 2). Appropriate biological reference standards of EV are required to allow accurate normalization (Valkonen et al., 2017).

6. Future perspectives & needs

Despite intensive research into the discovery of urinary biomarkers facilitating early diagnosis, accurate prognosis and prediction of therapy response in urological cancers, none of these markers has reached widespread use. Their implementation into daily clinical practice is hampered by a substantial degree of heterogeneity in performance characteristics and uncertainty about their reliability, clinical utility and cost-effectiveness, in addition to several technical limitations. Clinically relevant biomarkers for urogenital cancers, validated by multicenter prospective analysis, remain an unmet need.

EV biomarkers have the potential to overcome some of the limitations posed by classic proteomic and transcriptomic biomarkers. The field is however still in its infancy with the majority of published research focusing on discovery in small, heterogeneous patient and control populations. In addition, no guidelines or standardized procedures on biological sample processing, EV isolation, EV normalization and study design exist in order to conduct reliable and reproducible EV biomarker research (cfr. technical analysis). Also, the implementation of EV-based biomarkers will only be realized if test results can be returned to the clinician without delay. Classic EV isolation techniques limit their potential and the development of sensitive, selective and extensively validated capture platforms directed towards specific EV subpopulations are necessary before urinary EV biomarkers can enter routine clinical use.

Efforts to map the proteomic, genomic, transcriptomic, lipidomic and metabolomic content of urinary EV, isolated from prospectively collected clinical samples, using unbiased and unsupervised high-throughput discovery approaches will generate new candidate biomarkers with potential clinical value. Considering that their use to date remains mainly exploratory, the establishment of benchmark standards, assay optimization for clinical conditions and demonstration of analytical (technical sensitivity, technical specificity, robustness and limits of detection) and clinical validity (clinical sensitivity, clinical specificity, PPV and NPV) of biomarker assays are required to expand the clinical utility of high-throughput omics-based methods.

Aknowledgements

This work was supported by the Fund for Scientific Spearheads of Ghent University Hospital, Concerted Research Actions from Ghent University, “Stichting tegen Kanker”, the Fund for Scientific Research-Flanders (PhD grant: Jan Van Deun; Post-doctoral grant: An Hendrix)

and "Kom op tegen Kanker (Stand up to Cancer), the Flemisch cancer society" (Bert Dhondt: Emmanuel Vander Schueren Research Grant).

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