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Citation for published version:
Rimmer, MP, Gregory, CD & Mitchell, RT 2021, 'Extracellular vesicles in urological malignancies', *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, vol. 1876, no. 1, 188570.
https://doi.org/10.1016/j.bbcan.2021.188570

Digital Object Identifier (DOI):
10.1016/j.bbcan.2021.188570

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Biochimica et Biophysica Acta (BBA) - Reviews on Cancer

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Download date: 24. Sep. 2022

BBA - Reviews on Cancer 1876 (2021) 188570

Contents lists available at ScienceDirect

BBA - Reviews on Cancer

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

Review

Extracellular vesicles in urological malignancies

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ARTICLE INFO

Keywords:
Extracellular vesicles
Male reproductive tract
Prostasomes
Epididymosomes
Cancer
Biomarker
Liquid biopsy

ABSTRACT

Extracellular vesicles (EVs) are small lipid bound structures released from cells containing bioactive cargoes. Both the type of cargo and amount loaded varies compared to that of the parent cell. The characterisation of EVs in cancers of the male urogenital tract has identified several cargoes with promising diagnostic and disease monitoring potential. EVs released by cancers of the male urogenital tract promote cell-to-cell communication, migration, cancer progression and manipulate the immune system promoting metastasis by evading the immune response.

Their use as diagnostic biomarkers represents a new area of screening and disease detection, potentially reducing the need for invasive biopsies. Many validated EV cargoes have been found to have superior sensitivity and specificity than current diagnostic tools currently in use. The use of EVs to improve disease monitoring and develop novel therapeutics will enable clinicians to individualise patient management in the exciting era of personalised medicine.

1. Introduction

Extracellular vesicles (EVs) are small, cell-derived, lipid bilayer structures secreted by virtually all cell types [1]. Following their release, EVs constitute a heterogeneous population, incapable of replication. Initially believed to be a means of cellular waste removal, they are now understood to have numerous and significant functions [2,3]. The cargoes of EVs include mRNA, non-coding RNAs such as microRNA, long non-coding RNA, DNA, lipids, proteins and metabolites, the loading of which is influenced by the health, state and lineage of the parent cell [4–9]. Variable expression of these cargoes within EVs has led many to speculate their loading within EVs is a highly regulated process, however, although given this variation, the reverse may also be true [10–14]. Fundamental changes to cell and tissue DNA is reflected in EV cargoes, as demonstrated by Lee et al., showing DNA alterations in the tumour profile of bladder cancer was reflected in the DNA profile of urinary EVs [9]. Analysis of copy number variant between the tumour tissue and EVs demonstrated 12 somatic mutations were identified with an allele frequency of 65.6% between tissue and EV DNA [9].

Although the precise mechanisms which govern loading of individual proteins, metabolites and lipids into EVs has yet to be fully understood, RNA loading into EVs is thought to be based on recognition of a specific sequence within the nucleotide strand. On the identification of the 'loading' sequence motif, RNA incorporation within the EV is coordinated by the ubiquitously expressed RNA binding protein sumoylated heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) [15]. Variable expression of hnRNPA2B1 alters RNA loading in EVs which further demonstrates the dynamic nature of this process [15]. More recent work on RNA loading into EVs by Temoche-Diaz et al., has identified two distinct pathways in which microRNAs are loaded into EVs by a metastatic breast cancer cell line. Both a non-selective and selective pathway were identified, the latter regulated by the activity of the RNA binding protein Lupus La governing miR-122 loading, believed to be primarily due to the sub-cellular origin of the EV [16].

Following their release into the extracellular environment, EVs interact with recipient cells via numerous receptors on the cell surface including, but not limited to, tetraspanins, clathrin and integrins [17–20]. Interactions may also be receptor-independent. Cellular targeting mechanisms fall broadly into four categories: 1) interaction with cell surface ligands; 2) fusion with cell membranes and release of cargo into the cell; 3) endocytic uptake and transport to lysosomes and 4) endocytic uptake and transport to a specific area of the cell [21,22]. It is through interaction with cell surface receptors or release of cargoes into cells that EVs mediate their effect on recipient cells (Fig. 1) [23]. Precisely how EVs are targeted to recipient cells, however, is yet to be elucidated in detail.

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https://doi.org/10.1016/j.bbcan.2021.188570
Received 10 March 2021; Received in revised form 10 May 2021; Accepted 13 May 2021
Available online 19 May 2021
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Attempts to characterise EVs have been met with challenges owing to their diverse size, function and biogenesis. Ranging in size from 50 nm – 5um they are known to be produced by one of three pathways [24]. The first are formed within multivesicular endosomes through inward budding of the early endosome, following fusion of the endosome with the plasma membrane, these vesicles are released into the extracellular space and termed exosomes [25]. Formation of exosomes within a MVB leads to a lipid bilayer enriched with lipid components including glycophospholipid, cholesterol, ceramide and sphingomyelin [26]. The biogenesis of exosomes is highly regulated by the endosomal sorting complex required for transport (ESCORT) machinery in addition to other factors; this in turn leads to a rich complement of tetraspanins identified on exosomes, due to their endosomal origin [27]. Frequently used markers to identify exosomes include cluster of differentiation (CD) 9, 63 and 81, tumour suppressor gene 101, ALG-2-interacting protein X (ALIX) and heat shock proteins 60, 70 and 90 [27,28].

The second species of EV is from direct budding of the plasma membrane to form microparticles resulting in a lipid bilayer enriched with phosphatidylserine. They are best characterised by their surface markers including annexin A5, integrins and selectins [29–31]. The third species of EV is formed through the blebbing of the cell membrane as it undergoes apoptosis to produce apoptotic bodies which often contain cellular organelles and nuclear fragments [32–34]. Although considered a distinct cohort of EVs and the largest sub-type, they are only released during apoptosis unlike exosomes or microparticles which are released by healthy cells [35,36]. Despite their release occurring during programmed cell death, apoptotic EVs have numerous roles in disease progression in particular the tumour microenvironment, including enhanced tumour progression and disease resistance [37,38].

Although derived through distinct biogenesis pathways, there is substantial overlap between exosomes, microparticles and apoptotic bodies in their characterisation based on size, surface markers and cargoes. Once released into the extracellular environment, identification of specific subsets of EVs is challenging given this overlap. To address this, the International Society of Extracellular vesicles has developed consensus statements on how to classify EVs, which advocates describing EVs based on their size, biogenesis, tissue of origin and the presence of surface markers, outlined in Fig. 1 [1]. A simpler nomenclature for EVs is now advocated and they can be referred to as either small EVs (<200 nM) or large EVs (>200uM) and referencing their known surface markers such as $CD81^{+ve}$/ $TSG101^{+ve}$ [1].

The preferential loading of cargoes into EVs frequently results in EV cargo being significantly different to that of their cell of origin [39–42]. The study of EVs, in particular the characterisation of these cargoes, has grown dramatically over the years in numerous pathologies, especially in understanding their role in cancer [43]. Of particular interest is their use as novel biomarkers, tracking disease progression and predicting response to treatment. There is limited knowledge as to their release from the male reproductive tract [44]. However, the role of EVs in the maturation of sperm and fertility potential should not be underestimated, as they confer essential modifications in cargo and function [45]. These include acquisition of forward motility, capability to fertilise an oocyte and protection from oxidative stress, conferred by EVs for the epididymis or epididymosomes. Transfer of cAMP and Ca^{2+} signalling machinery to sperm which facilitate motility and the ability to carry out the acrosome reaction is mediated by prostate EVs or prostasomes [46]. The importance of the interaction between EVs of the male reproductive tract and sperm is best seen when comparing the function of sperm in vasectomised men, compared to healthy controls, who have impaired capacitation, motility and fertilisation potential [47–51].

Interaction between EVs from the male reproductive tract not only alters the function of sperm but also the female reproductive tract. EVs trigger increased endometrial prolactin stimulation, enhanced decidualisation and endometrial receptivity to a developing embryo and ameliorate the local immune response to sperm, reducing phagocytotic activity of neutrophils and monocytes [52,53]. A brief overview of the role of EVs in the male reproductive tract, in both health and disease is outlined in Fig. 2.

EVs play an important role in the development of urological cancers [54]. Their sustained production and bespoke cargo loading implies their production is highly regulated and contributes to their ability to manipulate individual cells and tissue [55]. Their widespread distribution throughout the body via the circulatory and lymphatic systems gives them the potential to 'prime' various sites for future metastatic spread [20,56]. This is best characterised by their contribution to the development and maintenance of the pre-metastatic niche and regulation of the tumour microenvironment [57,58] through to manipulation of the immune system, attenuating its response to metastasising cancer cells [59,60]. Cancer-derived EVs mediate their effects through interaction with recipient cells, modulating their function to transform the tissue into a supportive pro-metastatic environment [61]. Some examples of this include the transformation of macrophages into tumour-supporting macrophages, promotion of angiogenesis through activation of endothelial cells and development of cancer-associated fibroblasts [61–68]. In addition to modification of the pre-metastatic environment, transformation of recipient cells by EV uptake leads to increased metastatic organotropism to these tissues, as demonstrated in a mouse model [19].

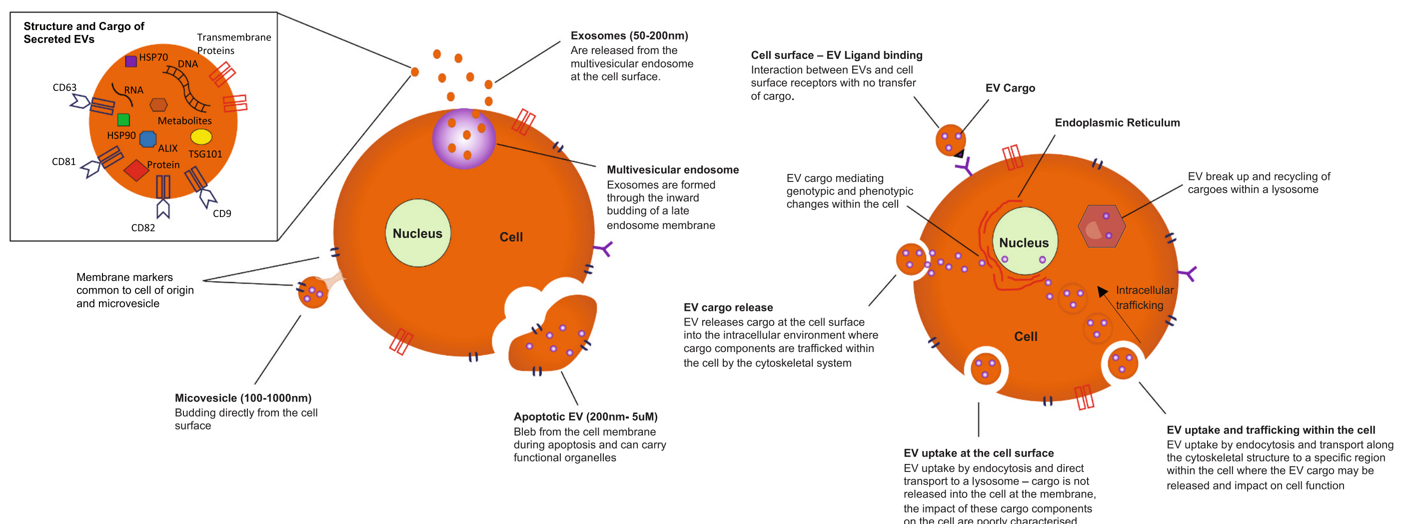

Fig. 1. Sites of EV production, interaction with recipient cells, EV intracellular fate and EV structure and cargo.

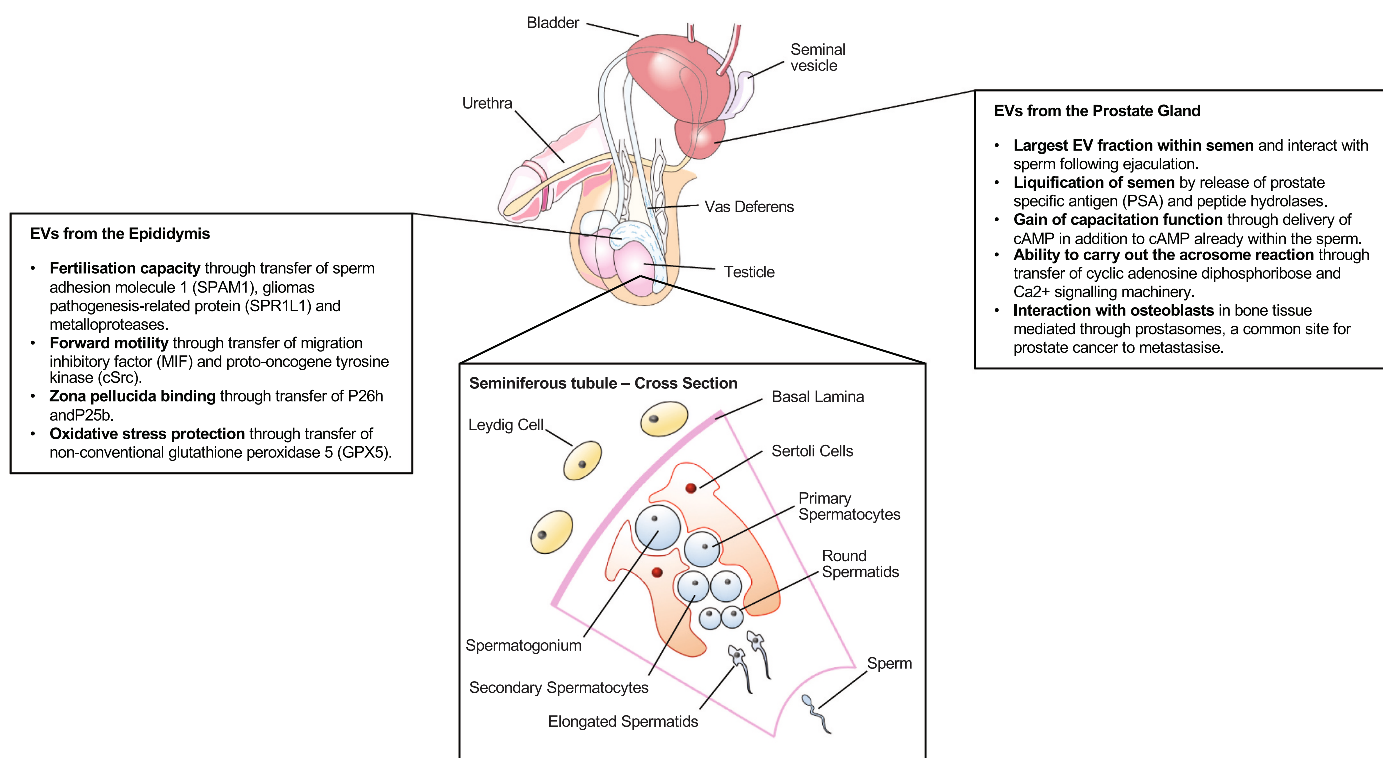

Fig. 2. Adult male reproductive tract, seminiferous tubule and summary of the release of EVs.

EVs confer chemotherapy resistance to recipient cells [69–71], through delivery of proteins such as ATP-binding cassette sub-family B member 1 (ABC1) [72] transfer of apoptosis inhibitors [73], increasing tumour invasiveness and metastasis [74,75]. Higher numbers of EVs released in response to chemotherapy highlight the surge in EV mediated intracellular communication in response to cellular stress [74]; potentially conferring pro-survival characteristics to recipient cells [76].

Many EV functions are mediated through their cargoes, the characterisation of which is growing rapidly with advances in their isolation and detection [77,78]. A growing number of studies have reported EV cargoes in relation to diagnostic accuracy, treatment prognosis, treatment response as well as numerous biological processes. At first, this may appear to herald a new era of precision medicine in relation to urological malignancies, however, the methodologies to isolate these EVs as well as likely co-precipitated molecules must be taken into account. A common EV isolation technique such as ultracentrifugation pellets EVs based on their biophysical properties, however, proteins of similar sizes, often reported as novel biomarkers, may also be precipitated. Variation in EV isolation methodologies is outlined in Tables 1 and 2 and discussed further below.

In cancer, the identification of EV cargoes has found applicability in diagnostics, disease monitoring and response to treatment [79–82]. Although EVs are unlikely to mediate all these processes in their entirety, the identification of cancer specific RNAs which are poorly related to their cell of origin outlines their potential role in cancer development [83–85]. Altered EV loading into cancer-derived EVs may be driven in part by enhanced expression of hnRNPA2B1, upregulated in several cancers including breast [86] and pancreatic [87]. The unique cargoes within cancer derived EVs and their distribution in plasma and urine makes them appealing targets for identification of novel biomarkers [88], similar to what has been achieved with circulating tumour cells and cell free DNA [89,90]. EVs themselves express numerous surface markers including proteins which may themselves be misattributed as biomarkers of disease, highlighting the importance of validation in larger cohorts and in comparison to non-cancer controls [91].In addition to characterisation of cargoes, increased EV production from cancer cells has led to speculation that this in itself may be of clinical utility to detect disease [92,93] and off-target effects of chemotherapy [94].

The role of EVs in many diseases has yet to be elucidated and the characterisation of their cargoes to identify novel diagnostic biomarkers has yet to be undertaken. In addition to this, the tissue of origin of many EVs is not always clear, whether released only from the diseased tissue or also from other tissues influenced by downstream signalling or systemic changes due to the disease. Evidence of the majority of EVs originating from malignant tissue, however, is demonstrated by a reduction in circulating EVs following removal of the diseased tissue [95]. Furthermore, lower pre- and post-operative EV levels correlate with greater survival [95]. Other potential sources of elevated EVs are from the systemic response to the disease or the subsequent medical treatment on diseased and non-diseased tissues [41]. The role of EVs in urogenital malignancies is poorly understood, especially in comparison to other diseases such as breast cancer. We provide an overview of the current level of understanding below.

2. Evidence acquisition

We undertook a literature search of PubMed articles written in English using 56 MeSH search terms, relating to extracellular vesicles, reproductive tissues and urological malignancies, as outlined in Supplementary Fig. 1. Search terms were combined using the Boolean operators AND/OR from inception until 3rd of February 2021.

Titles and abstracts of 9532 articles were screened against our inclusion criteria of studies reporting on EVs from male reproductive tract and in urogenital malignancies. We screened the bibliographies of review articles identified in our search to identify relevant studies not captured in out electronic search. We identified 91 original research articles reporting on EV cargoes in renal, prostate and bladder cancer. We report these studies narratively with a focus on lipids, proteins and RNAs, summarising EV cargoes and their identified cellular functions and clinical validation and use as potential biomarkers for disease.

Table 1
Extracellular vesicle cargo, known functions and clinical validation.

Disease	No. of Patients	No. of Controls	Source of EV	Isolation Techniques	EV Characterisation	Storage	Clinical Application / Role of Cargo	Cargo Generic	Cargo Specific	Cargo Identification	Sensitivity	Specificity	ROC- Area Under Curve	Reference
Renal Cancer	82	80	Serum	Centrifugation, Immuno-affinity beads & commercial EV precipitation kit	Flow cytometry & immunostaining	Serum frozen —80C pre-analysis	Diagnosis	miRNA	miR-210 miR- 1233	Candidate screening	miR-210 (70%) miR-1233 (81%)	miR-210 (62.2%) miR-1233 (76%)	miR-210 - 0.69 miR-1233 - 0.82	Zhang W, Ni M, Su Y, Wang H, Zhu S, Zhao A and Li G. MicroRNAs in Serum Exosomes as Potential Biomarkers in Clear-cell Renal Cell Carcinoma. *Eur Urol Focus*. 2018:4;412–419.
Renal Cancer	40	30	Serum	Centrifugation & commercial EV precipitation kit	TEM, Western Blot	Serum frozen —80C pre-analysis	Diagnosis	miRNA	miR-210	High throughput screening	82.50%	80%	0.8779	Wang X, Wang T, Chen C, Wu Z, Bai P, Li S, Chen B, Liu R, Zhang K, Li W, et al. Serum exosome miR-210 as a potential biomarker for clear cell renal cell carcinoma. *Journal of Cellular Biochemistry*. 2019:120;1492–1502.
Renal Cancer	28	18	Urine, Cell Lines	Commercial spin column for urinary EVs, commercial isoelectric precipitation	TEM	Urine frozen —80C pre-analysis	Diagnosis	miRNA	miR-126-3p miR-449a miR-34b-5p	Candidate analysis following high throughput screening	–	–	miR-126-3p - miR-449a: 0.84 miR-126-3p – miR-34b-5p: AUC: 0.79	Butz H, Nofech-Mozes R, Ding Q, Khella HWZ, Szabó PM, Jewett M, Finelli A, Lee J, Ordon M, Stewart R, et al. Exosomal MicroRNAs Are Diagnostic Biomarkers and Can Mediate Cell-Cell Communication in Renal Cell Carcinoma. *Eur Urol Focus*. 2016:2;210–218.
Renal Cancer	109	0	Plasma	Commercial EV precipitation kit	None	Plasma frozen —80C pre-analysis	Prognosis	miRNA	miR-let-7i-5p	Candidate analysis following high throughput screening	–	–	0.64	Du M, Giridhar KV, Tian Y, Tschannen MR, Zhu J, Huang CC, Kilari D, Kohli M and Wang L. Plasma exosome miRNAs-based prognosis in metastatic kidney cancer. *Oncotarget*. 2017:8;63,703–63,714.
Renal Cancer	108	0	Serum, Cell Lines	Centrifugation, Immuno-affinity beads & commercial EV precipitation kit	TEM, Western Blot	None	Progression, Prognosis	miRNA	miR-224	Candidate screening	–	–	Progression: 0.833 Prognosis: 0.857	Fujii N, Hirata H, Ueno K, Mori J, Oka S, Shimizu K, Kawai Y, Inoue R, Yamamoto Y, Matsumoto H, et al. Extracellular miR-224 as a prognostic marker for clear cell renal cell carcinoma. *Oncotarget*. 2017:8;109,877–109,888.
Bladder Cancer	28	12	Urine	Centrifugation	TEM, Western Blot, Flow cytometry	Urine supernatant stored at —80C before EV isolation	Diagnosis, discrimination between high and low grade disease	Protein	APOA1, CD5L, FGA, FGB, FGG, HPR, HP	Candidate analysis following high throughput screening	–	–	Range: 0.762–0.830	Chen CL, Lai YF, Tang P, Chien KY, Yu JS, Tsai CH, Chen HW, Wu CC, Chung T, Hsu CW, et al. Comparative and targeted proteomic analyses of urinary microparticles from bladder cancer and hernia patients. *J Proteome Res*. 2012:11;5611–29.
Bladder Cancer	80	80	Urine	Centrifugation		Urine supernatant	Diagnosis, Prognosis	lncRNA	MALT1, PCAT-1, SPRY4-IT1	Candidate screening	MALT1: 78.7%	MALT1: 67.5%	MALT1: 0.785 PCAT-1: 0.810	Zhan Y, Du L, Wang L, Jiang X, Zhang S, Li J, Yan K, Duan

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Table 1 (*continued*)

Disease	No. of Patients	No. of Controls	Source of EV	Isolation Techniques	EV Characterisation	Storage	Clinical Application / Role of Cargo	Cargo Generic	Cargo Specific	Cargo Identification	Sensitivity	Specificity	ROC- Area Under Curve	Reference
					TEM, Western Blot, NTA, Flow cytometry	stored at —80C before EV isolation					PCAT-1: 71.2% SPRY4-IT1: 87.5% Combined: 70.2%	PCAT-1: 80.0% SPRY4-IT1: 65.0% Combined: 85.6%	SPRY4-IT1: 0.799 Combined: 0.854	W, Zhao Y, Wang L, et al. Expression signatures of exosome long non-coding RNAs in urine serve as novel non-invasive biomarkers for diagnosis and recurrence prediction of bladder cancer. Mol Cancer. 2018:17;142.
Bladder Cancer	129	62	Urine	Centrifugation	TEM, Western Blot	Urine supernatant stored at —80C before EV isolation	Diagnosis, Prognosis	Protein	Alpha 1-antitrypsin, H2BK1	Candidate analysis following high throughput screening	Alpha 1-antitrypsin: 50.4% H2BK1: 62.0% Combined: 62.7%	Alpha 1-antitrypsin: 96.9% H2BK1: 92.3% Combined: 87.59%	Alpha 1-anti-trypsin: 0.736 H2BK1: 0.772 Combined: 0.87	Lin SY, Chang CH, Wu HC, Lin CC, Chang KP, Yang CR, Huang CP, Hsu WH, Chang CT and Chen CJ. Proteome Profiling of Urinary Exosomes Identifies Alpha 1-Antitrypsin and H2B1K as Diagnostic and Prognostic Biomarkers for Urothelial Carcinoma. *Sci Rep*. 2016:6;34,446.
Bladder Cancer	260	260	Serum	Commercial EV precipitation kit	TEM, NTA, Western Blot,	Urine supernatant stored at —80C before EV isolation	Diagnosis	lncRNA	PACT-1, UBC1, SNHG16	Candidate screening	Combined: 85%	Combined: 78%	PACT-1: 0.753 UBC1: 0.751 SNHG16: 0.681 Combined: 0.857	Zhang S, Du L, Wang L, Jiang X, Zhan Y, Li J, Yan K, Duan W, Zhao Y, Wang L, et al. Evaluation of serum exosome LncRNA-based biomarker panel for diagnosis and recurrence prediction of bladder cancer. *J Cell Mol Med*. 2019:23;1396–1405.
Bladder Cancer	59	49	Urine	Commercial EV precipitation kit	DLS, SEM, Western blot	Urine stored at 4C prior to EV isolation	Diagnosis	Protein	MAGE B4	Candidate screening	71.00%	66.00%	0.67	Yazarlou F, Mowla SJ, Oskooei VK, Motevaseli E, Tooli LF, Afsharpad M, Nekoohesh L, Sanikhani NS, Ghafouri-Fard S and Modarressi MH. Urine exosome gene expression of cancer-testis antigens for prediction of bladder carcinoma. Cancer Manag Res. 2018:10;5373–5381.
Bladder Cancer	206	36	Urine	Commercial EV isolation kit	None	Urine stored at —80C prior to EV isolation	Diagnosis	mRNA	SLC2A1, GPRC5A and KRT17	Candidate analysis following high throughput screening	SLC2A1: 64% GPRC5A: 54% KRT17: 58%	SLC2A1: 75% GPRC5A: 72% KRT17: 58%	SLC2A1: 0.70 GPRC5A: 0.64 KRT17: 0.64	Murakami T, Yamamoto CM, Akino T, Tanaka H, Fukuzawa N, Suzuki H, Osawa T, Tsuji T, Seki T and Harada H. Bladder cancer detection by urinary extracellular vesicle mRNA analysis. Oncotarget. 2018:9.
Bladder Cancer	85	45	Urine	Centrifugation	Western Blot	Urine supernatant stored at —20C before	Diagnosis	miRNA	miR-26a, miR-93, miR-191, and miR-940	High throughput screening	Combined: 70%	Combined: 84%	0.858	Long JD, Sullivan TB, Humphrey J, Logvinenko T, Summerhayes KA, Kozinn S, Harty N, Summerhayes IC,

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Table 1 (*continued*)

Disease	No. of Patients	No. of Controls	Source of EV	Isolation Techniques	EV Characterisation	Storage	Clinical Application / Role of Cargo	Cargo Generic	Cargo Specific	Cargo Identification	Sensitivity	Specificity	ROC- Area Under Curve	Reference
						EV isolation, EVs frozen at -80 after isolation Plasma frozen —80C pre-analysis, urine supernatant frozen —80C pre-analysis								Libertino JA, Holway AH, et al. A non-invasive miRNA based assay to detect bladder cancer in cell-free urine. Am J Transl Res. 2015:7;2500–9.
Bladder Cancer	6	3	Urine, Cell Line	Centrifugation	TEM, NTA, Western Blot	EVs stored at —80C prior to analysis	Diagnosis	miRNA	miR-21-5p	High throughput screening	75%	95.80%	0.9	Matsuzaki K, Fujita K, Jingushi K, Kawashima A, Ujike T, Nagahara A, Ueda Y, Tanigawa G, Yoshioka I, Ueda K, et al. MiR-21-5p in urinary extracellular vesicles is a novel biomarker of urothelial carcinoma. Oncotarget. 2017:8;24,668–24,678.
Bladder Cancer	16	8	Urine	Centrifugation, Microfluidic filtration	DLS, ELISA, TEM	None	Diagnosis	Protein	CD63 and EV signal intensity	Candidate screening	81.30%	90%	0.96	Liang L-G, Kong M-Q, Zhou S, Sheng Y—F, Wang P, Yu T, Inci F, Kuo WP, Li L-J, Demirci U, et al. An integrated double-filtration microfluidic device for isolation, enrichment and quantification of urinary extracellular vesicles for detection of bladder cancer. *Scientific Reports*. 2017:7;46,224.
Prostate Cancer	16	15	Urine	Centrifugation, Size exclusion filtration	TEM, DSL, Western Blot, Protein Quantification	EVs stored at —80C prior to analysis	Diagnosis	Protein	17 proteins including: ADIRF, TM256, PCYOX1, LAMTOR1	High throughput screening	–	–	TM256 and LAMTOR1: 0.94	Øverbye A, Skotland T, Koehler CJ, Thiede B, Seierstad T, Berge V, et al. Identification of prostate cancer biomarkers in urinary exosomes. Oncotarget. 2015;6 (30):30357–76.
Prostate Cancer	152	189	Urine	Centrifugation, Size exclusion filtration, commercial EV precipitation kit	TEM, NTA, Western Blot	Urine supernatant stored at —80C before EV isolation	Diagnosis, Discrimination between low and high grade tumours	Protein	TGM4, ADSV, CD63, GLPK5, PSA, PPAP, SPHM	Candidate screening	–	–	Diagnosis TGM4: 0.58 ADSV: 0.58 Combined: 0.65 Discrimination between high and low grade disease CD63: 0.65 GLPK5: 0.64 PSA: 0.66	Sequeiros T, Rigau M, Chiva C, Montes M, Garcia-Grau I, Garcia M, Diaz S, Celma A, Bijnsdorp I, Campos A, et al. Targeted proteomics in urinary extracellular vesicles identifies biomarkers for diagnosis and prognosis of prostate cancer. *Oncotarget*. 2017:8;4960–4976.

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Table 1 (*continued*)

Disease	No. of Patients	No. of Controls	Source of EV	Isolation Techniques	EV Characterisation	Storage	Clinical Application / Role of Cargo	Cargo Generic	Cargo Specific	Cargo Identification	Sensitivity	Specificity	ROC- Area Under Curve	Reference
													PPAP: 0.64 SPHM: 0.61 Combined: 0.70	
Prostate Cancer	89	106	Urine	Commercial EV concentrator	TEM, NTA, Western Blot	Urine stored 2-8C for up to 2 weeks prior to EV isolation, EVs stored at —80C prior to analysis	Discrimination between benign and high grade disease	RNA	PCA3, ERG	Candidate screening	–	–	Discrimination between high and low grade disease using: PSA, age, race, family history: 0.6723 RNA, PSA, age, race, family history: 0.803	Donovan MJ, Noerholm M, Bentink S, Belzer S, Skog J, O'Neill V, Cochran JS and Brown GA. A molecular signature of PCA3 and ERG exosome RNA from non-DRE urine is predictive of initial prostate biopsy result. *Prostate Cancer and Prostatic Diseases*. 2015:18;370–375.
Prostate Cancer	60	24	Urine, Serum	Centrifugation, Size exclusion concentration, Commercial EV precipitation kit	TEM, NTA, Western Blot, Protein quantification	Urine and serum supernatant stored at —80C before EV isolation	Diagnosis	miRNA	miR-1290, miR-145	Candidate screening	–	–	miR-1290: 0.613 miR-145: 0.623 miR-145 and PSA: 0.863	Xu Y, Qin S, An T, Tang Y, Huang Y and Zheng L. MiR-145 detection in urinary extracellular vesicles increase diagnostic efficiency of prostate cancer based on hydrostatic filtration dialysis method. *Prostate*. 2017:77;1167–1175.
Prostate Cancer	9	4	Urine	Centrifugation, Size exclusion concentration, Commercial EV precipitation kit	None	EVs stored at —80C prior to analysis	Diagnosis	isomiRNA	miR-21, miR-204, miR-375	Candidate analysis following high throughput screening	72.90%	88%	Combined isomiRs: 0.821 Combines PSA and isomiRs: 0.866	Koppers-Lalic D, Hackenberg M, de Menezes R, Misovic B, Wachalska M, Geldof A, Zini N, de Reijke T, Wurdinger T, Vis A, et al. Non-invasive prostate cancer detection by measuring miRNA variants (isomiRs) in urine extracellular vesicles. *Oncotarget*. 2016:7;22,566–78.
Prostate Cancer	78	28	Urine, Plasma	Filtration and size exclusion concentration	None	Plasma and urine supernatant stored at —80C prior to EV isolation	Diagnosis, identification of metastatic disease	miRNA	miR-141, miR-375, miR-107, miR574-3p	Candidate analysis following high throughput screening	miR-107: 67%	miR-107: 43%	miR-107: 0.62 miR-574-30: 0.66	Bryant RJ, Pawlowski T, Catto JW, Marsden G, Vessella RL, Rhees B, Kuslich C, Visakorpi T and Hamdy FC. Changes in circulating microRNA levels associated with prostate cancer. *Br J Cancer*. 2012:106;768–74.
Prostate Cancer	44	8	Cell Lines, Serum	Centrifugation & commercial EV precipitation kit	NTA	None	Tumour suppression	miRNA	miR-1246	High throughput screening	75%	100%	0.926	Bhagirath D, Yang TL, Bucay N, Sekhon K, Majid S, Shahryari V, Dahiya R, Tanaka Y and Saini S. microRNA-1246 Is an Exosomal Biomarker for Aggressive Prostate Cancer.

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Table 1 (*continued*)

Disease	No. of Patients	No. of Controls	Source of EV	Isolation Techniques	EV Characterisation	Storage	Clinical Application / Role of Cargo	Cargo Generic	Cargo Specific	Cargo Identification	Sensitivity	Specificity	ROC- Area Under Curve	Reference
														Cancer Res. 2018:78;1833–1844.
Prostate Cancer	30	49	Urine	Centrifugation	None	Urine supernatant stored at —80C before EV isolation	Diagnosis	lncRNA	lncRNA-p21	Candidate screening	67%	63%	0.663	Işın M, Uysaler E, Özgür E, Köseoğlu H, Şanlı Ö, Yücel Ö B, Gezer U and Dalay N. Exosomal lncRNA-p21 levels may help to distinguish prostate cancer from benign disease. *Front Genet*. 2015:6;168.
Prostate Cancer	60	10	Urine	Centrifugation	TEM	EVs stored at —80C prior to analysis	Diagnosis	miRNA	miR-21, miR-141, miR-375, miR-214, let-7c	Candidate screening	–	–	miR-21: 0.713 miR-141: 0.652 miR-214: 0.542 miR-375: 0.799 let-7c: 0.679	Foj L, Ferrer F, Serra M, Arévalo A, Gavagnach M, Giménez N and Filella X. Exosomal and Non-Exosomal Urinary miRNAs in Prostate Cancer Detection and Prognosis. *Prostate*. 2017:77;573–583.
Prostate Cancer	50	22	Plasma	Size exclusion chromatography, size exclusion concentration	TEM, NTA, Western Blot	Plasma supernatant stored at —80C before EV isolation	Diagnosis	miRNA	miR-200c-3p, miR-21-5p, Let-7a-5p	Candidate screening	–	–	miR-200c-3p: 0.68 miR-21-5p: 0.67 Let-7a-5p: 0.68	Endzeliņš E, Berger A, Melne V, Bajo-Santos C, Soboļevska K, Ābols A, Rodriguez M, Šantare D, Rudņickiha A, Lietuvietis V, et al. Detection of circulating miRNAs: comparative analysis of extracellular vesicle-incorporated miRNAs and cell-free miRNAs in whole plasma of prostate cancer patients. *BMC Cancer*. 2017:17;730.
Prostate Cancer	30	34	Cell Lines, Plasma	Centrifugation, Immuno-affinity beads, Commercial EV precipitation kit	TEM, DSL, Western Blot	Plasma supernatant stored at —80C before EV isolation	Diagnosis	lncRNA	SChLAP1, SAP30L-AS1	Candidate screening	SChLAP1: 87.9% SAP30L-AS1: 61.1%	SChLAP1: 76.7% SAP30L-AS1: 82.1%	SChLAP1: 0.8697 SAP30L-AS1: 0.6587 Combined: 0.9224	Wang YH, Ji J, Wang BC, Chen H, Yang ZH, Wang K, Luo CL, Zhang WW, Wang FB and Zhang XL. Tumour-Derived Exosomal Long Noncoding RNAs as Promising Diagnostic Biomarkers for Prostate Cancer. *Cell Physiol Biochem*. 2018:46;532–545.
Prostate Cancer	123	0	Plasma	Centrifugation, commercial EV precipitation kit	NTA	Plasma supernatant stored at —80C before EV isolation	Prognosis	miRNA	miR-1290, miR-1246, miR-375	High throughput screening	–	–	miR-1290 and miR-375: 0.68	Huang X, Yuan T, Liang M, Du M, Xia S, Dittmar R, Wang D, See W, Costello BA, Quevedo F, et al. Exosomal miR-1290 and miR-375 as prognostic markers in castration-resistant prostate cancer. *Eur Urol*. 2015:67;33–41.
Prostate Cancer	30	0	Urine	Centrifugation, size exclusion concentration	TEM	Urine supernatant stored at	Diagnosis	miRNA	PCA3	Candidate screening	–	–	0.534	Dijkstra S, Birker IL, Smit FP, Leyten GHJM, de Reijke TM, van Oort IM, Mulders

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Table 1 (*continued*)

Disease	No. of Patients	No. of Controls	Source of EV	Isolation Techniques	EV Characterisation	Storage	Clinical Application / Role of Cargo	Cargo Generic	Cargo Specific	Cargo Identification	Sensitivity	Specificity	ROC- Area Under Curve	Reference
						—80C before EV isolation								PFA, Jannink SA and Schalken JA. Prostate Cancer Biomarker Profiles in Urinary Sediments and Exosomes. *The Journal of Urology*. 2014:191;1132–1138.
Prostate Cancer	519	–	Urine	Size exclusion filtration, Commercial EV precipitation kit	None	Urine stored at 2-8C and —80C prior to EV isolation	Diagnosis, Discrimination between low and high grade disease	RNA	ERG, PCA3, SPDEF	Candidate screening	–	–	Combined: 0.74	McKiernan J, Donovan MJ, O'Neill V, Bentink S, Noerholm M, Belzer S, Skog J, Kattan MW, Partin A, Andriole G, et al. A Novel Urine Exosome Gene Expression Assay to Predict High-grade Prostate Cancer at Initial Biopsy. JAMA Oncol. 2016:2;882–9.
Prostate Cancer	503	–	Urine	Size exclusion filtration, Commercial EV precipitation kit	None	Urine stored at 4C prior to EV isolation and —80C after filtration	Diagnosis, Discrimination between low and high grade disease	RNA	ERG, PCA3, SPDEF	Candidate screening	–	–	Combined: 0.70	McKiernan J, Donovan MJ, Margolis E, Partin A, Carter B, Brown G, Torkler P, Noerholm M, Skog J, Shore N, et al. A Prospective Adaptive Utility Trial to Validate Performance of a Novel Urine Exosome Gene Expression Assay to Predict High-grade Prostate Cancer in Patients with Prostate-specific Antigen 2-10 ng/mL at Initial Biopsy. Eur Urol. 2018:74;731–738.
Prostate Cancer	20	9	Urine	Centrifugation, Size exclusion filtration	None	EVs stored at —80C post analysis, pre application	Diagnosis	miRNA	miR-196a-5p, miR-34a-5p, miR-143-3p, miR-501-3p and miR-92a-1-5p	High throughput screening	miR-196a-5p: 100%	miR-196a-5p: 89%	miR-196a-5p: 0.92 miR-143-3p: 0.72	Rodríguez M, Bajo-Santos C, Hessvik NP, Lorenz S, Fromm B, Berge V, Sandvig K, Linē A and Llorente A. Identification of non-invasive miRNAs biomarkers for prostate cancer by deep sequencing analysis of urinary exosomes. *Molecular Cancer*. 2017:16;156.
Prostate Cancer	51	40	Serum	Centrifugation, Size exclusion filtration, Commercial EV precipitation kit	TEM, Flow cytometry, Western Blot	None	Discriminating between local and metastatic disease	miRNA	miR-141	Candidate screening	80%	87.10%	0.8694	Li Z, Ma YY, Wang J, Zeng XF, Li R, Kang W and Hao XK. Exosomal microRNA-141 is upregulated in the serum of prostate cancer patients. Onco Targets Ther. 2016:9;139–48.
Prostate Cancer	50	21	Serum	Centrifugation, Size exclusion filtration	TEM, Western Blot	Serum supernatant stored at —80C before EV isolation,	Diagnosis, discrimination between prostate cancer and BPH	Protein	EphrinA2	Candidate screening	80.59%	88%	0.9062	Li S, Zhao Y, Chen W, Yin L, Zhu J, Zhang H, Cai C, Li P, Huang L and Ma P. Exosomal ephrinA2 derived from serum as a potential

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Table 1 (*continued*)

Disease	No. of Patients	No. of Controls	Source of EV	Isolation Techniques	EV Characterisation	Storage	Clinical Application / Role of Cargo	Cargo Generic	Cargo Specific	Cargo Identification	Sensitivity	Specificity	ROC- Area Under Curve	Reference
						EVs stored at —80C prior to analysis								biomarker for prostate cancer. J Cancer. 2018:9;2659–2665.
Prostate Cancer	15	13	Urine	Centrifugation, Size exclusion filtration	Protein and Lipid measurements	EVs stored at —80C following analysis of purity and yield	Diagnosis	Lipids	Lactosylceramide: Phosphatidylserine ratio	Candidate screening	–	–	0.989	Skotland T, Ekroos K, Kauhanen D, Simolin H, Seierstad T, Berge V, Sandvig K and Llorente A. Molecular lipid species in urinary exosomes as potential prostate cancer biomarkers. Eur J Cancer. 2017:70;122–132.
Prostate Cancer	35	35	Urine, Cell Lines	Centrifugation, Lectin induced precipitation	ATM, DSL, Western Blot	EVs stored at —80C prior to analysis	Diagnosis	miRNA	miR-574-3p, miR-141-5p, miR-21-5p	Candidate screening	miR-574-3p: 71% miR-141-5p: 66% miR-21-5p: 46%	–	miR-574-3p: 0.85 miR-141-5p: 0.86 miR-21-5p: 0.65	Samsonov R, Shtam T, Burdakov V, Glotov A, Tsyrlina E, Berstein L, Nosov A, Evtushenko V, Filatov M and Malek A. Lectin-induced agglutination method of urinary exosomes isolation followed by mi-RNA analysis: Application for prostate cancer diagnostic. *Prostate*. 2016:76;68–79.
Prostate Cancer	14	20	Urine	Centrifugation, Size exclusion filtration	TEM	Urine supernatant stored at —80C before EV isolation	Diagnosis	miRNA	miR-19b, miR-16	Candidate screening	miR-19b: 100% miR-16: 95%	miR-19b: 93% miR-16: 79%	–	Bryzgunova OE, Zaripov MM, Skvortsova TE, Lekchnov EA, Grigor'eva AE, Zaporozhchenko IA, Morozkin ES, Ryabchikova EI, Yurchenko YB, Voitsitskiy VE, et al. Comparative Study of Extracellular Vesicles from the Urine of Healthy Individuals and Prostate Cancer Patients. PLoS One. 2016:11;e0157566.
Prostate Cancer	19	16	Urine	Centrifugation	None	Urine stored 2-8C before EV isolation	Diagnosis	mRNA	GATA2	Candidate screening	–	–	0.74	Woo J, Santasusagna S, Banks J, Pastor-Lopez S, Yadav K, Carceles-Cordon M, Dominguez-Andres A, Den RB, Languino LR, Pippa R, et al. Urine Extracellular Vesicle GATA2 mRNA Discriminates Biopsy Result in Men with Suspicion of Prostate Cancer. J Urol. 2020:204;691–700.
Prostate Cancer	26	16	Urine	Centrifugation, Size exclusion filtration	Western Blot, ELISA	EVs stored at —80C following analysis	Diagnosis	Protein	TMEM256, flotillin 2, Rab3B, PARK7, LAMTOR1	Candidate screening	Flotillin 2: 88% Flotillin 2 and PARK7: 68%	Flotillin 2: 94% Flotillin 2 and PARK7: 93%	Flotillin 2: 0.91	Wang L, Skotland T, Berge V, Sandvig K and Llorente A. Exosomal proteins as prostate cancer biomarkers in urine: From mass spectrometry discovery to immunoassay-based

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Table 1 (*continued*)

Disease	No. of Patients	No. of Controls	Source of EV	Isolation Techniques	EV Characterisation	Storage	Clinical Application / Role of Cargo	Cargo Generic	Cargo Specific	Cargo Identification	Sensitivity	Specificity	ROC- Area Under Curve	Reference
														validation. European Journal of Pharmaceutical Sciences. 2017:98;80–85.
Prostate Cancer	24	23	Urine	Centrifugation	TEM, Western Blot	Urine supernatant stored at —80C before EV isolation	Diagnosis, Prognosis	Protein	11 proteins including: FABP5, Granulin, AMBP, CHMP4A, CHMP4C	High throughput screening	FABP5: 60%	FABP5: 100%	FABP5: 0.856	Fujita K, Kume H, Matsuzaki K, Kawashima A, Ujike T, Nagahara A, Uemura M, Miyagawa Y, Tomonaga T and Nonomura N. Proteomic analysis of urinary extracellular vesicles from high Gleason score prostate cancer. Scientific Reports. 2017:7;42,961.
Prostate Cancer	24	15	Cell Lines, urine	Centrifugation, Size exclusion filtration	TEM, Western Blot	Urine stored at 4C until processed	Diagnosis	RNA	AGR2 SV-G, AGR2 wt, AGR2 SV-G	Candidate screening	–	–	AGR2 SV-H: 0.96 AGR2 SV-G: 0.94 AGR2 wt: 0.91	Neeb A, Hefele S, Bormann S, Parson W, Adams F, Wolf P, Miernik A, Schoenthaler M, Kroenig M, Wilhelm K, et al. Splice variant transcripts of the anterior gradient 2 gene as a marker of prostate cancer. Oncotarget. 2014:5.
Prostate Cancer	47	39	Urine	Size exclusion filtration, Centrifugation	None	Urine stored at 4C prior to EV isolation	Diagnosis	RNA	TMPRSS2:ERG, BIRC5, ERG, PCA3, TMPRSS2	Candidate screening	–	–	BIRC5: 0.674 ERG: 0.785 PCA3: 0.681 TMPRSS2: 0.637 TMPRSS2:ERG: 0.744	Motamedinia P, Scott AN, Bate KL, Sadeghi N, Salazar G, Shapiro E, Ahn J, Lipsky M, Lin J, Hruby GW, et al. Urine Exosomes for Non-Invasive Assessment of Gene Expression and Mutations of Prostate Cancer. PLOS ONE. 2016:11;e0154507.

Abbreviations
ATM - Atomic force microscopy
DSL - Dynamic light scattering
CyroEM - Cryo electron microscopy.
TEM – Transmission electronic microscopy.
NTA – Nanoparticle tracking analysis.

Table 2
Extracellular vesicle cargo and known functions.

Disease	No. of Patients	No. of Controls	Source of EV	Isolation Techniques	EV Characterisation	Storage	Clinical Application / Role of Cargo	Cargo Generic	Cargo Specific	Reference
Renal Cancer	71	0	Plasma	Centrifugation, Size exclusion filtration	TEM, NTA, Western Blot	Plasma frozen —80C pre-analysis	Drug Resistance	lncRNA	lncARSR - lncRNA Activated in RCC with Sunitinib Resistance	Qu L, Ding J, Chen C, Wu ZJ, Liu B, Gao Y, Chen W, Liu F, Sun W, Li XF, et al. Exosome-Transmitted lncARSR Promotes Sunitinib Resistance in Renal Cancer by Acting as a Competing Endogenous RNA. *Cancer Cell*. 2016:29;653–668.
Renal Cancer	–	–	Cell Line	Centrifugation, concentration	Western Blot	Isolated EVs stored at -80C	Proliferation	Protein	HepaCAM	Jiang X, Zhang Y, Tan B, Luo C and Wu X. Renal tumour-derived exosomes inhibit hepaCAM expression of renal carcinoma cells in ap-AKT-dependent manner. *Neoplasma*. 2014:61;416.
Renal Cancer	–	–	Cell Line	Centrifugation, Size exclusion filtration, Density graded centrifugation, Immuno-affinity beads	Western Blot	None	Angiogenesis	Protein	Carbonic anhydrase IX	Horie K, Kawakami K, Fujita Y, Sugaya M, Kameyama K, Mizutani K, Deguchi T and Ito M. Exosomes expressing carbonic anhydrase 9 promote angiogenesis. *Biochemical and biophysical research communications*. 2017:492;356–361.
Renal Cancer	–	–	Cell Line	Centrifugation, Size exclusion concentration, Density graded centrifugation	TEM, Western Blot	None	Cell migration	Protein	CXCR4 & MM9	Chen G, Zhang Y and Wu X. 786–0 Renal cancer cell line-derived exosomes promote 786–0 cell migration and invasion in vitro. *Oncology letters*. 2014:7;1576–1580.
Renal Cancer	–	–	Cell Line	Centrifugation	TEM, size and zeta potential assessment	None	Angiogenesis and development of the pre-metastatic niche	mRNA, miRNA	miR-29a, miR-650, miR-15, miR-19b, miR-29c, miR-151	Grange C, Tapparo M, Collino F, Vitillo L, Damasco C, Deregibus MC, Tetta C, Bussolati B and Camussi G. Microvesicles released from human renal cancer stem cells stimulate angiogenesis and formation of lung premetastatic niche. *Cancer research*. 2011:71;5346–5356.
Renal Cancer	–	–	Cell Line	Centrifugation	Flow cytometry, NTA	Isolated EVs stored at -80C	Immune system modulation	Protein	HLA-G	Grange C, Tapparo M, Tritta S, Deregibus MC, Battaglia A, Gontero P, Frea B and Camussi G. Role of HLA-G and extracellular vesicles in renal cancer stem cell-induced inhibition of dendritic cell differentiation. *BMC cancer*. 2015:15;1–11.
Renal Cancer	36	36	Plasma	Centrifugation	TEM, Western Blot	None	Immune system modulation	Protein	TGF-β1	Xia Y, Zhang Q, Zhen Q, Zhao Y, Liu N, Li T, Hao Y, Zhang Y, Luo C and Wu X. Negative regulation of tumour-infiltrating NK cell in clear cell renal cell carcinoma patients through the exosome pathway. *Oncotarget*. 2017:8;37,783.

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Disease	No. of Patients	No. of Controls	Source of EV	Isolation Techniques	EV Characterisation	Storage	Clinical Application / Role of Cargo	Cargo Generic	Cargo Specific	Reference
Renal Cancer	29	23	Urine	Centrifugation, Density graded centrifugation	TEM, Western Blot	Urine frozen —80C pre-analysis and EVs frozen at -80 after isolation	Diagnosis	Protein	MMP9, CP, PODXL, DKK4, CAIX, AQP1, EMMPRIN, CD10, dipeptidase 1, syntenin-1	Raimondo F, Morosi L, Corbetta S, Chinello C, Brambilla P, Della Mina P, Villa A, Albo G, Battaglia C, Bosari S, et al. Differential protein profiling of renal cell carcinoma urinary exosomes. *Mol Biosyst*. 2013:9;1220–33.
Renal Cancer	8	8	Urine	Centrifugation, Size exclusion concentration	Western Blot	EVs frozen at -80 after isolation	Diagnosis	Lipids	Various Lipids	Del Boccio P, Raimondo F, Pieragostino D, Morosi L, Cozzi G, Sacchetta P, Magni F, Pitto M and Urbani A. A hyphenated microLC-Q-TOF-MS platform for exosome lipidomics investigations: application to RCC urinary exosomes. *Electrophoresis*. 2012:33;689–96.
Renal Cancer	–	–	Cell Line	Centrifugation, Size exclusion concentration, Density graded centrifugation	TEM	EVs frozen at -80 after isolation	Angiogenesis	mRNA	mRNA regulating VEGF expression	Zhang L, Wu X, Luo C, Chen X, Yang L, Tao J and Shi J. The 786–0 renal cancer cell-derived exosomes promote angiogenesis by downregulating the expression of hepatocyte cell adhesion molecule. *Mol Med Rep*. 2013:8;272–276.
Renal Cancer	33	22	Urine	Centrifugation, Size exclusion concentration	None	None	Transcription, Metabolism	esRNA	GSTA1, CEBPA and PCBD1	De Palma G, Sallustio F, Curci C, Galleggiante V, Rutigliano M, Serino G, Ditonno P, Battaglia M and Schena FP. The Three-Gene Signature in Urinary Extracellular Vesicles from Patients with Clear Cell Renal Cell Carcinoma. J Cancer. 2016:7;1960–1967.
Bladder Cancer	–	–	Cell Line	Centrifugation, Size exclusion filtration, Density graded centrifugation	TEM	None	Cell Migration, Angiogenesis	Protein	EDIL-3	Beckham CJ, Olsen J, Yin PN, Wu CH, Ting HJ, Hagen FK, Scosyrev E, Messing EM and Lee YF. Bladder cancer exosomes contain EDIL-3/Del1 and facilitate cancer progression. *J Urol*. 2014:192;583–92.
Bladder Cancer	–	–	Cell Line, Urine	Centrifugation, Density graded centrifugation	TEM, Flow cytometry, Western Blot	EVs frozen at -80 after isolation	Various functions including: Diagnosis & Cell Adhesion	Protein	353 proteins	Welton JL, Khanna S, Giles PJ, Brennan P, Brewis IA, Staffurth J, Mason MD and Clayton A. Proteomics analysis of bladder cancer exosomes. *Mol Cell Proteomics*. 2010:9;1324–38.
Bladder Cancer	–	–	Cell Line	Centrifugation	NTA, Western Blot	None	Tumour suppression	miRNA	miR23b, miR921, mmiR224	Ostenfeld MS, Jeppesen DK, Laurberg JR, Boysen AT, Bramsen JB, Primdal-Bengtson B, Hendrix A, Lamy P, Dagnaes-Hansen F, Rasmussen MH, et al. Cellular disposal of miR23b by RAB27-dependent exosome release is linked to acquisition of metastatic

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Disease	No. of Patients	No. of Controls	Source of EV	Isolation Techniques	EV Characterisation	Storage	Clinical Application / Role of Cargo	Cargo Generic	Cargo Specific	Reference
										properties. *Cancer Res*. 2014:74;5758–71.
Bladder Cancer	27	0	Urine, Plasma, Tumour	Centrifugation, Commercial EV isolation kit	None	Plasma frozen —80C pre-analysis, urine supernatant frozen —80C pre-analysis	Diagnosis	miRNA	miR 21, miR4454, miR720, miR205-5p, miR200c-3p, miR200-3p, miR21-5p, miR29b-3q, miR548ai, miR548aa, miR223, miR338-3p, miR378e, miR548n, miR1290, miR16, miR451a, let-7a-5p, let-7b-5p	Armstrong DA, Green BB, Seigne JD, Schned AR and Marsit CJ. MicroRNA molecular profiling from matched tumour and bio-fluids in bladder cancer. *Molecular Cancer*. 2015:14;194.
Bladder Cancer	–	–	Cell Line, Urine	Centrifugation, Commercial EV isolation kit	NTA, Western Blot, TEM	None	Prognosis	lncRNA	HOTAIR	Berrondo C, Flax J, Kucherov V, Siebert A, Osinski T, Rosenberg A, Fucile C, Richheimer S and Beckham CJ. Expression of the Long Non-Coding RNA HOTAIR Correlates with Disease Progression in Bladder Cancer and Is Contained in Bladder Cancer Patient Urinary Exosomes. *PLoS One*. 2016:11; e0147236.
Bladder Cancer	21	–	Urine, Cell Lines	Centrifugation, Commercial EV isolation kit	TEM, Western Blot	Urine supernatant stored at —80C before EV isolation	Diagnosis, Prognosis	miRNA	miR-200-3p	Baumgart S, Hölters S, Ohlmann CH, Bohle R, Stöckle M, Ostenfeld MS, Dyrskjøt L, Junker K and Heinzelmann J. Exosomes of invasive urothelial carcinoma cells are characterised by a specific miRNA expression signature. *Oncotarget*. 2017:8;58,278–58,291.
Bladder Cancer	34	9	Urine, Cell Lines	Centrifugation, Size exclusion filtration	NTA, TEM, Western Blot	None	Diagnosis, Prognosis	miRNA	miR-375 miR-let7c miR-194 miR-146a miR-30c-2	Andreu Z, Otta Oshiro R, Redruello A, López-Martín S, Gutiérrez-Vázquez C, Morato E, Marina AI, Olivier Gómez C and Yáñez-Mó M. Extracellular vesicles as a source for non-invasive biomarkers in bladder cancer progression. Eur J Pharm Sci. 2017:98;70–79.
Bladder Cancer	26	0	Urine	None	None	None	Diagnosis, Proliferation, Migration, Invasive Phenotype	miRNA	miR-141-3p miR-146b-5p miR-200a-3p miR-200b-3p	Baumgart S, Meschkat P, Edelmann P, Hartmann A, Bohle R, Pryalukhin A, Heinzelmann J, Stöckle M and Junker K. Invasion-associated miRNAs S as possible diagnostic biomarkers of muscle invasive bladder cancer in tumour tissues and urinary exosomes. *Journal of Urology*. 2018:199;e1038-e1038.
Bladder Cancer	89	50	Urine, Serum	Centrifugation	TEM, Western Blot	Urine and serum snap frozen in liquid nitrogen prior to EV isolation.	Clinical Staging, Survival	circRNA	circPRMT5	Chen X, Chen R-X, Wei W—S, Li Y—H, Feng *Z*-H, Tan L, Chen J-W, Yuan G-J, Chen S-L, Guo S-J, et al. PRMT5 Circular RNA Promotes Metastasis of Urothelial Carcinoma of the Bladder through Sponging miR-30c to Induce

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Table 2 (*continued*)

Disease	No. of Patients	No. of Controls	Source of EV	Isolation Techniques	EV Characterisation	Storage	Clinical Application / Role of Cargo	Cargo Generic	Cargo Specific	Reference
										Epithelial–Mesenchymal Transition. *Clinical Cancer Research*. 2018:24;6319–6330.
Bladder Cancer	–	–	Cell Line	Centrifugation, Size exclusion filtration, Commercial EV isolation kit	TEM, NTA, Western Blot	None	Proliferation, Migration, Invasion	lncRNA	lncRNA-UCA1	Xue M, Chen W, Xiang A, Wang R, Chen H, Pan J, Pang H, An H, Wang X, Hou H, et al. Hypoxic exosomes facilitate bladder tumour growth and development through transferring long non-coding RNA-UCA1. Mol Cancer. 2017:16;143.
Bladder Cancer	–	–	Cell Line	Centrifugation	Western Blot	None	Development of the pre-metastatic niche	Protein	ErbB2, CRK	Yoshida K, Tsuda M, Matsumoto R, Semba S, Wang L, Sugino H, Tanino M, Kondo T, Tanabe K and Tanaka S. Exosomes containing ErbB2/CRK induce vascular growth in premetastatic niches and promote metastasis of bladder cancer. Cancer Sci. 2019:110;2119–2132.
Bladder Cancer	8	11	Urine	Centrifugation, Size exclusion filtration	CryoEM, NTA,	Urine supernatant stored at —80C before EV isolation	Diagnosis	mRNA	LASS2, GALNT1, ARHGEF39, FOXO3	Perez A, Loizaga A, Arceo R, Lacasa I, Rabade A, Zorroza K, Mosen-Ansorena D, Gonzalez E, Aransay AM, Falcon-Perez JM, et al. A Pilot Study on the Potential of RNA-Associated to Urinary Vesicles as a Suitable Non-Invasive Source for Diagnostic Purposes in Bladder Cancer. Cancers (Basel). 2014:6;179–92.
Bladder Cancer	46	–	Urine	Centrifugation, Commercial EV isolation kit	None	Urine supernatant stored at 4C prior to EV isolation	Diagnosis	miRNA	miR-141-3p, miR-141-3p	Poli G, Egidi MG, Cochetti G, Brancorsini S and Mearini E. Relationship between cellular and exosomal miRNAs targeting NOD-like receptors in bladder cancer: preliminary results. *Minerva Urol Nefrol*. 2020:72;207–213.
Bladder Cancer	6	5	Urine, Plasma	Centrifugation	Western Blot	Urine supernatant stored at —80C before EV isolation, Urine micro pellet frozen at —80C prior to analysis	Diagnosis	Protein	Resistin, GTPase NRas, EPS8L2, Mucin 4, EPS8L1, Retinoic acid-induced protein 3, Alpha subunit of GsGTP binding protein, EH-domain-containing protein 4, Galectin-3-binding protein	Smalley DM, Sheman NE, Nelson K and Theodorescu D. Isolation and Identification of Potential Urinary Microparticle Biomarkers of Bladder Cancer. Journal of Proteome Research. 2008:7;2088–2096.
Bladder Cancer	–	–	Urine, Cell Line	Centrifugation	NTA, Western Blot	Urine supernatant stored at —80C before EV isolation	Invasive disease	Protein	Periostin	Silvers CR, Liu Y-R, Wu C—H, Miyamoto H, Messing EM and Lee Y—F. Identification of extracellular vesicle-borne periostin as a feature of muscle-invasive bladder cancer. Oncotarget. 2016:7.
Bladder Cancer	6	6	Urine	Centrifugation	TEM, NTA, Western Blot	Cell culture & urine supernatant stored at —80C prior to EV isolation	Cell membrane, extracellular matrix, inflammation &	Protein, mRNA	HEXB, S100A4, SND1, TALD01, EHd4	Silvers CR, Miyamoto H, Messing EM, Netto GJ and Lee Y—F. Characterisation of urinary extracellular vesicle proteins in

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Table 2 (*continued*)

Disease	No. of Patients	No. of Controls	Source of EV	Isolation Techniques	EV Characterisation	Storage	Clinical Application / Role of Cargo	Cargo Generic	Cargo Specific	Reference
							angiogenesis signalling pathways			muscle-invasive bladder cancer. Oncotarget. 2017:8.
Bladder Cancer	34	9	Urine	Size exclusion filtration, Centrifugation	TEM, NTA, Western Blot	None	Diagnosis, Prognosis	miRNA	miR-375, miR-146a, apoB	Andreu Z, Otta Oshiro R, Redruello A, López-Martín S, Gutiérrez-Vázquez C, Morato E, Marina AI, Olivier Gómez C and Yáñez-Mó M. Extracellular vesicles as a source for non-invasive biomarkers in bladder cancer progression. European Journal of Pharmaceutical Sciences. 2017:98;70–79.
Prostate Cancer	12	–	Tissue from metastatic disease	Tissue homogenisation, centrifugation	None	Tissue frozen prior to EV isolation	Diagnosis, Disease Progression, Angiogenesis	Protein	25 proteins including: annexin A1, A3, A5, DDAH 1	Ronquist KG, Ronquist G, Larsson A and Carlsson L. Proteomic analysis of prostate cancer metastasis-derived prostasomes. Anticancer Research. 2010:30;285–90.
Prostate Cancer	8	5	Cell Lines, Urine	Centrifugation	Western Blot	Urine supernatant stored at —80C before EV isolation	Diagnosis, Cell Adhesion, Cell Motility	Protein	ITGA3, ITGB1	Bijnsdorp IV, Geldof AA, Lavaei M, Piersma SR, van Moorselaar RJ and Jimenez CR. Exosomal ITGA3 interferes with non-cancerous prostate cell functions and is increased in urine exosomes of metastatic prostate cancer patients. J Extracell Vesicles. 2013:2.
Prostate Cancer	90	50	Urine	Centrifugation & commercial EV precipitation kit	SEM, Western Blot	None	Diagnosis	miRNA	miR-2909	Wani S, Kaul D, Mavuduru RS, Kakkar N and Bhatia A. Urinary-exosome miR-2909: A novel pathognomonic trait of prostate cancer severity. *J Biotechnol*. 2017:259;135–139.
Prostate Cancer	–	–	Cell Lines	Centrifugation, Density graded centrifugation	TEM, Western Blot	None	Diagnosis	Protein	ALIX, FASN, XPO1, ENO1	Duijvesz D, Burnum-Johnson KE, Gritsenko MA, Hoogland AM, Vredenbregt-van den Berg MS, Willemsen R, Luider T, Paša-Tolić L and Jenster G. Proteomic profiling of exosomes leads to the identification of novel biomarkers for prostate cancer. *PLoS One*. 2013:8;e82589.
Prostate Cancer	–	–	Cell Lines	Centrifugation	TEM, NTA, Western Blot	None	Cell Differentiation	Protein	Ets1, PTHrP	Itoh T, Ito Y, Ohtsuki Y, Ando M, Tsukamasa Y, Yamada N, Naoe T and Akao Y. Microvesicles released from hormone-refractory prostate cancer cells facilitate mouse pre-osteoblast differentiation. *J Mol Histol*. 2012:43;509–15.
Prostate Cancer	11	0	Urine	Centrifugation, Density graded centrifugation	Immunoelectron microscopy	None	Diagnosis, Disease Progression	miRNA	PCA3, ERG	Nilsson J, Skog J, Nordstrand A, Baranov V, Mincheva-Nilsson L, Breakefield XO and Widmark A. Prostate cancer-derived urine exosomes: a novel approach to

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Table 2 (*continued*)

Disease	No. of Patients	No. of Controls	Source of EV	Isolation Techniques	EV Characterisation	Storage	Clinical Application / Role of Cargo	Cargo Generic	Cargo Specific	Reference
										biomarkers for prostate cancer. Br J Cancer. 2009:100;1603–7.
Prostate Cancer	10	0	Cell Lines, Serum	Centrifugation	Western Blot	Serum supernatant stored at —80C before EV isolation, EVs stored at —20C before characterisation	Prediction of Chemotherapy Response	Protein	P-glycoprotein	Kato T, Mizutani K, Kameyama K, Kawakami K, Fujita Y, Nakane K, Kanimoto Y, Ehara H, Ito H, Seishima M, et al. Serum exosomal P-glycoprotein is a potential marker to diagnose docetaxel resistance and select a taxoid for patients with prostate cancer. *Urologic Oncology: Seminars and Original Investigations*. 2015:33;385.e15–385.e20.
Prostate Cancer	–	–	Cell Lines, Plasma	Centrifugation, Density graded centrifugation, commercial EV precipitation kit	TEM, Flow cytometry, Western Blot	EVs stored at —80C prior to analysis	Diagnosis, Tumour Progression, Anti-apoptosis, Cell Proliferation, Chemotherapy Resistance, Cell Migration, Angiogenesis	Protein	103 proteins, differentially expressed	Minciacchi VR, You S, Spinelli C, Morley S, Zandian M, Aspuria P-J, Cavallini L, Ciardiello C, Sobreiro MR, Morello M, et al. Large oncosomes contain distinct protein cargo and represent a separate functional class of tumour-derived extracellular vesicles. *Oncotarget*. 2015:6.
Prostate Cancer	70	51	Urine	Centrifugation, Size exclusion filtration	TEM, NTA, Western Blot	Urine supernatant stored at —80C before EV analysis	Diagnosis and Tumour Suppressive Effects	mRNA	CMTM3, CDH3	Royo F, Zuñiga-Garcia P, Torrano V, Loizaga A, Sanchez-Mosquera P, Ugalde-Olano A, González E, Cortazar AR, Palomo L, Fernández-Ruiz S, et al. Transcriptomic profiling of urine extracellular vesicles reveals alterations of CDH3 in prostate cancer. *Oncotarget*. 2016:7;6835–46.
Prostate Cancer	47	16	Plasma, Serum	Centrifugation, Commercial EV precipitation kit	Protein levels, Western Blot	Plasma cryopreserved prior to EV isolation, EVS stored at —80C prior to analysis	Diagnosis, Prognosis	Protein	Survivin	Khan S, Jutzy JMS, Valenzuela MMA, Turay D, Aspe JR, Ashok A, Mirshahidi S, Mercola D, Lilly MB and Wall NR. Plasma-Derived Exosomal Survivin, a Plausible Biomarker for Early Detection of Prostate Cancer. *PLOS ONE*. 2012:7; e46737.
Prostate Cancer	36	0	Plasma	Centrifugation & Commercial EV precipitation kit	None	Plasma supernatant stored at —80C before EV isolation	Resistance to Hormone Treatment	mRNA	Androgen receptor splice variant	Del Re M, Biasco E, Crucitta S, Derosa L, Rofi E, Orlandini C, Miccoli M, Galli L, Falcone A, Jenster GW, et al. The Detection of Androgen Receptor Splice Variant 7 in Plasma-derived Exosomal RNA Strongly Predicts Resistance to Hormonal Therapy in Metastatic Prostate Cancer Patients. Eur Urol. 2017:71;680–687.
Prostate Cancer	15	30	Plasma, Cell Lines	Centrifugation, Size exclusion filtration	NTA, Flow Cytometry	Plasma stored at —80C before EV isolation	Screening and Diagnosis	Protein	PSA	Logozzi M, Angelini DF, Iessi E, Mizzoni D, Di Raimo R, Federici C, Lugini L, Borsellino G, Gentilucci A, Pierella F, et al. Increased PSA

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Table 2 (*continued*)

Disease	No. of Patients	No. of Controls	Source of EV	Isolation Techniques	EV Characterisation	Storage	Clinical Application / Role of Cargo	Cargo Generic	Cargo Specific	Reference
										expression on prostate cancer exosomes in in vitro condition and in cancer patients. *Cancer Lett.* 2017:403;318–329.
Prostate Cancer	31	8	Serum, Cell Lines	Centrifugation, Size exclusion filtration, Immuno-affinity beads, size exclusion chromatography, Density graded centrifugation	Western Blot	EVs stored at —80C prior to analysis, Serum supernatant stored at —80C prior to EV isolation	Diagnosis	Protein	Gamma-glutamyltransferase 1	Kawakami K, Fujita Y, Matsuda Y, Arai T, Horie K, Kameyama K, Kato T, Masunaga K, Kasuya Y, Tanaka M, et al. Gamma-glutamyltransferase activity in exosomes as a potential marker for prostate cancer. BMC Cancer. 2017:17;316.
Prostate Cancer	31	14	Urine	Centrifugation, Size exclusion filtration	NTA, CryoEM	Urine supernatant stored at —80C before EV analysis	Disease monitoring	Metabolites	76 individual metabolites with differential abundance between prostate cancer a BPH	Clos-Garcia M, Loizaga-Iriarte A, Zuñiga-Garcia P, Sánchez-Mosquera P, Rosa Cortazar A, González E, Torrano V, Alonso C, Pérez-Cormenzana M, Ugalde-Olano A, et al. Metabolic alterations in urine extracellular vesicles are associated to prostate cancer pathogenesis and progression. J Extracell Vesicles. 2018:7;1,470,442.
Prostate Cancer	–	–	Cell Lines	Commercial EV precipitation kit	TEM, Western Blot	None	Cell Proliferation, Invasion	cicrRNA	circ_SLC19A1	Zheng Y, Li J-x, Chen C-j, Lin Z-y, Liu J-x and Lin F-j. Extracellular vesicle-derived circ_SLC19A1 promotes prostate cancer cell growth and invasion through the miR-497/septin 2 pathway. Cell Biology International. 2020:44;1037–1045.
Prostate Cancer	3	3	Urine	Centrifugation, Size exclusion filtration	TEM, Western Blot, NTA,	Urine stored at 4C prior to centrifugation and supernatant frozen in LN, isolated EVs stored at -80C	Diagnosis	Metabolites	11 metabolites specific to urinary EVs	Puhka M, Takatalo M, Nordberg ME, Valkonen S, Nandania J, Aatonen M, Yliperttula M, Laitinen S, Velagapudi V, Mirtti T, et al. Metabolomic Profiling of Extracellular Vesicles and Alternative Normalization Methods Reveal Enriched Metabolites and Strategies to Study Prostate Cancer-Related Changes. Theranostics. 2017:7;3824–3841.
Prostate Cancer	22	23	Urine, Tumour conditioned medium	Density graded centrifugation, Size based concentration, Size exclusion chromatography, commercial precipitation kit	NTA, TEM, Western Blot	Urine supernatant stored at —80C, EV pellets stored at —80C prior to analysis	Diagnosis, protein synthesis, nucleic acid synthesis, autophagy, immune system activation	Protein	705 differentially EV enriched proteins including HRAS, AKT1, CUL3, NKX3–1, PTEN	Dhondt B, Geeurickx E, Tulkens J, Van Deun J, Vergauwen G, Lippens L, Miinalainen I, Rappu P, Heino J, Ost P, et al. Unravelling the proteomic landscape of extracellular vesicles in prostate cancer by density-based fractionation of urine. Journal of Extracellular Vesicles. 2020:9;1,736,935.

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Table 2 (*continued*)

Disease	No. of Patients	No. of Controls	Source of EV	Isolation Techniques	EV Characterisation	Storage	Clinical Application / Role of Cargo	Cargo Generic	Cargo Specific	Reference
Prostate Cancer	16	15	Cell Lines, Urine	Centrifugation	Western Blot, TEM	None	Transcription, Diagnosis	Protein	Catenin	Lu Q, Zhang J, Allison R, Gay H, Yang W-X, Bhowmick NA, Frelix G, Shappell S and Chen Y—H. Identification of extracellular δ-catenin accumulation for prostate cancer detection. *The Prostate*. 2009:69;411–418.
Prostate Cancer	10	10	Cell Lines, Urine	Size exclusion filtration, Density graded centrifugation	Western Blot	None	Diagnosis, Disease Monitoring	Protein	PSA, PSMA, 5 T4	Mitchell PJ, Welton J, Staffurth J, Court J, Mason MD, Tabi Z and Clayton A. Can urinary exosomes act as treatment response markers in prostate cancer? Journal of Translational Medicine. 2009:7;4.
Prostate Cancer	–	–	Urine, plasma	Size exclusion filtration, size exclusion chromatography, centrifugation, Cryo-EM	NTA, Western Blot, ELISA	Urine supernatant and platelet free plasma stored at —80C before EV isolation	Prognosis	Protein	Differentia expression of 643 proteins including: protein S, kininogen-1, insulin-like binding proteins, Afamin, cardiotrophin-1	Welton JL, Brennan P, Gurney M, Webber JP, Spary LK, Carton DG, Falcón-Pérez JM, Walton SP, Mason MD, Tabi Z, et al. Proteomics analysis of vesicles isolated from plasma and urine of prostate cancer patients using a multiplex, aptamer-based protein array. Journal of Extracellular Vesicles. 2016:5;31,209.
Prostate Cancer	29	–	Urine	Centrifugation, Size exclusion filtration	None	Urine stored at 4C prior to EV isolation, EVs stroed at —70C following isolation	Diagnosis	mRNA	PCA3, ERG	Hendriks RJ, Dijkstra S, Jannink SA, Steffens MG, van Oort IM, Mulders PFA and Schalken JA. Comparative analysis of prostate cancer specific biomarkers PCA3 and ERG in whole urine, urinary sediments and exosomes. Clinical Chemistry and Laboratory Medicine (CCLM). 2016:54;483–492.
Prostate Cancer	46	17	Urine	Size exclusion filtration, Centrifugation	TEM	Urine supernatant stored at —80C before EV isolation	Diagnosis	RNA	PCA3, ERG	Pellegrini KL, Patil D, Douglas KJS, Lee G, Wehrmeyer K, Torlak M, Clark J, Cooper CS, Moreno CS and Sanda MG. Detection of prostate cancer-specific transcripts in extracellular vesicles isolated from post-DRE urine. The Prostate. 2017:77;990–999.
Prostate Cancer	4	4	Urine	Centrifugation, field flow fractionation	TEM, Western Blot	Urine stored at —80C prior to EV isolation	Diagnosis	Lipids	22:6/22:6-phosphatidylglycerol 16:0, 16:0 - diacylglycerol 16:1, 18:1-diacylglycerol Triacylglycerol species	Yang JS, Lee JC, Byeon SK, Rha KH and Moon MH. Size Dependent Lipidomic Analysis of Urinary Exosomes from Patients with Prostate Cancer by Flow Field-Flow Fractionation and Nanoflow Liquid Chromatography-Tandem Mass Spectrometry. Analytical Chemistry. 2017:89;2488–2496.

Abbreviations
ATM - Atomic force microscopy
DSL - Dynamic light scattering
CyroEM - Cryo electron microscopy.
TEM – Transmission electronic microscopy.
NTA – Nanoparticle tracking analysis.

3. Evidence synthesis

3.1. Renal cancer

EVs released from the kidney both in normal physiology, and in renal cancer (RC), have been identified in both plasma and urine [96]. Primary renal cancer is a heterogeneous spectrum of malignancies and accounts for around 90% of all kidney malignancies. Renal cancer preferentially metastasises to the lungs, bone, brain and lymph nodes, suggesting a potential role of EVs in signalling to distant tissues to prepare and maintain the metastatic niche [97–99].

3.2. RNA in renal cancer EVs

Although EV isolation from urine represents a truly non-invasive biopsy, one limitation of examining urinary EVs is the challenge in identifying their tissue of origin and many studies report on the isolation of EVs from plasma and serum. Despite this, urinary and serum RC EVs carry microRNAs capable of diagnosing RC with high levels of sensitivity and specificity (Tables 1 and 2) [100]. Further interrogation of their cargoes has identified numerous other micro-RNAs, lncRNAs, lipids and proteins with promising potential as biomarkers. MicroRNA cargoes with promising clinical diagnostic applications include miR-1233–1, the pro-angiogenic miR-210 and miR-224 with known roles in cell proliferation and migration [89,101–107]. Comparing micro-RNA EVs cargo from RC patients to healthy controls demonstrated the ability to distinguish RC patients with a sensitivity and specificity of 70% and 62.2% respectively for miR-210 and 81% and 76% respectively for miR-1233–1 [101]. Following tumour resection in a subset of 10 patients, 7-days post operatively, significantly lower exosomal levels of miR-210 and miR-1233 where found, $p < 0.01$ [101]. Although many micro-RNAs identified are upregulated in other diseases, miR-1233–1 has yet to be identified in pathologies other than RC. Similar findings were reported by Wang et al. outlining diagnostic potential for miR-210, with a sensitivity and specificity of 82.5% and 80% respectively, again reporting a reduction in serum exosomal miR-210 at 1, 4 and 12 weeks post-operatively [102].

To increase diagnostic efficacy, the use of micro-RNA combinations have been studied. Of particular interest, is miR-126-3p in combination with either miR-449a or miR-34b-5p, with an Area Under the Curve (AUC) of 0.84 and 0.79 respectively, in discriminating between RC patients and healthy controls [103]. Micro-RNAs have found utility in diagnostics and also survival prediction; in particular miR-let-7i-5p, combined with clinical and biochemical features, discriminates between patients with a survival of 14 vs 39 months, AUC 0.64 [89].

Similar findings and diagnostic accuracy have been reported following examination of the RNA cargoes of plasma EVs, notably micro-RNA miR-224, whose known cellular functions include cellular proliferation and migration [101,104]. Higher levels of EV miR-224 have been associated with poorer clinical outcomes in RC patients, and may have future prognostic biomarker applicability [104]. Other RNAs of interest in RC include lncARSR, a lncRNA associated with a poor response to the tyrosine kinase inhibitor sunitinib, which is used in treatment of RC. Intracellular exchange of lncARSR via EVs leads to upregulation of AXL and c-MET receptors, both of which positively corelate with sunitinib resistance [105].

In addition to miRNA cargos, EV-incorporated mRNA has been studied for its diagnostic biomarker potential. Three genes in particular were identified with lower urinary EV expression compared to healthy controls. The known functions of these genes GSTA1, CEBPA and PCBD1 include transcription and metabolism and warrant further investigation including validation in large patient cohorts [108]. Following radical nephrectomy, levels of GSTA1, CEBPA and PCBD1 within urinary EVs increased and were found to be similar to health controls, however, pointing to their release from malignant tissues [108]. Work by Zhang et al. identified tumour cell derived EVs co-culturered with human umbilical vein endothelial cells (HUVEC) showed increased vascular endothelial growth factor mRNA and protein expression, this in turn increased tubular formation on within HUVEC, which in vivo may promote angiogenesis [107]. Although this work was carried out in cell lines and validation of this finding in a patient population has yet to be undertaken.

3.3. Protein in renal cancer EVs

Despite substantial work to characterise RNA cargoes of EVs, proteins have also been studied revealing numerous biological roles of the identified cargoes. Raimondo et al. reported numerous protein cargoes within urinary EVs including matrix metallopeptidase-9 (MMP-9), podocalyxin-like *protein* 1 (PODXL), carbonic anhydrase IX (CAIX) and syntenin-1 [109]. Although their differential expression makes them potential biomarkers for diagnosis, validation in large patient cohorts is still required.

Interrogation of EVs released from renal cancer cell lines has also led to the identification of numerous cargoes. The RC cell line, OS-RC-2 mediate reduced expression of the tumour suppressor hepatic and glial cell adhesion molecule, which in turn causes a reciprocal increase in cell proliferation and disease progression [110]. In addition to RNA, RC EVs also transport proteins such as CAIX, a cellular hypoxia marker and common feature of the tumour microenvironment, which induces hypoxia inducible factor 1 (HIF1) [111]. CAIX is overexpressed in several cancers and in vitro studies have demonstrated it promotes endothelial tube formation, a prerequisite for angiogenesis [111]. Our understanding of the role of EVs in RC metastasis is generally limited. However, matrix MMP-9 and chemokine receptor CXCR-4 were both upregulated in the RC cell line 786-O following co-culture with EVs derived from the same malignant cell line [112]. This finding is suggestive of 'closed loop' communication between cells, driving malignant change in adjacent cells, through EVs. In addition to local changes, EVs promote development of the pre-metastatic niche. RC derived EVs from human CD105 positive cancer stem cells have pro-angiogenic RNA cargo when studied in vitro. Administration of these EVs lead to enhanced development of lung metastasis in a mouse model, when renal cancer cells were injected at a later time point [63].

As well as the direct action of EVs on local cells within the renal parenchyma or sites of metastatic spread, RC derived EVs also interact with the immune system, the modulation of which is an important feature of cancer progression. Impaired maturation of monocyte derived dendritic cells and T cell activation is mediated through transfer of HLA-G to these cells [113]. Dendritic cell infiltration into developing tumours results in a significant immune response leading to reduced progression of disease. Conversely, inhibition of dendritic call maturation, ameliorates this immune response and allows the cancer to progress unhindered [114]. EVs derived from the renal cell adenocarcinoma cell line, ACHN, co-cultured with the immortalised T cell line, Jurkat, induced apoptosis in a dose dependent manner via the caspase pathway. EVs derived from ACHN cells carry Fas Ligand, a type-II transmembrane protein of the tumour necrosis factor (TNF) receptor family known to induce caspase activation and apoptosis. EVs treated with soluble Fas reduced EV mediated apoptosis in Jurkat T cells. Natural Killer (NK) cells form part of the innate immune system and have strong anti-tumorigenic properties through their ability to discriminate between self and cancerous ligands on the cell surface. This can be disrupted, however, by the EV-mediated immunosuppressive cytokine TGF- β. Following interaction between RC derived EVs and NK cells, NK cell function is disrupted, leading to reduced cytotoxic activity towards cancer cells [115].

3.4. Lipids in renal cancer EVs

Although less frequently reported on than RNAs and proteins, EV-incorporated lipid cargoes can be utilised in clinical applications. Del

Boccio et al. identified differences in the lipid components of urinary EVs between RC patients and healthy controls [106]. Although this work was undertaken in a clinical cohort, the small numbers of patients and controls ($n = 8$ in each group) means this work must be validated in a larger sample size prior to its use as a lipid-based diagnostic biomarker.

Together, this evidence demonstrates the significant role EVs play in RC, highlighting numerous opportunities to exploit them as novel diagnostic biomarkers and potentially to risk-stratify patients with RC in response to treatment and prognosis. Validation of these markers in large clinical cohorts has the potential to facilitate superior monitoring and guide treatment for individual patients, with greater accuracy than is currently achievable.

3.5. Bladder cancer

Bladder cancer is the most common malignancy of the urinary tract and diagnosed by cystoscopy and tissue biopsy, which are invasive and carries associated procedural risk to the patient [116]. Although urinary cytology has been explored as an alternative diagnostic tool, the variation in sensitivity has limited its clinical use. As such, there is an urgent unmet need for screening test to facilitate early disease detection and non-invasive diagnostic biomarkers to reduce the need for invasive testing.

One potential avenue for this is through interrogation of the EVs released from bladder cancer identified to be elevated in both plasma and urine, compared to healthy controls [117] (Tables 1 and 2).

EVs derived from bladder cancer cell lines confer tumorigenic changes in cells though a range of pathways inducing genome instability and increased invasiveness. Interrogation of their cargoes has led to the identification of several potential biomarkers [118]. Despite previous reports indicating that EVs return to their pre-malignant levels both in terms of composition and cargo [95], a persistent cancer phenotype was recently identified by Hiltbrunner et al. Over-expression of exosomal proteins regulating glycolysis and glycolytic shift were identified, in patients post cystectomy, both of which play important roles in cancer metabolism [119]; altered EV mediated glucose metabolism also being a known feature of the pre-metastatic niche [120]. This was in a small cohort of 13 patients however, and must be confirmed in larger cohorts and for different pathologies.

3.6. RNA in bladder cancer EVs

The lncRNA transcriptase antisense RNA, HOTAIR, has been identified within urinary EVs in patients with bladder cancer. HOTAIR facilitates tumour progression and is associated with poor prognosis and high recurrence rates in patients with invasive high-grade disease [121]. Epigenetic modification and silencing of miR-205 by HOTAIR leads to increased cell proliferation, migration and invasion of bladder cancer cell lines and represents a potential prognostic marker for disease activity and recurrence. Screening other candidate lncRNAs within EVs has identified metastasis-associated lung adenocarcinoma transcript 1, prostate cancer associated transcript-1 (PCAT-1) and sprouty receptor tyrosine kinase signalling antagonist 4-intronic transcript 1 as diagnostic and prognostic biomarkers [122]. Combining these markers has led to the development of a diagnostic panel with a sensitivity and specificity of 70.2% and 85.6% respectively in discriminating bladder cancer from controls [122].

As with RC, examination of urinary exosomes has revealed several candidate markers for prognosis in bladder cancer. Of particular interest are the EV cargoes H2BK1 and alpha 1-antitrypsin as both prognostic and diagnostic biomarkers, with a combined sensitivity and specificity of 62.7% and 87.59% respectively in discriminating patients with bladder cancer from controls [90]. The diagnostic potential of lncRNAs incorporated in serum EVs was investigated by screening 11 lncRNAs against 200 cases of bladder cancer. Following validation of these lncRNAs, three were found to have a high diagnostic accuracy for bladder cancer: PCAT-1, upregulated in bladder cancer 1 (UBC1) and small nucleolar RNA host gene 16 (SNHG16). A screening panel of all three lncRNAs showed they were highly diagnostic of bladder cancer with an AUC of 0.857 [123]. However, widespread validation of these lncRNAs in clinical studies has yet to be undertaken despite a sensitivity and specificity of 80% and 75%, respectively (AUC: 08.26), discriminating between patients with bladder cancer and healthy controls, when validated in an independent training set [123]. Another combined RNA panel including miR-26a, miR-93, miR-191 and miR-940, had a sensitivity of 70%, specificity of 84% and diagnostic value of AUC: 0.858 for identifying bladder cancer in a cohort of 85 patients and 45 controls [124].

Further EV cargoes explored for their diagnostic potential include three mRNAs identified through RNAseq analysis and further validated in a cohort of 206 patients and 36 controls. The diagnostic value of these RNAs was SLC2A1 AUC 0.70, GPRC5A AUC 0.64 and KRT17 AUC 0.64, when these mRNAs were combined with conventional urinary cytology, their diagnostic accuracy rose to AUC 0.93 [125]. This highlights the importance of utilising established diagnostic methods in conjunction with newly established markers to improve test accuracy.

Interestingly EVs released from bladder cancer tissue express high levels of the tumour suppressor micro-RNAs miR-23b and miR-921 [126]. This finding suggests that malignant cells have exploited EVs to dispose of intracellular tumour-supression molecules, thus allowing the malignancy to evade cellular checkpoints which would typically slow or stop the progression of disease. Characterisation of micro-RNAs in EVs from serum, tissue, urine and white blood cells in bladder cancer patients revealed 19 upregulated micro-RNAs of which miR-2 and miR-4454 were upregulated in EVs from all sources [127].

In addition to RNA cargo, EVs derived from bladder malignancies and isolated from urine, carry endothelial locus 1 (EDIL-3), a glycoprotein secreted by endothelial cells mediating endothelial cell attachment and migration [128]. EDIL-3 has been identified in other malignancies including hepatocellular carcinoma and pancreatic cancer, conferring poor prognosis in both. Studies of EDIL-3 in high-grade bladder cancer EVs promoted cell migration and angiogenesis in bladder cancer cells. This was confirmed with short hairpin RNA interference to knockdown EDIL-3, which inhibited migration and angiogenesis [128].

A feasibility study by Perez et al. identified LAG1 homolog ceramide synthase-2 and Polypeptide *N*-acetylgalactosaminyltransferase 1 were upregulated in urinary EVs in a small cohort of bladder cancer patients, while chromosome 9 open reading frame 100 (ARHGEF39) and FOXO3 were found only in EVs derived from benign tissue [129]. A further urinary EV mRNA, miR-21-5p was validated for its diagnostic potential and found to be highly sensitive with an AUC of 0.89, however this was only in a small cohort of 6 patients with 3 controls [130].

EV cargoes not only have diagnostic potential but also a possible role in disease monitoring and progression. Andreu et al. examined miRNAs within urinary EVs identifying miR-30c-2 was predictive for disease relapse whereas the EV incorporated miRNAs let-7c, miR-375 and miR-194 where both found to be downregulated in bladder cancer patients while miR-146a was significantly upregulated [131]. Both miR-375 and miR-146a were validated as potential biomarkers for high grade and low grade disease respectively in small cohort of patients.

3.7. Proteins in bladder cancer EVs

Initial examination of EV cargo released by bladder cancer cell lines revealed over 350 protein cargoes, with 72 previously unidentified in other human EV proteomic studies [132]. Further validation of these cargoes revealed a subset of 7 proteins: apolipoprotein A1, CD5 antigen-like, fibrinogen alpha chain, fibrinogen beta chain, fibrinogen gamma chain, phosphocarrier protein and haptoglobin, which could discriminate between low- and high-grade disease [133]. Although the function of many of these proteins in bladder cancer has yet to be defined, EVs

released from invasive bladder cancer increase migration of urothelial cells co-cultured with these EVs, a crucial transitionary step from localised to invasive disease [134]. Further pro-oncogenic roles identified within EVs include promotion of the pre-metastatic niche, identified through the effects of the protein C10 regulator of kinase (CRK) and mRNA ErbB2 [135]. Knock down of CRK impaired disease progression, reducing local and metastatic tumour progression in a mouse cell line [135].

In bladder cancer cells lines, unique EV cargoes have been identified, including MAGE B4 which was overexpressed in urinary exosomes of transitional cell carcinoma of the bladder [136]. One notable role of MAGE B4 is inhibition of apoptosis and tumour cell survival [137], however urinary exosome levels of MAGE B4 in bladder cancer patents was lower than that identified in small cohort ($n = 8$) of BHP patient controls, outlining other roles for MAGE B4 in different tissues and also the risk of false positives when screening for bladder cancer. The small numbers used in these validation studies mean their results should be interpreted with caution, and larger cohorts are needed to further validate these markers.

Despite the identification of numerous EV cargoes derived from both bladder cancer tissue and cell lines, there is limited scope for their routine use in clinical practice at present. Further validation of these potential markers as well as the feasibility of their use with established diagnostic tools must be undertaken to better define their applicability in a clinical setting.

3.8. Prostate cancer

Prostate cancer is a common malignancy diagnosed in aging men and a significant cause of mortality. Although survival is improving through early detection of latent disease and improving treatment outcomes, for many patients, their disease is first recognised once it has metastasised. The subjective and poorly specific nature of digital rectal examination and serum prostate specific antigen (PSA) means there is an urgent unmet need for sensitive and specific biomarkers.

Both benign and malignant prostate cells release EVs which have been detected in plasma and urine with significant diagnostic potential [138,139]. The term prostasome has been used to describe EVs released by the prostate and these likely represent a mixed cohort of EVs derived from numerous pathways [43,140–144].

Initial studies characterising the cargo of these EVs have identified over 200 potential candidates for biomarkers with various roles in cell adhesion, differentiation, migration and transcription [145–151]. Exploration of their diagnostic ability has shown them to discriminate between benign and malignant disease, but also high and low grade disease using a panel of EV incorporated proteins including transglutaminase-4, adseverin, cluster differentiation 63, putative glycerol kinase 5, prostate-specific antigen, prostatic acid phosphatase and N-sulphoglucosamine sulphohydrolase [147].

The function and impact of prostasomes as potent mediators in the development of cancer should not be underestimated. EV incorporated circ_SLC18A1, increased in prostate cancer derived EVs, downregulated miR-497 with a reciprocal rise in expression of miR-497's target gene septin 2 [152]. This over expression leads to important EV regulated phenotypic changes associated with prostate cancer progression, notably cell growth. In addition to impacts on local tissue, prostate cancer derived EVs play an important role in disease progression in tissues remote from the prostate. The mechanisms by which prostate cancer metastasises to bone is yet to be fully elucidated but the development of the pre-metastatic nice is, in part, developed through the action of prostasomes. Several examples of how these EVs impact metastasis and their mechanisms have been identified. Dai et al., demonstrated prostasome transfer of pyruvate kinase M2 (PKM2) to bone marrow stromal cells in culture. Transfer of PKM2 to recipient cells effectively 'primes' the tissue for subsequent metastatic spread through up-regulation of CXCL12 [153]. In addition to direct protein transfer to tissues, EV incorporated microRNAs also play a crucial role, including has-miR-940 which promoted osteogenic differentiation of human mesenchymal stem cell to osteoblasts in culture [154]. siRNA knockdown of potential candidate genes through which has-miR-940 mediates its action revealed osteogenesis was regulated through ARHGAP1 and FAM134A [154]. Another microRNA identified in the regulation of osteoblastic activity is miR-141-3p,previously identified in as a biomarker for prostate cancer (Table 1 and Table 2). Ye et al., demonstrated EV mediated delivery of miR-141-3p showed preferential targeting to bone tissues and suppressed the gene DLC1, which in turn activates the p38MAPK pathway leading to promotion of osteoblast activity through increased expression of osteoprotegerin [155].

EV cargo loading is dynamic and reflective of their cell of origin, this feature continues as these cells mutate both in terms of acquisition of malignant potential but also other features such as development of chemotherapy resistance. Corcoran et al., demonstrated docetaxel-resistant cell lines conveyed this resistance through transfer of MDR-1/P-gp to docetaxel-sensitive cells. In addition to enhanced cell proliferation and invasion from cells incubated with docetaxel-resistant EVs, correlations were identified between cellular response and patients response to docetaxel treatment, when cells were cultured with patients EVs [156].

3.9. RNA in prostate cancer EVs

The diagnostic potential of urinary EV-incorporated RNA has also been explored. Of particular note are the RNA panels: miR-572, miR-1290, miR-141, miR-145 [157] and miR-21, miR-204, miR-375 [158] combined with serum PSA, which demonstrate good discrimination between benign and malignant disease with an AUC of 0.863 and 0.866 respectively. The use of miR's in addition to PSA offered superior diagnostic potential than PSA alone. Using a combination of urinary EV mRNA levels of Erythroblast transformation-specific related gene (ERG), *prostate cancer* antigen 3 (PCA3) and PSA with patient characteristics including age, race and family history improved the diagnostic accuracy from AUC 0.6723 to 0.803 [159] highlighting the applicability of ERG, PCA3 and PSA as diagnostic biomarkers [160]. In addition to the biomarker potential of microRNAs, miR-1908 interaction with spermidine synthase (SRM) was found to regulate EV release in prostate cancer cells, confirmed by reduced secretion following SRM knockdown [161].

Numerous other micro-RNAs and lncRNAS with altered expression in prostate cancer have been identified in both urine [157–160,162] and plasma [163–166] (Tables 1 and 2). Some of these micro-RNAs have been investigated for their correlation with the Gleason score as alternative diagnostic biomarkers, although none have been as robustly validated as ERG. Gleason scores are used as a prognostic marker of prostate adenocarcinoma with scores <6 considered to have good prognosis, while scores >8 imply aggressive disease. Other RNA molecules with good correlation to the Gleason score include: (a) elevated miR-145 in the EVs in both serum and urine predictive of a score > 8 [157] and (b) lower levels of urinary let-7c, an RNA precursor which correlates well with clinical staging [162]. Serum EV cargoes also show good discrimination between patients with high and low grade disease despite it representing only a small fraction of circulating RNA. In particular, levels of let-7a-5p were able to discriminate between patients with Gleason scores of <6 and those with scores of >8 with an AUC of 0.68 [164].

Interestingly, not all micro-RNAs identified within cancer-derived EVs are upregulated. The downregulation of tumour suppressor micro-RNAs including miR-1246 [165] and miR-214, which have numerous roles, has been identified compared to disease free, or benign prostatic hyperplasia controls [162]. Despite their potential, the routine use of EVs to detect urogenital malignancies is not yet commonplace, and further work must be undertaken to determine their clinical applicability and utility. To date the combination of the lncRNAs SChLAP1 and SAP30L-AS1 have the highest diagnostic accuracy in prostate cancer

with a AUC of 0.9224, when compared to PSA alone which has a sensitivity and specificity ranging from 78 to 100% and 6–66% respectively [166,167]. Serum exosomal levels of miR-141 were elevated in patients with metastatic disease compared to localised disease and [168]. This is likely due to the presence of more diseased tissue, but also makes miR-141 a potential target for further evaluation in monitoring response to treatment or disease progression.

Urinary EV-incorporated ERG, PCA3, SPDEF was validated in two studies for their diagnostic potential to discriminate between low and high grade disease with an AUC of 0.74 [169] and 0.70 [170]. Additional work on the diagnostic potential of urinary EV cargoes was undertaken by Bryzgunova et al. identifying miRNA-19b and miR-16 to have a sensitivity and specificity of 100%/93% and 95/79% respectively in distinguishing prostate cancer from healthy controls [171].

Examination of the potential roles of EV cargoes has identified their ability to alter the function of the immune system. Angelis and Spiliotis et al. identified circular RNA, specifically circ_SLC19A1, within EVs interacts with miR-497, regulating the expression of septin 2, the dysregulated expression of which is identified in numerous cancers [172]. EV mediated circ_SLC19A1 indirectly alters the regulation of septin 2, promoting growth and invasion of prostate cancer cells [152]. Modulation of the immune system was reported by Salimu et al. reporting EV mediated delivery of prostaglandin E_2 to antitumour CD8+ T cells suppressed their dendritic function [173]. Further EV - T cell interaction leads to downregulation of the transmembrane receptor NKG2D on NK cells and $CD8^+$ T cells [174,175]. While EV-mediated transfer of adenosine reduces T cell response to IL-2 [176].

3.10. Proteins in prostate cancer EVs

The protein cargoes of EVs have been reported by numerous authors with validation for diagnosis, discrimination between low- and high-grade disease and disease progression.

In EVs isolated from serum, prostasomal EphrinA2, a member of the protein-tyrosine kinase family has been validated in a cohort of 50 patients and 21 controls showing a high sensitivity (80.59%) and specificity (88%) in discriminating between prostate cancer and benign prostate hyperplasia with an AUC of 0.9062 [177]. Other serum and plasma EV markers identified within prostasomes include PSA, survivin and P-glycoprotein with potential roles in diagnosis screening and predicting response to chemotherapy [178–180]. Gamma-glutamyltransferase 1 levels, initially identified within EVs released in cell culture has subsequently been identified in serum EVs derived from prostate cancer patients and may have potential use as a diagnostic marker [181]. In addition to diagnostic markers, EV cargoes have the potential to discriminate between malignant and benign prostate disease. Khan et al. demonstrated the apoptosis inhibitor, survivin, was significantly raised in EVs derived from prostate cancer patents compared to a cohort of patients with benign prostatic hyperplasia (BPH) and healthy controls [179]. In addition to survivin, EV-incorporated PSA can also be used to screen for prostate cancer when compared to healthy and BPH controls [178].

None of these markers have been validated in large patient cohorts, limiting their clinical applicability at present. A proteomic analysis undertaken by Minciacchi et al. revealed 103 differentially expressed proteins with numerous known functions including cell proliferation, transmembrane transport, vesicle mediated transport, angiogenesis and chemotherapy resistance [182]. As with other cargoes the numerous functions identified within EVs signals their potential use not only clinically but also give important clues as to potential mechanisms at a cellular level through which they mediate their effects. Although the clinical utility of these markers is important and has great potential to advance diagnostics, screening and disease monitoring, identification of mechanistic pathways may lead to new treatment to disrupt these pathways. Mass spectrometry-based proteomic assessment of urinary EVs by Dhondt et al. demonstrated 1134 enriched proteins within EVs including known drivers of prostate cancer HRAS, AKT1, CUL3, NKX3–1 and PTEN [183]. Comparisons between prostate cancer patients and healthy controls revealed 705 of these proteins were differentially enriched within EVs. Following resection of prostate disease, analysis of urinary EVs demonstrated an increase in the number containing bladder and kidney markers, suggesting prostate cancer leads to increased EV release into urine. This work was carried out in a small cohort, however, and requires further validation prior to clinical application.

As well as their identification within clinical samples, cultured cell-line EVs have been found to carry protein cargoes such as ALIX, FASN, XPO1 and ENO1 [148]. Further work by Itoh et al. also revealed Ets1 and PTHrP were incorporated within EVs [149]. These were found to promote cell differentiation within osteoblasts in a mouse model and give important insights as to the possible function of prostasomes within other tissues and may be of significant clinical relevance, given prostate cancer preferentially metastasises to bone.

3.11. Lipids in prostate cancer EVs

Lipid cargoes of prostasomes have received little attention in comparison to protein and RNA, however Skotland et al. corelated the ratio between Lactosylceramideand Phosphatidylserine in a small cohort of 15 prostate cancer patients and 13 healthy controls, with an AUC of 0.989 [184]. The use of mass spectrometry in healthcare settings is currently limited to the identifcaiton of specific markers, rarther than large scale screening for numerous compounds which will limit the use of EV lipid profiling, despite its high diagnostic accuracy. The small cohort size included in this study however means that despite promising results, they require validation in larger cohorts prior to their use in clinical settings. However, this is the first study to report the use of EV incorporated lipids as a possible biomarker for prostate cancer diagnosis.

3.12. Metabolites in prostate cancer EVs

Changes in EV incorporated metabolites have been identified in a cohort of 31 prostate cancer patients and 14 controls. Of these metabolites, 76 showed differential expression levels within EVs. Of particular interest are two androgen precursors 3beta-hydroxyandors-5-en-17-one-3-sulphate and estrone sulphate, implicated in the progression of prostate cancer [185]. A small study of urine samples from three patients pre- and post-prostatectomy also identified differential expression of EV incorporated metabolites compared to healthy controls [186]. However, as with many EV studies, these findings are reported in a small number of patients and require validation in larger cohorts.

3.13. Testicular cancer

Although EVs are reported in renal, bladder and prostate cancer, EV identification and functional appreciation have yet to be achieved in testicular cancer. Testicular cancer is the commonest cancer in young men globally. The majority of cases arise due to the persistence of foetal germ cells which should differentiate into spermatogonia during fetal and early postnatal life, but instead transform into pre-neoplastic Germ Cell Neoplasia In-Situ (GCNIS). Interestingly, the presence of GCNIS cells in early postnatal life, and the peak age of onset for testicular germ cell tumours being 25–29 years, means that the pre-malignant phase of this cancer can last over 20 years. Prognosis for testicular cancer is good once diagnosed, but an effective diagnostic test prior to malignant transformation during the pre-malignant phase has yet to be developed. The potential capability of EVs in providing material for detecting testicular cancer is currently unknown. Although a unique micro-RNA signature has been identified in testicular cancer, its presence within EVs has not been investigated [187].

3.14. Methodological challenges in EV isolation and sample preparation

The heterogeneous nature of EVs means their isolation from a range of biological media including serum, plasma and urine as well as cell culture supernatant leads to challenges in achieving replicable standards. As no 'gold stand' methodology exists, efforts to build consensus driven reporting on EVs have been led by the International Society for Extracellular Vesicles (ISEV), developing two minimum consensus positions [1,188]. The majority of this heterogeneity is based on the separation techniques used and as such is of upmost importance, as this effectively generates the material to be analysed and will influence results and conclusions drawn [189]. Frequently used techniques include ultracentrifugation, size exclusion chromatography, filtration, immunoaffinity capture, microfluidic separation and polymer precipitation [190–194]. As well as the EV separation technique used, numerous other factors can influence EV purity include pre-analytical variables such as the medium in which EVs are to be isolated from, sample acquisition and storage. Many of these issues were apparent in the studies included in this review and are outlined in Tables 1 and 2.

3.15. Pre-analytical variables

Despite development of a consensus position on EV isolation and characterisation, substantial variation exists when reporting biomarker discovery and validation. In addition to the heterogeneity from EVs themselves, inherent pre-analytical variation can impact on the results obtained [195].

How samples are obtained and stored can affect their molecular and proteomic profiles. This is of great significance given reporting of novel biomarkers is in part based on the measurement of these factors [196]. Many studies included in their review used EVs in blood to conduct their analysis. However, the way blood is processed varies greatly. Additives within the phlebotomy bottles into which blood is collected varies greatly and often based on the intended use of the clinical sample. These additives can include ethylenediaminetetraacetic acid which is suitable for a range of DNA and protein-based studies whereas heparin binds to numerous proteins and may impact protein-based assays.

[195,197]. In addition to interaction between additives and presumptive EV cargoes, the number of EVs identified within a blood sample can be altered after only a few hours, with Lacroix et al. reporting a 80% increase in EVs after 4 h [198]. Variation in the number of additional EVs generated through interaction with additives to transport blood may be reduced by storing and transporting samples of serum as opposed to plasma. This would require further sample preparation which may delay downstream analysis to isolate EVs and inadvertently lead to unwanted sample variation. In many studies reported in this review, often a single sample of blood was obtained as opposed to repeated samples, accounting for variation in sample acquisition or unknown physiological processes which may impact EVs number [199].

In addition to blood, urine is a frequently collected clinical sample in which to separate EVs for further analysis. The bladder, unlike many organs, changes in volume and contents throughout the day, the contents of which originate from the kidney as it filters waste from blood. As such it represents a potentially rich and diverse source of EVs, either through the direct release of EVs from the bladder and kidneys or through co-contamination with semen comprising testicular, prostate and seminal fluid EVs. Many authors reported using freshly voided urine often centrifuging the sample to remove large pellets of co-contaminants, reduced the heterogeneity of the sample and the presumed effect of urine pooled within the bladder for longer periods. A notable contaminant of urine is the presence of uromodulin, a major protein component of urine with the potential to interact with EVs [200]. Uromodulin has been demonstrated to interact and bind to EVs, forming a uromodulin-EV complex, this is both larger and heavier than single EVs, and thus size based EV isolation methods may not accurately capture these particles [201]. How uromodulin interacts with EVs when stored post void, is currently unknown.

Acquisition of clinical samples is further compounded by a lack of consensus driven protocols in how to obtain these samples and process them. This variation, often compounded by a small cohort size leads to challenges in the reliability of the results obtained with much work reported in this review requiring validation in larger patient cohorts.

3.16. EV separation from liquid biopsy and cell culture media

In addition to pre-analytical variables impacting EV yield and quality, the aforementioned methodologies to separate EVs from these samples often produces results with variable EV heterogeneity, structure and co-precipitates. Many of these factors in isolation, let alone in relation to other poorly controlled for variables, have the potential to confound any subsequent analysis [202]. This in part can be due to the nature of the sample preparation but also the technique used such as the shear stresses placed on EVs during pellet formation in ultracentrifugation, as well as the co-precipitation of non-vesicular debris [203]. Many of these techniques depend on the purity of the sample with size exclusion chromatography or density graded centrifugation reliant on the size profile of EVs, leading to the risk of co-precipitation of other particles with a similar size [204]. Despite the intention of differential ultracentrifugation being to pellet larger debris e.g. cells, with a higher buoyant density, while EVs remain suspended in the media to be pelleted in subsequent centrifugation steps, this methodology is somewhat limited in its ability generate a homogenous sample of EVs [205]. Particles of a similar size may be co-precipitated and contaminate the EV sample, assessing the size of the particles obtained through nanoparticle tracking analysis for example, may lead to false confidence in the purity of the sample [192]. Sample purity can be improved somewhat by using differential ultracentrifugation, followed by additional purification steps to allow large volumes of sample to be processed and remove co-precipitated particles; however, this is both time consuming and associated with large equipment costs and inevitably leads to reduced numbers of EVs obtained by the final preparation [206,207].

Gradient centrifugation again reduces sample heterogeneity and uses a dense medium through which the sample passes with particles of similar density held within the sample. Filtration of samples through membranes with a pre-specified pore size allows a low cost alternative to centrifugation and is more suited to processing of smaller samples and has been reported by some to produce EVs with greater functionality than centrifugation [208]. However, membrane deterioration allowing the passage of larger particles as well as mechanical stress to EVs passing through the membrane, can lead to contaminants within the sample. Although this technique does exclude larger particles, it fails to produce a concentrated sample of EVs as with centrifugation, thus additional steps may be required in order to prepare the EVs for analysis [209]. Isolation techniques which place EVs under less stress during their preparation include immunoaffinity capture and precipitation which make use of the known surface markers of EVs and their lipid bilayer membrane [210]. Immunoaffinity requires the use of high cost antibodies but can facilitate the capture of certain populations of EVs and with high purity. Precipitation also generates a high yield of EVs; however, this can be contaminated with protein aggregates and other lipid based molecules. More recently, microfluidic devices have been developed to isolate EVs from a range of suspensions including cell culture media, plasma and urine [211]. These devices are diverse and make use of the EV size, EV density and surface antibodies to separate them from their suspension as they flow through the device. Preferable for small sample sizes, microfluidic isolation of EVs is costly yet offers both a high yield of EVs and low co-capture of contaminates.

3.17. EV storage and analysis

Following sample acquisition or EV isolation many studies included in this review stored samples at different temperatures and for different

lengths of time. Methods of storage for serum, plasma and urine prior to EVs isolation varies greatly between studies and may impact the quantity and integrity of the EVs isolated. While this may be due to practicalities of obtaining clinical samples, variation in their storage at different temperatures can affect both quantity and integrity of EVs. Following the isolation of EVs, there is variation as to when they were characterised and interrogated for their cargoes. The impact of freeze-thawing is disputed with some authors reporting minimal impact on EV yield [212], others identifying a reduction in EV number, but minimal impact on EV uptake [213], and others reporting the addition of the cryoprotectant DMSO as improving EV yield and function [214].

Work to establish the optimal storage conditions for EVs has suggested −80C may be suitable with Enderle et al. reporting the isolation of EVs from plasma with minimally degraded RNA levels after 12 years of storage at −80C [215]. Interestingly, EVs stored at 4C were found to have a greater reduction in concentration than those stored at 60C, −20C and −80C [213]. Many of the studies included in this review stored EVs at -80C; however, several studies reported storage at 4C and this may impact on the reliability of the results obtained.

Following the presumptive isolation of EVs, substantial variation in validation of EV yield and purity has been identified. The most commonly used technique is transmission electron microscopy which enables the size and morphological appearance of the EVs to be identified. This was frequently accompanied by western blotting for common EV surface markers. Interestingly, some studies did not undertake any specific analysis of their sample to assess the yield of EVs or ensure their presumptive EVs were not contaminated. This raises issues with subsequent analysis and conclusions drawn as cargoes reportedly within EVs may have been identified by samples with a low number of EVs. Comparisons of identified cargoes to the EV depleted fraction should also be undertaken to ensure similar results or novel findings aren't reported in fractions which do not contain, or are not meant to contain, EVs. Again, lack of consistent reporting across studies and variation in the methodologies used means the identification of many biomarkers require further validation in larger cohorts and reliable identification using standardised techniques.

Variable reliability and cost associated with current EV isolation and storage techniques outlined above have led to the development of platforms which directly analyse EVs from biological samples without prior need for EV isolation steps. Novel platforms to profile EVs from patient blood samples have been developed using antibody capture EVs for identification of colorectal cancer allowing analysis within 2 h and requiring only 5 μL of serum [216]. More recently, microfluid EV capture devices have been developed again using small volume (100 μL – 5 mL) blood samples from glioblastoma patients utilising antibody based capture of EVs to isolate tumour specific EV incorporated RNA within 3 h [217]. Although the aforementioned studies do not focus on urological malignancies, similar devices have been developed for the characterisation of EVs in prostate cancer. Sunkara et al., developed a device utilising both sequential and tangential flow filtration of 30 μL blood samples to characterise mRNA of EVs between prostate cancer and healthy controls. Comparison of this technique to ultracentrifugation demonstrated superior EV isolation mRNA yield, with sample analysis only taking 40 min [218]. These platforms, should they be by clinically reliable and financially viable, may lead to development of novel multi-disease screening or point of care testing platforms for application in numerous health care settings.

4. Conclusions

The field of EVs has grown in the understanding of their widespread distribution, identification in numerous body fluids and their role in health and disease. The advent of technologies, to characterise EVs in vivo, but also isolate and study them in vitro, has led to significant advances in our understanding of their potential. Appreciation and application of the roles EVs play in cancer development, progression and response to chemotherapy are exciting and fast-moving areas.

Advances in the characterisation of their cargo has revolutionised our understanding of EVs over the past 10 years. Proteomic, metabolomic and RNAseq studies have enabled us to appreciate individual molecules leading to further research on the properties of individual proteins and micro-RNAs. Identification of unique cargo within EVs offers new opportunities to develop novel diagnostic tools in order to recognise diseases which currently lack sensitive and specific tests. Variation in methodologies used and the way in which biomarkers are identified means some novel reported cargoes should be treated with caution and validated in larger patient cohorts, prior to their consideration for use in healthcare settings. Of the 91 primary studies identified in our review, only 5 made references to ISEV standards when reporting data on EVs [182,219–222]. This highlights further work is required to standardise reporting across studies and fully implement minimum reporting standards.

Increasing use of EVs both in science and healthcare, rapidly expanding cataloguing of their cargo and falling costs of underlying technologies, will likely remove barriers to the routine use of EVs in healthcare settings to facilitate early disease detection and monitoring in the future. The correlation of EV cargo to current diagnostic techniques has demonstrated the exciting potential of EVs in ushering in a new era of precision medicine in relation to cancer diagnostics. This is of particular importance for urological malignancies which remain a significant health care burden. In addition to their diagnostic potential, EVs are a growing area of therapeutic interest. The widespread distribution and the development of new techniques to manipulate them through artificial incorporation of molecules such as siRNA or modulating their interaction with cells represents a new potential field of clinical therapeutics.

Author contributions

MPR – conceived the idea for the article, undertook the literature search, wrote the manuscript, developed the figures and approved the manuscript for submission.

CDG & RTM – conceived the idea for the manuscript, undertook editorial changes and approved it for submission.

Funding

MPR and RTM are funded by a MRC Centre for Reproductive Health Grant No: MR/N022556/1.

RTM is funded by a UK Research and Innovation (UKRI) Future Leaders Fellowship MR/S017151/1.

Declaration of Competing Interest

Michael P Rimmer, Christopher D Gregory and Rod T Mitchell have no decelerations of interest to declare.

Appendix A. Supplementray data

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