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DOI:
10.3233/BLC-160054

Document Version
Peer reviewed version

Citation for published version (Harvard):
D'Costa, J, Goldsmith, J, Wilson, J, Bryan, R & Ward, D 2016, 'A systematic review of the diagnostic and prognostic value of urinary protein biomarkers in urothelial bladder cancer', *Bladder Cancer*, vol. 2, no. 3, pp. 301-317. https://doi.org/10.3233/BLC-160054

A systematic review of the diagnostic and prognostic value of urinary protein biomarkers in urothelial bladder cancer

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Running title: Urinary protein biomarkers in urothelial bladder cancer

Keywords: bladder cancer, biomarker, urine, protein.

ABSTRACT

For over 80 years, cystoscopy has remained the gold-standard for detecting tumours of the urinary bladder. Since bladder tumours have a tendency to recur and progress, many patients are subjected to repeated cystoscopies during long-term surveillance, with the procedure being both unpleasant for the patient and expensive for healthcare providers. The identification and validation of bladder tumour-specific molecular markers in urine could enable tumour detection and reduce reliance on cystoscopy, and numerous classes of biomarkers have been studied. Proteins represent the most intensively studied class of biomolecule in this setting.

As an aid to researchers searching for better urinary biomarkers, we report a comprehensive systematic review of the literature and a searchable database of proteins that have been investigated to date. Our objective was to classify these proteins as: 1) those with robustly characterised sensitivity and specificity for bladder cancer detection; 2) those that show potential but further investigation is required; 3) those unlikely to warrant further investigation; and 4) those investigated as prognostic markers. This work should help to prioritise certain biomarkers for rigorous validation, whilst preventing wasted effort on proteins that have shown no association whatsoever with the disease, or only modest biomarker performance despite large-scale efforts at validation.

INTRODUCTION

Non-muscle-invasive bladder cancer (NMIBC: stages Ta/T1/Tis) is characterised by a high incidence of recurrence and a risk of progression to muscle-invasive disease (MIBC: stages T2+) [1]. Long-term surveillance thus remains the cornerstone of long-term management, and cystoscopy has represented the gold standard modality for over 80 years. However, the cystoscopic approach is both burdensome for patients and expensive for healthcare providers, and so there has been a decades-long search for non-invasive urinary biomarkers that can match or even improve upon the sensitivity and specificity of cystoscopy. However, current guidelines do not recommend the use of urinary biomarkers in the management of bladder cancer patients [2]. Despite this, urine cytology is often used as an adjunct to cystoscopy and whilst visual detection of cancer cells in urine is a very specific test with high sensitivity for high-grade bladder cancer, sensitivity for low-grade bladder cancer is only 4-31% [3]. Thus any new biomarkers might be considered useful if they outperform cytology i.e show very high specificity and high sensitivity for both low and high-grade bladder cancer.

Many classes of biomolecule have been investigated as urinary biomarkers but the majority of studies have analysed proteins with hundreds of published reports where specific proteins have been measured in urine as potential indicators of bladder cancer. In this review we focus entirely on soluble proteins (those that can be measured in the supernatant rather than the pellet following centrifugation). Most proteins can be measured reliably using inexpensive immunoassays. These may be rapid and qualitative, enabling point-of-care testing, or laboratory based quantitative immunoassays but in either

case, multiplex testing for a panel of protein biomarkers (if such a panel was defined) should be relatively straightforward to implement.

The proteins measured in biomarker studies will have been selected based on prior knowledge of the biology of bladder cancer or the biology of the particular protein or based on data from hypothesis generating approaches such as gene expression profiling and proteomic analyses of bladder cancer tissue, cell lines or indeed urine itself. In most cases, the measurements will have been made using a validated antibody-based assay such as an ELISA. The reliability of such assays is dependent on the specificity of the antibodies used; certain types of assay which rely on the specificity of a single antibody may be more susceptible to interference than 'sandwich assays' (those that rely on the specificity of a pair of antibodies). However, in many bladder cancer biomarker studies, patient selection is of more concern than the assay used. That is to say, "do the patients (and the non-cancer subjects) being used in the study represent the patient population where the biomarker test would be applied in the real world?". Ideally in studies of biomarkers for first presentation diagnosis both "cases" and "controls" should be patients undergoing investigation for suspected bladder cancer e.g. patients from haematuria clinics. For surveillance markers both cases and controls should be patients undergoing surveillance for disease recurrence. However, in many biomarker studies we see bias introduced by enrichment for high-grade and advanced disease which is likely to increase apparent sensitivity (% cases correctly identified) and inclusion of healthy volunteers which is likely to increase apparent specificity (% controls correctly identified), or the use of patients with large-primary tumours when the goal is to detect small recurrent tumours [4].

Another major pitfall in the measurement of urinary biomarkers for bladder cancer is haematuria [5]: haematuria is a symptom and sign of bladder cancer but is not the biological cause of bladder cancer. Thus, any protein present in blood may appear to act as a biomarker in case-control studies where haematuria is not matched, but will not be bladder cancer-specific.

Reviews of urinary biomarkers for bladder cancer (e.g. [6, 7]) tend to focus on the biomarkers that have been most extensively validated for detecting disease, especially those with FDA approval (NMP22, BTA, UroVysion, ImmuoCyt), but also others such as MMP9 which have been extensively measured but fall short of clinical utility, and perhaps a handful of 'promising candidates'. In the current review we attempt to comprehensively review all proteins which have been investigated as urinary biomarkers for bladder cancer. Our main rationale for doing so is to generate a useful resource for researchers that may indicate the potential (or otherwise) of a particular urinary protein under investigation. A secondary aim of is to collate and assess the literature on prognostic urinary biomarkers, an area which is often neglected but which could be incorporated into risk stratification algorithms and so aid patient management.

The biomarker studies reviewed are heterogeneous in terms of the populations studied. The non-bladder cancer control cohorts vary from healthy controls to non-malignant urological disorders to non-bladder urological malignancies and patients undergoing surveillance for bladder cancer recurrence with no detectable disease (or a mix of all four). The bladder cancer cases vary in stage and grade (which we have partially controlled for with our selection criteria, see below) but are also either primary or recurrent tumours or a mix of both (or unspecified) in different studies. Thus, to be inclusive, we have used the

term “detection biomarker” and present sensitivity for bladder cancer versus non-bladder cancer throughout this review rather than attempting to distinguish between proposed diagnostic and surveillance roles for biomarkers in individual studies.

MATERIALS AND METHODS

Systematic review methods were employed to find primary studies that reported test results on measured soluble protein biomarkers in urine. The search was conducted in Medline via the Pubmed search platform on the 25th August 2015 using the following search terms: *(("urinary bladder neoplasms"[MeSH Terms] OR ("urinary"[All Fields] AND "bladder"[All Fields] AND "neoplasms"[All Fields]) OR "urinary bladder neoplasms"[All Fields] OR ("bladder"[All Fields] AND "cancer"[All Fields]) OR "bladder cancer"[All Fields]) AND ("urine"[Subheading] OR "urine"[All Fields] OR "urine"[MeSH Terms])) AND ("biological markers"[MeSH Terms] OR ("biological"[All Fields] AND "markers"[All Fields]) OR "biological markers"[All Fields] OR "biomarker"[All Fields]).* Studies retrieved from the PubMed search were assessed for eligibility by two people (JD & JG) using the title and abstract or where necessary the full text. Disagreements regarding inclusion were resolved by discussion and moderation by the rest of the team. Papers were included if they reported on tests that measured soluble protein biomarkers in the urine of bladder cancer patients (any stage). We excluded papers that did not measure protein biomarkers in urine, measured only enzyme activities, or that analysed urinary cell pellets. Prognostic biomarker studies were manually identified from the full set of included publications.

The included papers underwent a quality filter step to separate the better designed and reported studies from those with poor reporting or design. Studies were categorized into "unequivocal" or "equivocal" categories based on whether they met the following selection criteria:

- $\geq$ 20 cancer patients
- $\geq$ 20 controls or more
- Specificity and sensitivity presented
- ≥25% pTa bladder cancer
- ≥ 15% grade 1 bladder cancer

The thresholds for the percentage of pTa and grade 1 tumours are well below the percentages for incident bladder cancer in the UK [8] but enable identification of studies with bias towards reporting protein biomarkers associated with high-grade/stage bladder cancer. Studies which were not available to view or purchase online were also excluded. The proteins reported in unequivocal studies were further categorised into those in 3 or more unequivocal studies (**validated detection biomarkers**), those with a combined sensitivity and specificity (sensitivity+specificity/2) $\geq$ 80% (**possible biomarkers**), and those with a combined sensitivity and specificity $\leq$ 80% (**unlikely biomarkers**). The value of 80% for combined specificity and sensitivity was selected on the basis that it is close to the performance of cystoscopy, the gold standard to which biomarkers are usually referenced (which is the only method of detection of bladder cancer recommended by NICE [2]). The reported sensitivity and specificity of white light cystoscopy vary greatly but a recent meta-analysis arrived at values of 85 and 87% [9]. The full search and categorisation strategy is shown in Figure 1.

RESULTS

Search Result Overview

Our initial literature search identified 1310 publications. From these, we identified 350 reports in which one or more proteins were measured in the urine of bladder cancer patients. Of the 350 reports, only 49 meet our criteria to be considered as "unequivocal". The remaining 301 studies either have too few cases or controls, do not report a high enough proportion of pTa and/or grade 1 tumours, do not provide values for sensitivity and specificity, or are not readily accessible. In total, we found evidence that 161 proteins have been investigated as urinary biomarkers for bladder cancer (Table 1 and Supplemental Information Table S1).

Validated detection biomarkers

Of the 161 proteins investigated, 27 were investigated in one or more "unequivocal studies" (Table 1). Only 4 proteins appear in 3 or more of the unequivocal studies and thus meet our criteria as "validated detection biomarkers". These are the well-known biomarkers: nuclear matrix protein 22 (**NMP22**), bladder tumour antigen (**BTA**), and the cytokeratin-based tests urinary bladder carcinoma antigen (**UBC**) and **Cyfra 21-1**. NMP22, BTA and UBC are commercially available as both quantitative and point-of-care assays. NMP22 (gene symbol: *NUMA1*) is a nuclear matrix protein overexpressed in bladder cancer cells and has been measured in 25 unequivocal studies with weighted mean sensitivity and specificity of 61.8% and 80.3%, respectively (4528 cancer patients vs. 7728 non-cancer). The antigen recognised in the BTA assay is reported as complement factor H related protein which is released from bladder cancer cells [10]: the weighted mean sensitivity and specificity for using BTA to detect bladder cancer across 23 unequivocal studies are 64.0% and 76.6%, respectively (2258 cancer patients v 2994 non-cancer). The UBC test measures soluble fragments of

cytokeratins 8 and 18 and has been reported in 11 unequivocal studies with a mean sensitivity and specificity of 64.4% and 80.3%, respectively (753 cancer patients v 1072 non-cancer). The Cyfra 21-1 test measures soluble fragments of cytokeratin 19 and has been reported in 3 unequivocal studies with a mean sensitivity and specificity of 64.4% and 85.5%, respectively (293 cancer patients v 331 non-cancer).

The mean sensitivities and specificities for the 4 well validated biomarkers across multiple studies are all very similar, in accordance with studies performing side-by-side comparisons: the results of such studies vary as to which biomarker performs best but seldom find substantial differences between them [11-13]. All 4 validated biomarkers also show a similar dependence on stage and grade with high sensitivity for high-grade and muscle-invasive disease but lower sensitivity for low-grade disease. Based on data from those studies listed in Table 1 which present sensitivity for different grades of disease, the mean sensitivities for grade 1/grade 3 are 53.4%/77.4% for NMP22, 51.4%/87.5% for BTA, 48.5%/76.0% for UBC and 55.7%/91.9% for Cyfra 21-1. Thus, these markers do have higher sensitivity for low-grade disease than cytology (albeit with lower specificity) but fall short of cystoscopy in terms of both sensitivity and specificity [3, 9]. Whilst there may be some utility for these commercially-available biomarkers in the surveillance of HR-NMIBC (when index tumour grade and stage are known), they cannot be solely relied upon for the diagnosis of incident disease.

Possible biomarkers

There are 11 proteins that have been evaluated in an unequivocal study and have a combined sensitivity and specificity (sensitivity+specificity)/2) of greater than 80%. We have

designated these as “possible biomarkers”. They are **apolipoprotein A4, calprotectin, CD147, CEACAM1, clusterin, coronin-1A, DJ-1, fibronectin, reg-1, stathmin-1,** and **Y-synuclein**. Several of these possible biomarkers are also supported by evidence from “equivocal studies”. Each protein is discussed briefly below. These are proteins that may merit further investigation: all require independent validation in appropriate patient cohorts.

The utility of urinary **fibronectin** for detecting bladder cancer reported in 2 unequivocal studies [14, 15] is supported by 9 “equivocal” studies: 5 of these studies present moderately high sensitivities and specificities with weighted means across the studies of 82.5% and 80.2%, respectively (390 cases and 520 controls) [16-20]. Although there is substantial evidence that increased urinary fibronectin is indicative of bladder cancer, Alias-Melgar [21] found that urinary fibronectin is increased in urolithiasis, and EIssa [17] reported that in side-by–side comparison NMP22 slightly outperforms fibronectin.

The utility of urinary **clusterin** is also reported by 2 unequivocal studies [22, 23] and 1 equivocal study [24] with the latter reporting a sensitivity of 73% but only 55% specificity. Clusterin is a multifunctional chaperone protein with alternatively spliced forms exhibiting different cellular locations and functions [25]. Hazzaa et al [22] found increased clusterin gene expression in bladder cancer, particularly in invasive disease, and that high clusterin expression was associated with poor prognosis. Although clusterin is widely expressed and found in all body fluids, which may limit it’s specificity [26], measurement of individual splice variants rather than total clusterin levels might merit further research.

CEACAM1 (Carcinoembryonic antigen-related cell adhesion molecule 1, also known as CD66a) was reported as a novel urinary marker for bladder cancer by Tilki et al [27]. In

this study comparing 93 cases (72 NMIBC, 17 low-grade) with 82 controls (30 healthy subjects, 10 with benign disease and 42 with a history of bladder cancer but no evidence of disease) urinary CEACAM1 generated a sensitivity of 74% at 95% specificity. As with most urinary biomarkers sensitivity was higher for MIBC than NMIBC. It is not stated whether the tumours were incident or recurrent and subjects with diabetes were excluded from the study but CEACAM1 appears to merit further investigation. Tilki et al [27] also reported that CEACAM1 immunostaining was detected on endothelial cells rather than cancer cells in bladder tumours. It has not been reported whether the urinary CEACAM1 is expressed as a soluble isoform (lacking the transmembrane domain) or if the ectodomain is shed by proteolytic cleavage.

Ebbing et al [28] reported that urinary **calprotectin** (a heterodimer of S100A8 and S100A9 proteins with antimicrobial properties) can be used to detect bladder cancer with 80% sensitivity at 92% specificity in a study with 46 cases (38 NMIBC, 25 low-grade) and 40 healthy controls. The median calprotectin level was 10-fold higher in bladder cancer patients than healthy controls and less than 2-fold increased in patients with prostate and renal cancers (although their inclusion in the data analysis slightly decreased specificity). Calprotectin has been reported as a prognostic indicator in bladder cancer and to be upregulated both in tumours and sera [29, 30]. However, calprotectin is released by neutrophils during inflammation which may compromise its role as a tumour marker.

Two urinary proteins, **stathmin-1** and **CD147** make the “possible biomarkers” category on the basis of a study by Bhagirath et al [31] analysing the urine of 30 bladder cancer cases (21 NMIBC, 13 low-grade) and 30 controls (15 healthy, 15 benign prostatic hyperplasia). Sensitivities and specificities were 90% and 87% for stathmin-1, respectively (also known as

oncoprotein-18) and 97% and 100% for CD147, respectively (also known as basigin or EMMPRIN). Although a small single study, both stathmin-1 and CD147 are known cancer-related proteins and there are reports that overexpression of stathmin-1 [32] and CD147 [33] are associated with aggressive bladder cancer and a poor prognosis.

Another 4 possible biomarkers were reported in a study by Kumar et al [34]: **ϒ-synuclein** (87.5% sensitivity at 90.0% specifity), **DJ-1** (83.3% sensitivity at 100% specificity), **apolipoprotein A4** (79.2% sensitivity at 100% specificity) and **Coronin-1A** (66.7% sensitivity at 100% specificity) based on 173 cases (110 NMIBC, 89 low-grade) and 212 controls (66 healthy, 91 other malignancy, 121 assorted chronic conditions). Various apolipoproteins have been reported as increased in the urine of bladder cancer patients [35-37]; however, being moderately abundant in plasma, their specificity is not assured and, in the case of apolipoprotein A4, Chen et al [38] found no evidence of elevation in bladder cancer patients. ϒ-synuclein was also investigated by one equivocal study which reported only 40.2% sensitivity albeit at 96.5% specificity [39] (112 cancer and 230 controls). Although DJ-1 has been reported to be overexpressed in aggressive high-grade bladder cancer [40], no other urine studies have been published. We have performed a pilot study and found urinary DJ-1 to be significantly increased only in MIBC, disputing a high sensitivity for all stages and grades of bladder cancer (Ward, unpublished data). Coronin-1A is a cytoskeletal protein that has not been otherwise reported in bladder cancer. It seems likely that Kumar et al's study [34] may have overestimated the performance of these 4 possible biomarkers.

Using proteomics, Orenes-Piñero et al [41] identified a protein at increased levels in the urine of bladder cancer patients as **Reg-1 (**lithostathine-1-alpha). Immunohistochemistry showed Reg-

1 overexpression in bladder tumours and measurement in 32 cases (16 NMIBC, 5 low-grade) and 48 controls (cystoscopy negative) gave 81% sensitivity at 81% specificity.

Unlikely biomarkers

Sixteen of the 27 proteins evaluated in unequivocal studies have a combined sensitivity and specificity of less than 80% (including the 4 validated detection biomarkers). As the diagnostic performance of these proteins is well below that achieved by flexible cystoscopy it is unlikely that any of these would find widespread clinical use as standalone biomarkers for detecting bladder cancer. Nonetheless, the evidence suggests that some cases of bladder cancer result in an increase in the urinary concentration of these proteins and consequently they may be diagnostically useful when used in combination with one another or other markers.

Equivocal biomarkers

We found that the majority of the biomarker studies we reviewed could not be classified as unequivocal. This is due to missing information (stage/grade/sensitivity/specificity) or due to a non-representative patient population which is likely to inflate the estimated sensitivity and specificity. Fifteen proteins that have not been investigated in an unequivocal study have been evaluated in at least 5 equivocal studies (Figure 2), and some of the equivocal studies report high sensitivities and specificities. With the caveat that these "equivocal biomarkers" may not be robust in the clinical setting, we briefly discuss those reported in 5 or more studies below.

Urinary carcinoembryonic antigen (**CEA**) has been investigated in 28 equivocal studies. The weighted mean values for sensitivity and specificity are 54.0% and 90.5% respectively (814 cases and 578 controls) based on accessible studies reporting sensitivity and specificity [42-47]. Most of the CEA studies were published in the 1970s and 1980s (Figure 3) and interest presumably waned due to the sensitivity being too low for clinical utility. However, as expected for an oncofetal antigen, specificity appears to be high and therefore urinary CEA could prove useful in the context of a multimarker panel.

The role of matrix metallopeptidase 9 (**MMP9**) as a urinary biomarker has been investigated in 16 equivocal studies with 8 presenting values for sensitivity and specificity [48-55]. The weighted mean values for sensitivity and specificity are 72.1% and 77.2%, respectively (based on 707 cases and 917 controls). MMP9 is biologically plausible as a biomarker for invasive bladder cancer and urinary levels are clearly elevated in many cases of bladder cancer; however, modest sensitivity and specificity (especially for low-grade disease) limit its usefulness.

Urinary vascular endothelial growth factor (**VEGF**) has been investigated in 13 equivocal studies with 6 presenting sensitivity and specificity data [48, 54, 56-59]. The weighted mean values for sensitivity and specificity are 71.4% and 78.1%, respectively (based on 509 cases and 389 controls).

Tissue polypeptide antigen (**TPA**, a complex of cytokeratins 8, 18 & 19) has been investigated in 10 equivocal studies with 3 presenting sensitivity and specificity data [46, 60, 61]. The weighted mean values for sensitivity and specificity are 84.1% and 96.6%, respectively (based on 277 cases and 311 controls). However, Stefanovic et al reported that TPA lacks diagnostic accuracy [62], and Carbin et al reported that TPA is only effective if 24

hour urine samples are analysed [63]. Additionally, although the averaged sensitivity/specificity appear higher than the averaged sensitivity/specificity for other cytokeratin based tests (UBC and Cyfra 21-1), in a direct comparison of TPA and Cyfra 21-1 Sanchez-Carbayo found TPA to be slightly inferior to Cyfra 21-1, indicating that the equivocal studies have overestimated the performance of TPA [61, 62].

Survivin has been investigated in 9 equivocal studies with 6 reporting sensitivity and specificity data [58, 64-68]. The weighted mean values for sensitivity and specificity are 69.4% and 88.3%, respectively (based on 437 cases and 313 controls).

Matrix metallopeptidase 2 (**MMP2**) has been investigated in 8 equivocal studies with 4 presenting sensitivity and specificity data [49, 52, 53, 69]. The weighted mean values for sensitivity and specificity are 68.2% and 88.8%, respectively (based on 345 cases and 681 controls).

A number of interleukins have been repeatedly investigated in the urine of bladder cancer patients. Interleukin-8 (**IL-8**) has been investigated in 10 equivocal studies with 4 presenting sensitivity and specificity data [54, 56, 70, 71]. The weighted mean values for sensitivity and specificity are 66.4% and 83.1%, respectively (based on 225 cases and 273 controls). Interleukins 2 and 6 (**IL-2** and **IL-6**) have been measured in 5 and 7 equivocal studies respectively. However most of these studies focus on response to BCG treatment rather than bladder cancer detection [72]. With only one study [56] reporting sensitivity and specificity data for IL-6 (67% and 63%), and none for IL-2, there is no evidence that either is likely to be useful for detecting bladder cancer.

CA19-9 has been investigated as a urinary biomarker in 8 equivocal studies, but with only two reporting sensitivity and specificity (83.3% and 50.8% [73], and 71.6% and 91.6% [74], respectively). Strictly speaking, this is a glycan biomarker rather than a glycoprotein biomarker [75]. CA19-9 may be a useful biomarker for bladder cancer when interpreted with reference to secretor phenotype [76, 77].

BCLA-4 has been investigated as a urinary biomarker in 6 equivocal studies. BCLA-4 was first reported in 1996 as a spot in 2D-electrophoresis analyses of nuclear matrix extracts that was more intense in bladder cancer than normal urothelium [78]. The authors then partially sequenced the protein in the gel spot and generated antibodies to a synthetic peptide (EISQLNAG), despite the sequence not matching any known human protein sequences. The antibodies were used to generate immunoassays and BCLA-4 measured in the urine of 54 bladder cancer patients (predominantly high-grade) and 51 control subjects, generating a sensitivity of 96.4% at 100% specificity [79]. In 2004 the same group identified BCLA-4 as a member of the ETS transcription family [80] and then expanded their study to 70 cancer subjects and 147 controls (89% sensitivity and 95% specificity) [81]. However, both of these papers have now been retracted [82, 83]. Despite this, BCLA-4 has been widely reported as a 'promising biomarker' in previous reviews and a further study in China using antibodies raised against the EISQLNAG sequence have reported very high sensitivity and specificity [84]. Thus, BCLA-4 has a chequered history and although BCLA-4 ELISAs appear to detect bladder cancer better than other urinary markers, BCLA-4 has neither gained regulatory approval or been widely adopted as a urinary biomarker for bladder cancer detection.

Apolipoproteins A1 (**APOA1**) and E (**APOE**) and have been investigated in 5 and 6 equivocal studies respectively. The 5 APOA1 papers are from 2 research groups with both reporting

high sensitivity and specificity (89.2% and 84.6% [85-87], and 94.6% and 92.0% [38, 88], respectively). Apolipoproteins are abundant in plasma and hence urinary concentrations will be influenced by haematuria. The study by Chen et al [88] included 13 control subjects with haematuria and their urinary APOA1 was slightly elevated; the authors concluded that urinary APOA1 might need to be interpreted with reference to haematuria. As a urinary biomarker, APOE appears less useful than APOA1 [36-38, 48].

We were unable to find any consistent evidence of high sensitivity or specificity for any of the remaining proteins mentioned in 5 unequivocal studies (**ICAM-1**, **β-gonadotropin, E-cadherin, carbonic anyhdrase IX**), although we note that carbonic anhydrase IX urinary mRNA has recently been reported as a potentially useful biomarker [89].

In summary, none of the biomarkers investigated in $\geq$ 5 equivocal studies has the sensitivity and specificity required to act as a standalone biomarker for detecting bladder cancer. Finally, in our search for unproven but possible biomarkers we manually searched the reports for the proteins presented in <5 equivocal studies for those the highest sensitivity and specificity. MMP3 and TIMP2 showed high sensitivity and specificity in studies carried out in Egypt [49, 51]. These results should be treated with caution as in Egypt many cases of bladder cancer are bilharzial SCC. Additionally, TIMP2 has been reported to have lower sensitivity and specificity in other studies. A small study by Gecks et al [90] suggested that Tenascin-C can be used to detect recurrent bladder cancer with 91% sensitivity at 80% specificity. In a study using urine from 68 cases (mixed stage and grade), 68 healthy controls and 16 patients with cystitis Lorenzi et al [91] reported that measuring the serine peptidase HTRA-1 gave 92.7% sensitivity and 95.6% specificity for bladder cancer detection. At the

time of writing no further studies have corroborated (or refuted) the biomarker potential of HTRA1-1.

Prognostic urinary biomarkers

Urinary biomarkers have the potential to inform not only on the presence or absence of bladder cancer, but also to provide prognostic information. Such a biomarker would provide information on outcome and could guide choices between conservative and radical treatment regimens. The word 'prognostic' has been applied variably to urinary biomarkers. For example, high levels of biomarker post resection are often reported as a poor prognostic indicator, but are most likely just indicative of residual disease. A truly prognostic indicator should indicate outcome in patients with tumours; not versus those without tumours. The majority of urinary protein biomarkers considered in this review increase in concentration with both stage and grade of disease and could therefore be considered as prognostic indicators. However, very few studies have directly investigated the association between urinary biomarker levels at presentation and outcome, and even fewer have investigated whether urinary biomarkers can provide prognostic information over and above that provided by standard clinicopathological factors (Table 2). Indeed, at the time of writing, only BTA, CEA, MMP9, tenascin-C, cystatin-B and the soluble extracellular domains of EGFR and EpCAM have been reported as independent prognostic indicators (Table 2) and these data require independent validation. The two NMP22 prognostic studies listed in Table 2 confuse disease detection and prognosis, although further literature searching identified a large study by Shariat et al [92] which found that including pre-treatment urinary NMP22 levels slightly improved the ability of nomograms to predict later recurrence.

Biology of urinary biomarkers

The proteins that have been shown to be increased in concentration in the urine of bladder cancer patients are highly diverse in terms of their biological activities, the pathways that they are involved with and their cellular compartmentalisation. They include, amongst others, proteases, lipid binding/transport proteins, cytoskeletal components and cytokines. The most significantly over represented biological processes include "regulation of cell migration", "response to wounding", "regulation of apoptosis" and "inflammatory response" [93]. Eight of the proteins are in the KEGG_PATHWAY "Pathways in Cancer" (survivin, E-cadherin, fibronectin, IL6, MMP2, MMP9, PDGFR and VEGF). Other proteins are less obviously mechanistically linked to cancer with Apo-A1, apo-A4, apoE, clusterin, fibrinogen, fibronectin, thrombin and α1-antitrypsin all classed as plasma proteins and with α1-antitrypsin, thrombin, IL6 and fibronectin also being classed as acute phase proteins. Over half of the proteins are *bona fide* secreted proteins, but there are also 7 cytoplasmic/cytoskeletal and 6 plasma membrane proteins and 2 nuclear proteins (NMP22 and EN2) (Figure 4).

DISCUSSION

We have systematically reviewed the literature concerning urinary proteins as biomarkers for bladder cancer. We focussed solely on proteins which are measured in solution in urine, rather than proteins present in cancer cells in the cell pellet or DNA, RNA or metabolite biomarkers. Thus, all of the biomarkers discussed above can be measured by immunoassay

in a single urine sample which may enable biomarker multiplexing and point-of-care testing in the future.

We found that the majority of urine biomarker studies use patient populations enriched for high-grade and high-stage disease which is likely to inflate estimates of sensitivity and specificity and/or do not provide sufficient information to be thoroughly evaluated. It is clear that the urinary concentrations of the vast majority of proteins investigated as biomarkers positively correlate with both stage and grade of disease. Thus, low stage and grade disease is not easily detected. As low and high-grade bladder cancer can be considered separate entities at the genomic level [94], it might be expected that different urinary biomarkers would be required to detect each. By definition, the cancer cells are still relatively normal, both genomically and phenotypically, in low-grade disease so that although some alterations in gene expression have been noted [95], the processes involved in releasing proteins into the urine may be essentially normal. With high-grade and invasive disease it is likely that many of these processes are unregulated such that proteins released directly from the cancer cells, as breakdown products from the extracellular matrix, as a result of inflammatory responses or plasma proteins all find their way into the urine. Thus, although high-grade bladder cancer is a heterogeneous disease, it is perhaps not surprising that many of the urinary proteins reviewed can detect high-grade and stage disease with high sensitivity. A panel of protein markers, carefully selected on the basis of close associations with the various molecular sub-types of bladder cancer might offer the way forward in facile non-invasive detection of bladder cancer.

Our review shows that the use of pre-treatment urinary biomarker levels for prognostication has been addressed in very few studies compared with disease detection.

This may be due to the long-term follow up of patients required for such studies, the lack of a perceived need for such biomarkers or the fact that in patients with a confirmed tumour molecular markers can be measured directly in the tumour rather than in urine. It is however possible that processes which release proteins from tumours (secretion, leakage, shedding) and degradation of the surrounding urothelium are important prognostic indicators and are more effectively measured in urine than in the tumour itself.

In accord with others, we find that the "validated detection markers" (NMP22, BTA, UBC, Cyfra 21-1) do not rival flexible cystoscopy in terms of sensitivity and specificity [96]. We also find that many other biomarkers with are often reported as "promising" are not genuinely promising as their sensitivity or specificity is too low, or the evidence supporting their utility is equivocal. Finally, we have highlighted a small number of proteins that might warrant further validation and hopefully be confirmed as clinically useful biomarkers.

There are several limitations related to the review methodology. Firstly we may have introduced publication bias as we couldn't access all of the publications online, however, as most were published before the year 2000 we reasoned that if that particular biomarker was significant, then the publication trail would have continued beyond 2000. Secondly, the review may be skewed to more positive data due to publication bias present in the literature i.e. only positive results get published. Thirdly, as we only included studies which reported both sensitivity and specificity, we could have been affected by outcome reporting bias i.e. where studies report selected outcomes usually those that are positive [97]. Despite these limitations we believe this review presents a comprehensive summary of the literature and a searchable database of proteins that have been investigated to date (Table S1), which will aid researchers searching for better urinary biomarkers. We conclude that:

- The majority of urine biomarker studies contain bias or are insufficiently reported.
- The urinary concentrations of a large number of proteins are increased by the presence of bladder cancer, but most proteins are not increased in all cases and are not specific to bladder cancer.
- NMP22, BTA, UBC and Cyfra 21-1 are the only well-validated urinary protein biomarkers and their sensitivity and specificity are well below those of cystoscopy.
- Fibronectin, clusterin, CEACAM1, apolipoprotein A4, calprotectin, CD147, coronin-1A, DJ-1, reg-1, stathmin-1, and ϒ-synuclein may be considered as possible biomarkers.
- Biomarkers supported by multiple "equivocal" studies include CEA, MMP9, VEGF, TPA, survivin, CA19-9, APOA1 and BCLA-4. Of these, only BLCA-4 reportedly has high-sensitivity, but is mired in controversy.
- Biomarkers supported by a single "equivocal" report of high sensitivity and specificity include MMP3 and HTRA.

None of the urinary protein biomarkers investigated to date can be used for accurate non-invasive detection of bladder cancer. Current efforts to combine protein biomarkers to improve test accuracy also fail to reach clinically useful sensitivity and specificity [48, 98]. DNA/RNA-based markers may supersede protein biomarkers in the near future [99-103]; however, with currently-available technology, these are more complex, expensive and time-consuming to measure than protein markers with little potential for point-of-care testing in the immediate future. Notwithstanding, we hope that the increasing understanding of bladder cancer at the molecular-genomic level may enable selection of the correct cancer-specific proteins (or variants of proteins) to underlie a clinically applicable biomarker panel.

Conflict of Interest

The authors have no conflict of interest to report.

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Table1.

Protein name	Gene symbol	Sensitivity (%)	Specificity (%)	Cancers (n)	Controls (n)	Refs
Alpha-1-anti-trypsin	*SERPINA1*	70.6	71.8	206	102	[104]
Angiogenin	*ANG*	66	75	50	20	[23]
Apolipoprotein A4	*APOA4*	79.2	100	110	66	[34]
Autocrine motility factor receptor	*AMFR*	84	75	45	62	[105]
BIGH3	*TGFBI*	70	80	30	30	[31]
Bladder tumour antigen (BTA)	*BTA*[#]	64	76.6	2258	2994	[11, 12, 55, 106-125]
Calprotectin	*S100A8 & S100A9*	80.4	92.5	46	135	[28]
Cathepsin B	*CTSB*	55.7	56.1	122	107	[126]
Cathepsin L	*CTSL*	71.3	74.8	122	107	[126]
CCL18	*CCL18*	70.4	67.7	206	102	[104]
CD147 (EMMPRIN)	*BSG*	96.7	100	30	30	[31]
CEACAM1	*CEACAM1*	74	95	93	82	[27]
Clusterin,	*CLU*	76.3	86.5	168	151	[22, 23]
Coronin-1A	*CORO1A*	66.7	100	110	66	[34]
CYFRA21-1	*KRT19*	64.4	85.5	293	331	[127-129]
DJ-1	*PARK7*	83.3	100	110	66	[34]
EN2	*EN2*	82	75	466	52	[130]
FDP	*FGA & FGB*	52	91	57	139	[107]
Fibronectin	*FN1*	89	85.6	126	41	[14, 15]
NMP22	*NUMA1*	61.8	80.3	4528	7728	[11, 12, 107-112, 116-118, 120, 123, 126, 131-141]
PDGFRβ	*PDGFRB*	70.6	81.2	117	68	[142]
Prothrombin	*F2*	71.1	75.0	76	80	[143]
Reg-1	*REG1A*	81.3	81.2	32	48	[41]
Semenogelin -2	*SEMG2*	66.7	80	110	66	[34]
Stathmin-1	*STMN1*	90.0	86.7	30	30	[31]
Urinary bladder carcinoma antigen (UBC)	*KRT8 & KRT18*	64.4	80.3	753	1072	[11, 12, 106, 112, 113, 131, 144-148]
γ-synuclein,	*SNCG*	87.5	90.0	110	66	[34]

Table 2.

Marker	No. of studies	No. of patients	Comments	Refs
BTA	1	97	Independent prognostic indicator	[149]
Carcinoembryonic antigen (CEA)	3	425	Independent prognostic indicator	[44, 47, 150]
β-HCG	1	52	Prognostic in MIBC	[151]
EGFR	1	436	Independent prognostic indicator	[152]
EpCAM	1	607	Independent prognostic indicator	[152]
MMP9	1	188	Independent prognostic indicator	[153]
NMP22	2	333	Detection-prognosis	[109, 154]
Plasminogen Activator Inhibitor type I (PAI-1)	1	244	Not prognostic	[155]
PDGFRβ	1	185	Predicts recurrence in NMIBC	[142]
Tenascin-C	1	66	Independent prognostic indicator	[156]
Tissue polypeptide antigen (TPA)	1	97	Prognostic	[157]
Urinary sFas	1	128	Predicts recurrence in NMIBC	[158]
Urine tumour-associated trypsin inhibitor(TATI)	1	157	Not prognostic	[159]
Cystatin-B	1	47	Independent prognostic indicator	[160]

LEGENDS.

Table 1. Summary of unequivocal biomarker studies. For proteins with multiple studies, sensitivity and specificity are presented as means weighted according to sample size in each study. [#]indicates potentially several genes. NMP22, BTA and UBC data include studies using quantitative and point-of-care versions of the assay.

Table 2. Prognostic urinary biomarkers for bladder cancer.

Figure 1. Search strategy outline and results.

Figure 2. Numbers of publications for the most commonly investigated urinary protein biomarkers. Papers providing measurement data included, reviews excluded.

Figure 3. The history and lifecycle of bladder cancer biomarkers. The number of publications for each biomarker with >10 publications in total is shown for each half-decade from 1971. CEA peaks in the 1970s and TPA in the 1980s. BTA and cyfra 21-1 peak in 1996-2000 whilst fibronectin, NMP22 and UBC peak in 2001-2005. The rate of publication of all of these biomarkers are now declining whereas MMP9 and VEGF continue to rise.

Figure 4. Cellular compartmentalisation of protein biomarkers reported ≥ 1 unequivocal or ≥5 unequivocal biomarker studies.

Figure 1.

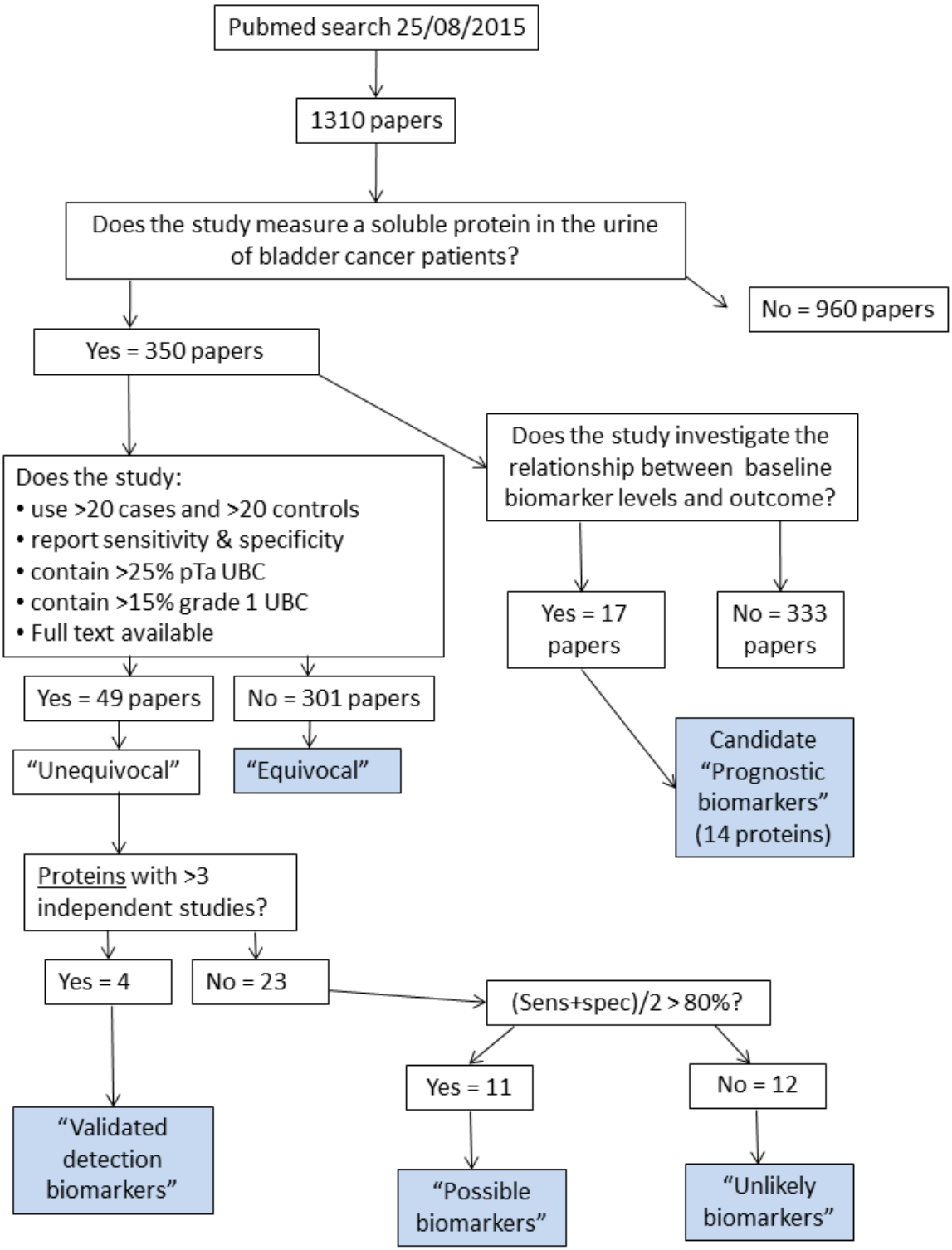

Pubmed search 25/08/2015
1310 papers
Does the study measure a soluble protein in the urine of bladder cancer patients?
No = 960 papers
Yes = 350 papers
Does the study investigate the relationship between baseline biomarker levels and outcome?
Does the study:
• use >20 cases and >20 controls
• report sensitivity & specificity
• contain >25% pTa UBC
• contain >15% grade 1 UBC
• Full text available
Yes = 17 papers
No = 333 papers
Yes = 49 papers
No = 301 papers
"Unequivocal"
"Equivocal"
Candidate "Prognostic biomarkers" (14 proteins)
Proteins with >3 independent studies?
Yes = 4
No = 23
(Sens+spec)/2 > 80%?
Yes = 11
No = 12
"Validated detection biomarkers"
"Possible biomarkers"
"Unlikely biomarkers"

Figure 2.

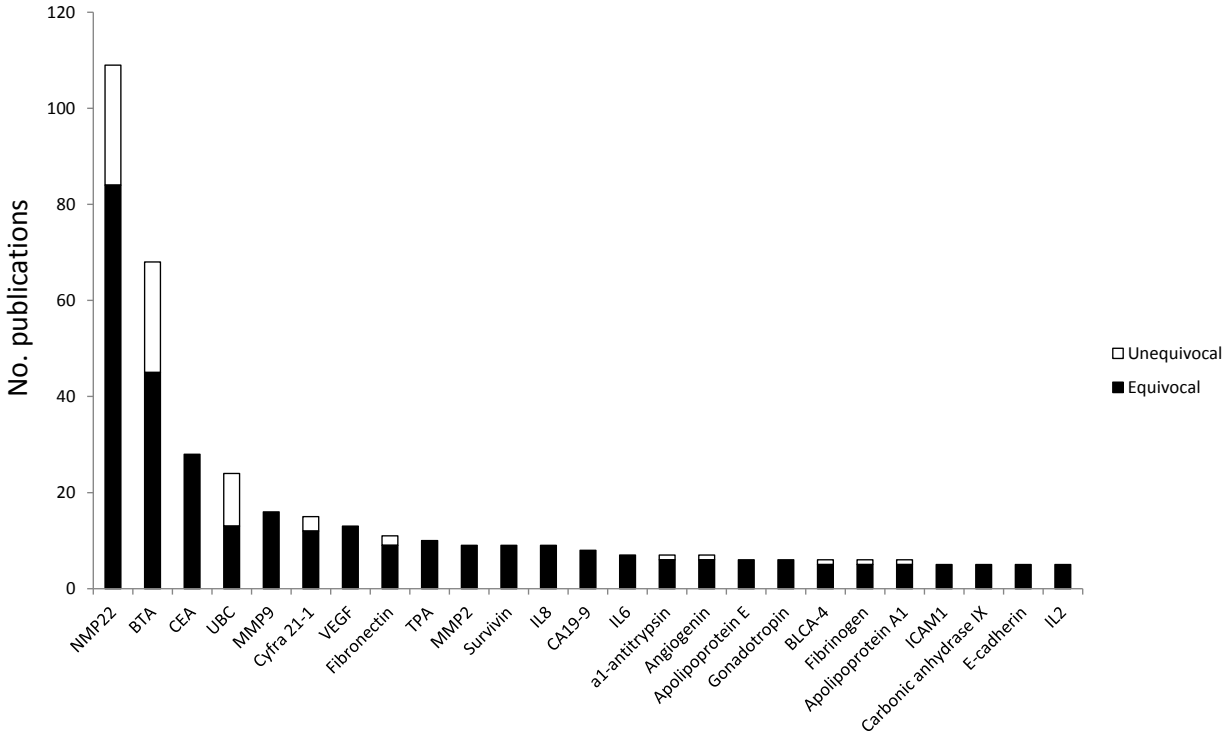

120
100
80
60
40
20
0
No. publications
Unequivocal
Equivocal
NMP22
BTA
CEA
UBC
MMP9
Cyfra 21-1
VEGF
Fibronectin
TPA
MMP2
Survivin
IL8
CA19-9
IL6
a1-antitrypsin
Angiogenin
Apolipoprotein E
Gonadotropin
BLCA-4
Fibrinogen
Apolipoprotein A1
ICAM1
Carbonic anhydrase IX
E-cadherin
IL2

Figure 3.

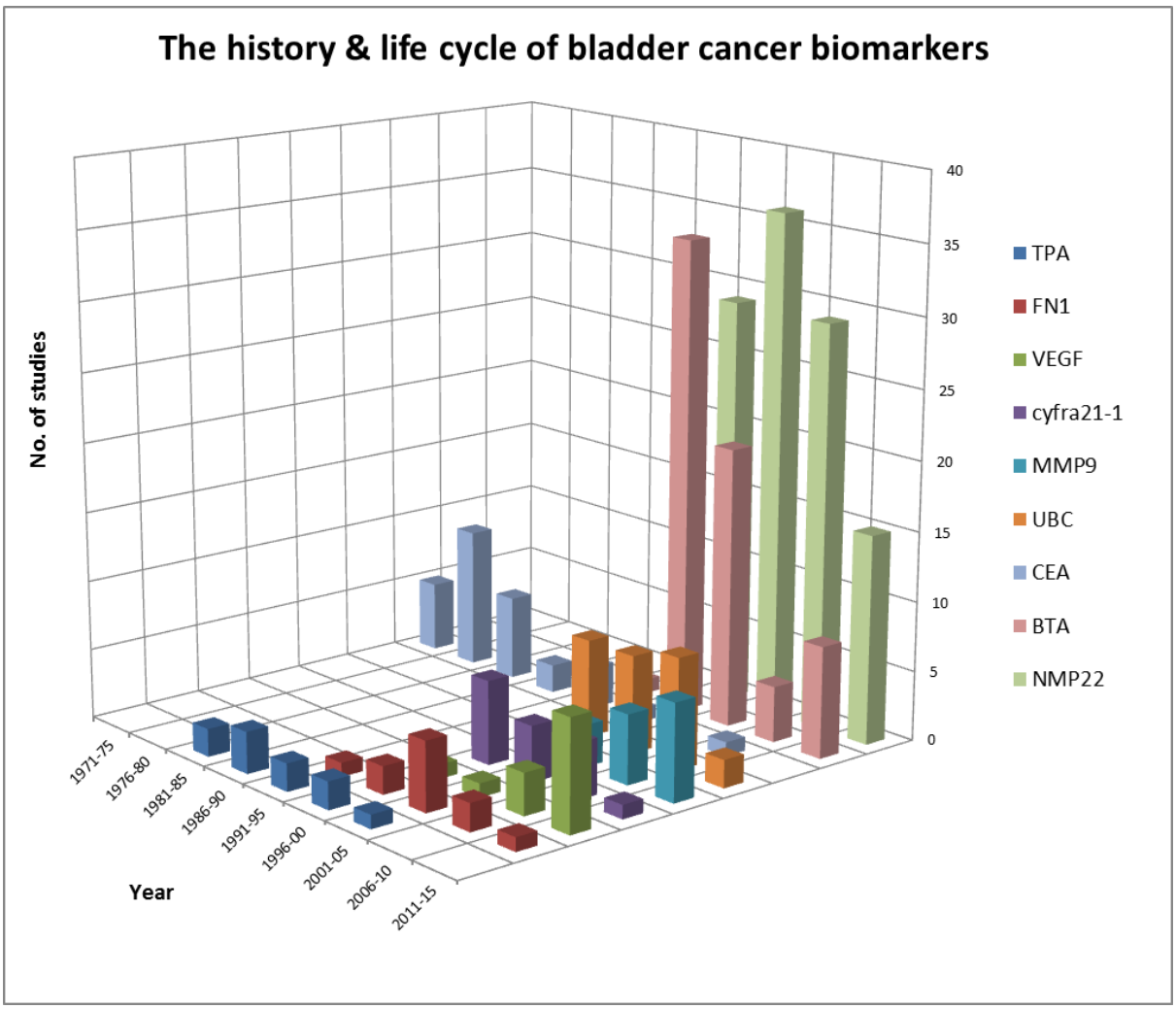
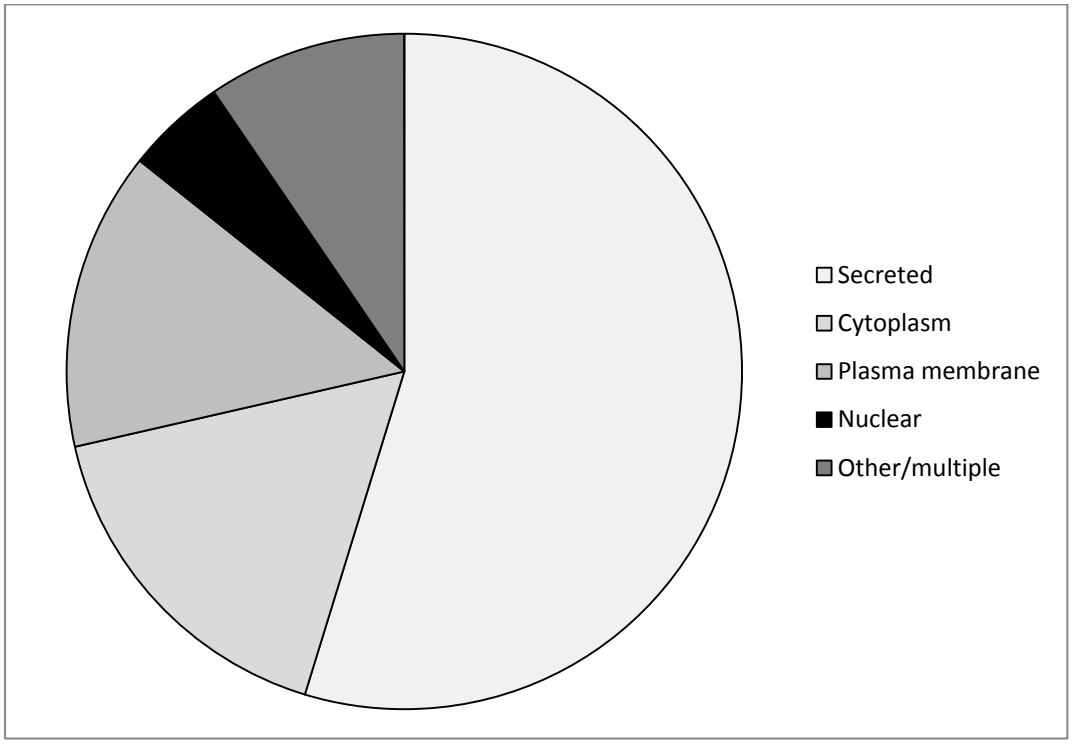

The history & life cycle of bladder cancer biomarkers
No. of studies
40
35
30
25
20
15
10
5
0
1971-75
1976-80
1981-85
1986-90
1991-95
1996-00
2001-05
2006-10
2011-15
Year
TPA
FN1
VEGF
cyfra21-1
MMP9
UBC
CEA
BTA
NMP22

Figure 4.


Secreted
Cytoplasm
Plasma membrane
Nuclear
Other/multiple

Supplemental Information Table S1. List of unequivocal biomarker studies.

Gene names are provided where available. Assays for which the biomarker cannot be unambiguously identified as the product of a single particular gene are indicated with an asterisk.

Proteins	Gene symbols	Reference
alpha-1-antitrypsin	SERPINA1	Yang, Feng et al. 2011
IL8, MMP9, MMP10, SERPINA1, VEGFA, ANG, CA9, APOE, SDC1, and SERPINE1	IL8 MMP9 MMP10 SERPINA1 VEGFA ANG CA9 APOE SDC1 SERPINE1	Chen, Chang et al 2014
Acidic Fibroblast growth factor	FGFA	Chopin, Caruelle et al. 1993
ADAM28, SPINK5, PTP1	ADAM28 SPINK5 PTP1	Tyan, Yang et al. 2011
Afamin, Alpha-1-anti-trypsin, Alpha-2-HS-glycoprotein,Angiotensinogen, Apolipoprotein A-II precursor, Apolipoprotein L1, Complement C9, nter-alpha-trypsin inhibitor HC, Plasminogen, Thrombospondin-1 , Transferrin	AFM SERPINA1 AHSG AGT APOA2 APOL1 C9 ITIH-X PLG THBS1 TF	Chen, Chen et al 2012
alpha-1-antitrypsin Apoloprotein E	SERPINA1 APOE	Urquidi V, Goodison S, et al 2012
Angiogenin	ANG	Eissa, Kenawy et al. 2004
Angiogenin, VEGF, Carbonic anyhdrase IX, BTA	ANG VEGF* CA9 Complement_factor_H_related_protein*	Urquidi V, Goodison S, et al 2012
Apo-A1	APOA1	Li, Li et al 2011
Apo-A1	APOA1	Li, Li et al 2013
Apo-A1	APOA1	Li, Li et al. 2014
APOA1 APOA4 Heparin cofactor II Peroxiredoxin-2	APOA1 APOA4 SERPIND1 PRDX2	Chen, Chen et al 2010
APOA1, APOA2, APOB, APOC3, APOC2, APOE, APOA4, TIM, SAA4, ProEGF	APOA1 APOA2 APOB APOC3 APOC2 APOE APOA4 SAA4 TIM EGF	Chen, Lin et al. 2013
APOA2	APOA2	Chen, Chen et al. 2015
Autocrine motility factor (AMF)	Autocrine_motility_factor*	Guirguis, Schiffmann et al. 1988
Basic fetoprotein	Basic_fetoprotein*	Ichikawa, Nakayama et al. 2000
Basic Fetoprotein	Basic_fetoprotein*	Tsujii, Yonese et al. 1990
BLCA-4	BLCA-4_(unknown)	Van Le, Miller et al. 2005 RETRACTED
beta human chorionic gonadotrophin	gonadotropin*	McLoughlin, Pepera et al. 1993
Beta-2 Microglobulin	B2M	Engström 1988
Bikunin	AMBP	Tsui, Tang et al. 2010
BLCA-4	BLCA-4*	Feng, Wang et al. 2011
BLCA-4	BLCA-4*	Konety, Nguyen et al 2000

BLCA-4	BLCA-4*	Konety, Thu-Suong et al 2000
BLCA-4	BLCA-4*	Myers-Irvin, Landsittel et al 2005
BLCA-4	BLCA-4*	Shiff, Veltri et al. 2006
BTA	Complement_factor_H_related_protein*	Blumenstein, Ellis et al. 1999
BTA	Complement_factor_H_related protein*	Chautard, Daver et al 2000
BTA	Complement_factor_H_related_protein*	Gutiérrez Baños , Martín García et al 1998
BTA	Complement_factor_H_related_protein*	Gutiérrez Baños , Martín García et al 1998
BTA	Complement_factor_H_related_protein*	Heicappell, Wettig et al. 1999
BTA	Complement_factor_H_related_protein*	Heicappell, Müller et al. 2000
BTA	Complement_factor_H_related_protein*	Irani,Desgrandchamps et al 1999
BTA	Complement_factor_H_related_protein*	Kirillos, McDermott et al 1997
BTA	Complement_factor_H_related_protein*	Khaled, Abdel-Salam et al 2001
BTA	Complement_factor_H_related_protein*	Nasuti, Gomella et al. 1999
BTA	Complement_factor_H_related_protein*	Raitanen, Kaasinen et al. 2001
BTA	Complement_factor_H_related_protein*	Raitanen, Hellström et al. 2001
BTA	Complement_factor_H_related_protein*	Takashi, Schenck et al. 1999
BTA	Complement_factor_H_related_protein*	Yogi, Ikeuchi et al. 1991
BTA	Complement_factor_H_related_protein*	Gomez, Rodriguez et al, 2002
BTA	Complement_factor_H_related_protein*	Quek, Chin et al. 2002
BTA	Complement_factor_H_related_protein*	Wang, Xu et al. 1999
BTA	Complement_factor_H_related_protein*	Miyanaga, Akaza et al. 1997
BTA	Complement_factor_H_related_protein*	The United Kingdom and Eire Bladder Tumour Antigen Study Group 1997
BTA NMP22	NUMA1 Complement_factor_H_related_protein*	Gutiérrez Baños, Rebollo Rodrigo et al. 2000
BTA	Complement_factor_H_related_protein*	Rodríguez Martínez, Escaf Barmadah et al. 2000
BTA, Survivin	Complement_factor_H_related_protein* BIRC5	Davies, Chen et al. 2005
BTA, UBC	Complement_factor_H_related_protein* KRT8 KRT18	Babjuk, Soukup et al. 2008
BTA, Urinary Bladder cancer antigen	Complement_factor_H_related_protein* KRT8 KRT18	Hazzaa, Elashry et al 2010,
BTA, Urinary Bladder cancer antigen	Complement_factor_H_related_protein* KRT8 KRT18	Vlahou, Giannopoulos et al. 2004
BTA, NMP22, Survivin, CD44, VEGF	NUMA1 Complement_factor_H_related_protein* BIRC5 CD44 VEGF*	Sun, He et al. 2006
BTA	Complement_factor_H_related_protein*	Krupski, Moskaluk et al 2000
BTA	Complement_factor_H_related_protein*	Mattioli, Seregni et al. 2000
CA 19-9	CA19-9*	Vestergaard, Wolf et al. 1998
CA19-9	CA19-9*	Noto, Fujime et al. 1997
CA19-9	CA19-9*	Pal, Roy et al. 2011
CA19-9	CA19-9*	Roy, Dasgupta et al. 2013
CA19-9	CA19-9*	Chuang & Liao 2004
CA19-9, Carcinoembryonic	Carcinoembryonic_antigen*	Cicigoi, Rocca Rossetti et al. 1986

antigen		
CA19-9, Carcinoembryonic antigen (CEA), Tissue polypeptide antigen (TPA)	CA19-9* Carcinoembryonic_antigen* Tissue_polypeptide_antigen*	Casetta, Piana et al. 1993
CA19-9, DU-PAN-2	CA19-9* DUPAN2*	Nagao, Itoh et al. 2007
Calreticulin	CALR	Kageyama, Isono et al. 2004
Calreticulin	CALR	Kageyama, Isono et al. 2009
Calreticulin annexin A2 annexin A3	CALR ANXA2 ANXA3	Lu, Lin et al. 2014
Calreticulin, Catechol-o-methyltransferase, γ-synuclein, BTA	CALR COMT SNCG Complement_factor_H_related_protein*	Iwaki, kageyama et al 2004
Carcinoembryonic antigen	Carcinoembryonic_antigen*	Colleen, Ek et al 1979
Carcinoembryonic antigen	Carcinoembryonic_antigen*	Coombers, Hall et al. 1975
Carcinoembryonic antigen	Carcinoembryonic_antigen*	Saied, El-Metenawy et al. 2007
Carcinoembryonic antigen	Carcinoembryonic_antigen*	Wahren, Edsmyr et al. 1975
Carcinoembryonic antigen	Carcinoembryonic_antigen*	Wahren & Edsmyr 1978
Carcinoembryonic antigen	Carcinoembryonic_antigen*	Wahren, Nilsson et al. 1982
Carcinoembryonic antigen	Carcinoembryonic_antigen*	James, Alroy et al. 1980
Carcinoembryonic antigen	Carcinoembryonic_antigen*	Nilsson, Wahren et al. 1982
Carcinoembryonic antigen (CEA)	Carcinoembryonic_antigen*	Nevile, Nery et al 1973
Carcinoembryonic antigen (CEA) /p +IgG, IgA and IgM /p	Carcinembryonic_antigen* Immunoglobulins*	Huland, Otto et al. 1983
Carcinoembryonic antigen (CEA), Ferritin, Tissue polypeptide antigen (TPA)	Carcinoembryonic_antIgen* ferritin* tissue_polypeptide_antigen*	Halim, el-Ahmady et al. 1992
Carcinoembryonic antigen FDP	Carcinoembryonic_antigen* FDP	Wajsman, Merrin et al. 1975
Cathepsin B	CTSB	Eiján, Sandes et al 2000
Cathepsin B	CTSB	Kotaska, Dusek et al. 2012
Carcinoembryonic antigen	Carcinoembryonic_antigen*	Fraser, Ravry et al. 1975
Carcinoembryonic antigen	Carcinoembryonic_antigen*	Gadja, Tyloch et al. 1995
Carcinoembryonic antigen	Carcinoembryonic_antigen*	Guinan, McKiel et al. 1978
Carcinoembryonic antigen	Carcinoembryonic_antigen*	Ionescu, Romas et al. 1976
Carcinoembryonic antigen	Carcinoembryonic_antigen*	Jakse, Rauschmeier et al. 1983
Carcinoembryonic antigen	Carcinoembryonic_antigen*	Klippel, Axt et al. 1983
Carcinoembryonic antigen	Carcinoembryonic_antigen*	Korsetn, Persijn et al. 1976
Carcinoembryonic antigen	Carcinoembryonic_antigen*	Murphy, Vandevoord et al 1977
Carcinoembryonic antigen	Carcinoembryonic_antigen*	Oshiumi, Yagi et al. 1978
Carcinoembryonic antigen	Carcinoembryonic_antigen*	Oshiumi, Yagi et al. 1979
Carcinoembryonic antigen	Carcinoembryonic_antigen*	Tailly, Cornelissen et al. 1983
Carcinoembryonic	Carcinoembryonic_antigen*	Zimmerman, Wahren et al.

antigen		1980
CEA & TPA	Carcinoembryonic_antigen* tissue_polypeptide_antigen*	Stefanović, Mitić-Zlatković et al. 1999
CEA, TPA and CA 19-9	Carcinoembryonic_antigen* tissue_polypeptide_antigen* CA19-9*	Tizzani, Cassetta et al. 1987
Chorionic Gonadotropin(CG),	gonadotropin*	Iles, Jenkins et al. 1989
Chorionic Gonadotropin(CG),	gonadotropin*	Iles, Persad et al. 1996
Chorionic Gonadotropin(CG), Chorionic Gonadotropin β-Subunit [GCβ](Core fragment as well in Urine)	gonadotropin*	Hotakainen, Haglund et al. 2002
Clusterin	CLU	Stejskal & Fiala 2006
CXCL1	CXCL1	Kawanishi, Matsui et al. 2008
Cyfra 21-1	KRT19	El-Ahmady, Halim et al. 1999
Cyfra 21-1	KRT19	Nisman, Yutkin et al 2009
Cyfra 21-1	KRT19	Senga, Kimura et al 1996
Cyfra 21-1 UBC TPA NMP22	KRT8 KRT18 KRT19 NUMA1 Tissue_polypeptide_antigen*	Sánchez-Carbayo, Herrero et al. 1999
CYFRA 21-1, VEGF	KRT19 VEGF*	Bian and Xu 2007
CYFRA21-1	KRT19	Dittadi, Barioli et al 1996
CYFRA21-1	KRT19	Pariente, Bordenave et al 1997
CYFRA21-1 FDP NMP22 UBC	KRT19 FGA&FGB NUMA1 KRT8 KRT18	Jeong, Park et al 2012
CYP1A1	CYP1A1	Dörrenhaus,Müller et al 2007
Cystatin B	CSTB	Feldman, Banyard et al. 2009
cytokeratin	cytokeratin*	Basta, Attallah et al. 1988
Cytokeratin	cytokeratin*	Helmy, Seddek et al. 1991
D-dimer, IL-6, IL-8, sFAS, VEGF, BTA, NMP22	FGA&FGB IL6 CXCL8 FAS VEGF* Complement_factor_H_related_protein* NUMA1	Abogunrin, O'Kane et al 2012
DEK	DEK	Datta, Adelson et al. 2011
E-Cadherin	CDH1	Banks, Porter et al. 1995
E-Cadherin	CDH1	Protheroe, Banks et al 1999
E-cadherin	CDH1	Shi, Laudon et al. 2008
EGFR, EpCAM	EGFR EpCAM	Bryan, Regan et al. 2015
EpCAM	EpCAM	Bryan, Shimwell et al. 2014
Epidermal growth factor (EGF)	EGF	Messing and Murphy-Brooks 1994
epithelial membrane antigen, NMP52	MUC1 NMP52	Attallah, El-Far et al. 2015
Fas	FAS	Yang, Li et al 2013
FDP, NMP22, BTA	FGA&FGB NUMA1 Complement_factor_H_related_protein*	Oeda and Manabe 2001)
Ferritin ,Carcinoembryonic antigen (CEA) Beta-2 Microglobulin	Ferritin* Carcinoembryonic_antigen* B2M	Ohashi, Tohjoh et al. 1983
Fibronectin	FN1	Wunderlich, Reichelt et al. 2001
FGB, APOE, Alpha-1-antitrypsin and LRG1	FGB APOE LRG1 SERPINA1	Lindén, Lind et al. 2012
Fibronectin	FN1	Eissa, Zohny et al 2010

Fibronectin	FN1	Malmstrom, Larson et al 1993
Fibronectin	FN1	Ménendez, Fernández-Suárez et al 2005
Fibronectin, CK18	FN1 KRT18	Sánchez-Carbayo, Urrutia et al. 2000
glutathione S-transferase P1	GSTP1	Lafuente, Rodriguez et al. 1998
Heparin-binding epidermal growth factor–like growth factor (HB-EGF) ,Epidermal growth factor (EGF)	EGF HBEGF	Keay, Zhang et al. 2001
HIP/PAP	REG3A	Nitta,Konishi et al 2012
Histone H2B NIF-1	Histone_H2B* ZNF335	Frantzi, Zoidakis et al. 2013
HSP60, HSP70, HSP90, Interferon-γ, Tumour necrosis factor-α, Tumour growth factor-β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13	HSPD1 HSP70 HSP90AA1 IFNG TNF TGFB IL1B IL2 IL4 IL5 IL6 CXCL8 IL10 IL13	Margel, Pevsner-Fischer et al. 2011
HtrA1	HTRA1	Lorenzi, Lorenzi et al. 2013
ICAM1	ICAM1	Chow, Cheng et al. 1998
ICAM1	ICAM1	Shi, Goya et al 1998
IL-11	IL11	Wu, Tao et al. 2013
IL-18, IL-2, IFN-gamma, IL-12, IL-4	IL18 IFNG IL12 IL4	Eto, Koga et al. 2005
IL-1b,	IL1B	Martins, Darlin et al. 1994
IL-1b, IL- 2, IL- 6, IL- 8, IL-10, IL-12, TNF-a, and IFN-gamma	IL1B IL2 IL6 CXCL8 IL10 IL12 TNFA IFNG	Watanabe, Matsuyama et al. 2003
IL-1b, IL- 2, IL- 6, IL- 8, IL-10, IL-12, TNF-a, IFN-gamma,Intercellular adhesion molecule-1 (ICAM-1)	IL1B IL2 IL6 CXCL8 IL10 IL12A TNFA IFNG ICAM1	Jackson, Alexandroff et al. 1995
IL2, IL6, IL8, TNF Alpha, CYFRA 21-1, NMP22	IL2 IL6 CXCL8 TNFA NUMA1 KRT19	Sanchez-Carbayo, Urrutia et al. 2001
Il-6, IL-10	IL6 IL10	Cai, Mazzoli, et al 2012
IL-6, IL-8, VEGF	IL6 CXCL8 VEGF*	Reid, Stevenson et al. 2012
IL-8	CXCL8	Sheryka, Wheeler et al 2003
IL-8, MMP-9 and 10, PAI-1, VEGF, ANG, CA9 APOE	IL-8 MMP9 MMP10 PAI-1 VEGF* ANG CA9 APOE	Rosser, Ross et al. 2013
IL-8, MMP9, MMP10, SDC1, CCL18, PAI-1, CD44, VEGF, ANG, CA9,A1AT,OPN, PTX3, APOE	CXCL8 MMP9 MMP10 SDC1 CCL18 SERPINE1 CD44 VEGF* ANG CA9 SERPINA1 SPP1 PTX3 APOE	Goodison, Chang et al. 2012
IL-8, MMP9, Syndecan, BTA	CXCL8 MMP9 SDC1 Complement_factor_H_related_protein*	Urquidi, Chang et al. 2012
IL-8, MMP9, VEGFA	CXCL8 MMP9 VEGFA	Rosser, Dai et al. 2014
Insulin-like growth factor 2	IGF2	Watson, Burling et al. 2009
intercellular adhesion molecule-1, NMP 22, Monocyte chemoattractant protein-1.	ICAM1 NUMA1 CCL2	Parekattil, Fisher et al. 2003
Interleukin-2	IL2	Fleischmann, Toossi et al. 1989
Keratin (CK1K10 antibody)	cytokeratin*	Attallah, Helmi et al. 1991
Laminin P1	Laminin*	Abou Farha, Meneheers et

		al. 1993
Midkine, HAI-1, ULBP2	MDK SPINT1 ULBP2	Shimwell, Bryan et al. 2013
MMP1, MMP10	MMP1 MMP10	Du, Lin et al. 2014
MMP2, MMP9	MMP2 MMP9	Gerhards, Jung et al. 2001
MMP2, MMP9, Cathepsin B, UPA	MMP2 MMP9 CTSB PLAU	Sier, Casetta et al. 2000
MMP2, MMP9, Fibronectin	MMP2 MMP9 FN1	Saito, Kimoto et al. 2005
MMP2, MMP9, MMP2/NGAL, MMP9/TIMP, ADAMTS	MMP2 MMP9 ADAMTS TIMP1 NGAL	Mohammed, Seleim et al. 2013
MMP2, MMP9, NGAL	MMP2 MMP9 LCN2	Fernández, Wszolek et al 2009
MMP2, MMP9, TIMP-2	MMP2 MMP9 TIMP2	Eissa, Ali-Labib et al. 2007
MMP2, MMP9, UBC, TPS, NMP22	MMP2 MMP9 KRT8 KRT18 NUMA1 tissue-polypeptide-specific antigen*	Di Carlo, Terracciano et al. 2006
MMP3, MMP9	MMP3 MMP9	El-Sharkawi, El Sabah et al. 2014
MMP7	MMP7	Jäger, Tschirdewahn et al. 2013
MMP7	MMP7	Szarvas, Singer et al. 2011
MMP9	MMP9	Eissa, Labib et al 2003
MMP9	MMP9	Offersen, Knap et al. 2010
BTA	Complement_factor_H_related_protein*	Thomas, Leyh et al 1999
MMP-9, MMP-2, ADAMTS 7, MMP-9/NGAL complex, MMP9/TIMP complex and MMP-9 dimer	MMP9 MMP2 ADAMTS7 NGAL TIMP1	Roy, Louis et al. 2008
MUC1	MUC1	Xiang, Zhou et al. 2005
nicotinamide N-methyltransferase	NNMT	Sartini, Muzzonigro et al. 2013
NMP2, BTA	NUMA1 Complement_factor_H_related_protein*	Abd El Gawad, Moussa et al. 2005
NMP22	NUMA1	Arora, Sarunban et al. 2010
NMP22	NUMA1	Atsu, Ekici et al. 2002
NMP22	NUMA1	Akaza, Miyanaga et al. 1997a
NMP22	NUMA1	Akaza, Miyanaga et al. 1997b
NMP22	NUMA1	Casella, Huber et al 2000
NMP22	NUMA1	Chahal, Darshane et al. 2001
NMP22	NUMA1	Chang, Wu et al. 2004
NMP22	NUMA1	Hutterer, Karakiewicz et al. 2008
NMP22	NUMA1	ippe, Pandrangi et al. 1999
NMP22	NUMA1	Kapila, Kehinde et al 2008
NMP22	NUMA1	Kumar, Kumar et al. 2006
NMP22	NUMA1	Kundal, Pandith et al 2010
NMP22	NUMA1	Landman, Change et al 1998
NMP22	NUMA1	Lahme, Bichler et al 2000
NMP22	NUMA1	Lekili, Sener et al. 2004
NMP22	NUMA1	Menendez, Filella et al 2000
NMP22	NUMA1	Moonen, Kiemeney et al. 2005
NMP22	NUMA1	O'Sullivan, Sharples et al

		2012
NMP22	NUMA1	Oge, Atsu et al. 2001
nmp22	NUMA1	Onal, Han et al. 2015
NMP22	NUMA1	Paoluzzi,Cuttano et al 1999
NMP22	NUMA1	Pérez García, Escaf Barmadah et al 2000
NMP22	NUMA1	Ponsky, Sharma et al. 2001
nmp22	NUMA1	Raina, Pahlajani et al. 2008
NMP22	NUMA1	Sagnak, Ersoy et al. 2011
NMP22	NUMA1	Sánchez-Carbayo, Herrero et al 1999
NMP22	NUMA1	Serreta, Lo Presti et al 1998
NMP22	NUMA1	Serretta, Presti et al 1998
NMP22	NUMA1	Shariat, Savage et al. 2011
NMP22	NUMA1	Shariat, Zippe et al. 2005
NMP22	NUMA1	Schlake, Crispen et al. 2012
NMP22	NUMA1	Soloway, Briggman et al 1996
NMP22	NUMA1	Srivastava, Arora et al. 2012
NMP22	NUMA1	Thomas, Leyh et al 1999
NMP22	NUMA1	Todenhöfer, Hennenlotter et al. 2014
NMP22	NUMA1	Lotan, Elias et al. 2009
NMP22	NUMA1	Lotan, Capitanio et al. 2009
NMP22	NUMA1	Ueda, Kawaguchi et al. 2009
NMP22	NUMA1	Rodríguez , Justo et al. 2008
NMP22	NUMA1	Mansoor, Calam et al. 2008
NMP22	NUMA1	Ihm, Kim et al. 2007
NMP22	NUMA1	Chen, Han et al. 2007
NMP22	NUMA1	Hautmann, Eggers et al. 2007
NMP22	NUMA1	Fatela-Cantillo, Fernandez-Suarez et al 2007
NMP22	NUMA1	Darenkov, Perlin et al. 2006
NMP22	NUMA1	Xin, You et al. 2006
NMP22	NUMA1	Kitukawa, Yamamoto et. 2006
NMP22	NUMA1	Yokoyama, Sekigawa et al. 2004
NMP22	NUMA1	Su, Yang et al. 2003
NMP22	NUMA1	Perez-Garcia, Eyo et al. 2002
NMP22	NUMA1	Miyoshi, Matsuzaki et al. 2001
NMP22	NUMA1	Zippe, Pandrangi et al. 1999
NMP22	NUMA1	Zippe, Pandrangi et al. 1999
NMP22	NUMA1	Lahme, Bichler et al. 2001
NMP22	NUMA1	Stampfer,Carpinito et al 1998
NMP22 BTA	NUMA1 Complement_factor_H_related_protein*	Casetta, Gontero et al. 2000
NMP22 BTA	NUMA1 Complement_factor_H_related_protein*	Friedrich, Hellstern et al. 2002
NMP22 BTA	NUMA1 Complement_factor_H_related_protein*	Friedrich, Hellstern et al. 2003

NMP22, BTA, Basic Fetoprotein(BFP)	NUMA1 BFP* Complement_factor_H_related_protein	Miyanaga, Akaza et al. 2003
NMP22, cytokeratin-18	NUMA1 KRT18	Song, Du et al. 2009
NMP22 MCM5	NUMA1 MCM5	Kelly, Dudderidge et al. 2012
NMP22, BTA	NUMA1 Complement_factor_H_related_protein*	Abbate, D'Introno et al. 1998
NMP22, BTA	NUMA1 Complement_factor_H_related_protein*	Miyake, Nakai et al 2014
NMP22, Fibronectin	NUMA1 FN1	Eissa, Swellam et al 2002
NMP22,BTA	NUMA1 Complement_factor_H_related_protein*	Serretta, Pomara et al 2000
NMP22,E-cadherin, cathepsin D	NUMA1 CDH1 CTSD	Salama, Selem et al. 2012
NMP22, BTA	NUMA1 Complement_factor_H_related_protein*	Bhuiyan, Akhter et al. 2003
NMP22, BTA	NUMA1 Complement_factor_H_related_protein*	Sharma, Zippe et al 1999
NMP22, Urinary Bladder Cancer test(UBC)	NUMA1 KRT8 KRT18	Kibar, Goktas et al. 2006
NMP52	NMP52*	Attalah, Sakr et al. 2005
Oncofetal fibronectin	FN1	Alías-Melgar, Neave-Sánchez et al. 2013
Orosomucoid(ORM), zinc-alpha2-glycoprotein(ZAG)	ORM1 AZGP1	Irmak, Tilki et al. 2005
PAI-1, BTA, CD44, CCL18	SERPINE1 CD44 CCL18 Complement_factor_H_related_protein*	Urquidi V, Kim et al 2012
Plasminogen Activator Inhibitor type I (PAI-1)	SERPINE1	Becker, Szarvas et al. 2010
Prothymosin-alpha	PTMA	Tzai, Tsai et al. 2006
Pro-u-PA	PLAU	Lin , Tsui et al. 2006
Psoriasin	S100A7	Celis, Rasmussen et al. 1996
sCD14	CD14	Jackson, Lien et al. 1997
Serine/threonine-protein kinase PLK2	PLK2	Tan, Chen et al. 2010
SH3BGRL3	SH3BGRL3	Chiang, Pan et al. 2015
Soluble carbonic anhydrase IX (s-CAIX)	CA9	Hyrsl, Zavada et al. 2009
Soluble E-Cadherin	CDH1	Shariat, Matsumoto et al. 2005
Soluble intercellular adhesion molecule-1	ICAM1	Aboughalia 2006
Soluble Met (sMet)	MET	McNeil, Sorbellini et al. 2014
survivin	BIRC5	Abd El-Halim, El-Shafie et al. 2014
Survivin	BIRC5	Hausladen, Wheeler et al. 2003
Survivin	BIRC5	Li, Wang et al 2013
Survivin	BIRC5	Srivastava, Singh et al. 2013
Survivin	BIRC5	Sharp, Hausladen et al. 2002
Survivin, NMP22	BIRC5 NUMA1	Shariat, Casella et al. 2004
Survivin, CYFRA 21-1	BIRC5 KRT19	Ohsawa, Nishimura et al. 2004
Syndecan-1	SDC1	Miyake, Lawton et al. 2014
TACSTD2	TACSTD2	Chen, Lai et al 2012
Tenascin C(B and C domains)	TNC	Richter, Tost et al. 2009
Tenascin-C	TNC	Gecks, Junker et al. 2011
Tenascin-C	TNC	Guan, Zeng et al. 2014

TGFα, VEGF	TGFA VEGFA	Hameed and el-Metwally 2008
Thromboxane receptor	TBXA2R	Moussa et al. 2011
TIMP-2 survivin	TIMP2 BIRC5	Eissa, Shabayek et al. 2010
Tissue factor	F3	Lwaleed, Francis et al. 2000
Tissue Polypeptide Antigen	Tissue_polypeptide_antigen*	Costello and Kumar 1985
Tissue polypeptide antigen (TPA)	Tissue_polypeptide_antigen*	Carbin, Ekman et al. 1989
Tissue polypeptide antigen (TPA)	Tissue_polypeptide_antigen*	Mack, Scheiber et al. 1987
Tissue polypeptide antigen (TPA)	Tissue_polypeptide_antigen*	Kumar, Costello et al. 1981
Tissue polypeptide antigen (TPA), HER-2/neu(EGF), Urokinase-type Plasminogen Activator Receptor (uPAR) ,TP53 Mutation	Tissue_polypeptide_antigen* EGF PLAUR	Ecke, Schlechte et al. 2005
tissue polypetide specific antigen	tissue-polypeptide-specific antigen*	Boman, Hedelin et al. 2001
tissue-polypeptide-specific antigen (TPS)	tissue-polypeptide-specific antigen*	Sánchez-Carbayo, Urrutia, et al 2000
tissue-polypeptide-specific antigen (TPS)	tissue-polypeptide-specific antigen*	Yao, Chang et al. 1995
UBC	KRT8 KRT18	Hedelin, Jonsson et al. 2006
UBC	KRT8 KRT18	Heicappell, Schostak et al. 2000
UBC	KRT8 KRT18	Sánchez-Carbayo, Herrero et al 1999
UBC	KRT8 KRT18	Gacci, Serni et al. 2006
UBC CYFRA 21-1 NMP22	KRT8 KRT18 KRT19 NUMA1	Sánchez-Carbayo, Urrutia et al. 2001
Urinary bladder carcinoma antigen (UBC), Urine tumor-associated trypsin inhibitor(TATI), CYFRA 21-1	KRT8 KRT18 KRT19 SPINK1	Gkialas, Papadopoulos et al 2008
Urinary gonadotropin peptide (UGP)	gonadotrophin*	El-Ahmady, Halim et al 1996
Urinary sFas	FAS	Srivastava, Singh et al 2014
Urinary sFas, NMP22	FAS NUMA1	Svatek, Herman et al. 2006
Urine tumor-associated trypsin inhibitor(TATI), NMP22	SPINK1 NUMA1	Shariat, Herman et al. 2005
Urine tumour-associated trypsin inhibitor(TATI)	SPINK1	Kelloniemi, Rintala et al. 2003
Urokinase-type Plasminogen Activator(uPA),Urokinase-type Plasminogen Activator Receptor (uPAR)	PLAU PLAUR	Casella, Shariat et al. 2002
Urokinase-type Plasminogen Activator(uPA),Urokinase-type Plasminogen Activator Receptor (uPAR), NMP22	PLAU PLAUR NUMA1	Shariat, Casella et al. 2003
Uroplakin	Uroplakin*	Lai, Ye et al. 2010
VEGF	VEGF*	Crew, O'Brien et al 1999
VEGF	VEGF*	Jeon, Lee et al 2001
VEGF, angiogenin	VEGF* ANG	Urquidi, Goodison, et al 2012

Vitamin D binding protein	GC	Li, Chen et al
Fibronectin	FN1	Hegele, Heidenreich et al. 2003