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DOI:
10.1016/j.euf.2019.02.012

PROGNOSTIC DNA METHYLATION BIOMARKERS IN HIGH-RISK NON-MUSCLE INVASIVE BLADDER CANCER: A SYSTEMATIC REVIEW TO IDENTIFY LOCI FOR PROSPECTIVE VALIDATION

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Key words: High risk non-muscle-invasive bladder cancer; prognostic markers; biomarkers; molecular markers; DNA; methylation; systematic review

Word count: 4705 (+ 300 for Abstract)

Declarations: RT Bryan has contributed to advisory boards for Olympus Medical Systems with regard to narrow band imaging cystoscopy.

ABSTRACT

Context: High-risk non-muscle-invasive bladder cancer (HR-NMIBC) represents over 30% of all incident urothelial bladder cancers (BCs); patients are at risk of progression, and 20-30% will die from BC within 5years. Current guidelines recommend induction and maintenance intravesical BCG or upfront radical cystectomy for highest risk disease, treatments with markedly different morbidity, mortality and patient burden. There are no validated biomarkers to facilitate such treatment decisions. Alterations in DNA methylation are commonplace in BC; hence, measurable changes in DNA methylation represent an opportunity for the discovery of such biomarkers.

Objective: To systematically assess the evidence regarding DNA methylation markers as prognosticators for HR-NMIBC.

Evidence acquisition: Standard systematic review methods were employed with searches undertaken in MEDLINE and EMBASE and PubMed up to December 2018. Studies that included patients with HR-NMIBC and investigated the utility of DNA methylation biomarkers as prognostic tools were included.

Evidence synthesis: Of 63 prognostic biomarker studies identified, 21 met the protocol-driven inclusion criteria and were directly relevant to HR-NMIBC patient outcomes: tumour recurrence (TR), tumour progression (TP), disease-specific survival (DSS), overall survival (OS). These studies described 140 methylation markers; of these, the most promising were: *CDH13* (Hazard ratios (HRs): 5.1 for TR, 6.6 for TP, 3.8-8.0 for OS)*, PCDHs* (HRs: 4.7.for TR, 2.5 for TP, 3.0-4.8 for OS)*, RUNX3* (HR: 5.1 TP), *HOXA9* (HR: 1.9 for TR), *ISL1* (HRs: 1.7 for TR, 3.3 for TP), and *PAX6* (HR: 2.2 for TR).

Conclusions: This systematic review identifies a number of potentially useful prognostic methylation markers for HR-NMIBC. These loci (*CDH13*, *PCDH*s, *RUNX3*, *HOXA9*, *ISL1*, and *PAX6*) should be validated in prospective studies in order to translate benefit to patients.

Patient summary: Early bladder cancer represents a more complex spectrum of disease than can be assessed by conventional methods. Emerging studies on molecular markers will improve our understanding of this disease and may enable more precise and personalised treatment.

1. INTRODUCTION

Bladder cancer (BC) is a common cancer worldwide with an estimated 430,000 new cases in 2012, and with highest incidence in Southern and Western Europe, North America, as well as in certain countries in North Africa and Western Asia [1]. Over 75% of cases present as non-muscle invasive bladder cancer (NMIBC: stages Tis/Ta/T1) at diagnosis, but NMIBCs are a heterogeneous group of cancers with variable risks of recurrence and progression [2, 3]. Currently, the European Organisation for Research and Treatment of Cancer (EORTC) and the Club Urologico Español de Tratamiento Oncolȯgiro (Spanish Urological Oncology Group, CUETO) risk calculators, incorporating clinical and pathological parameters, are available for predicting the risks of recurrence and progression [4, 5]. To ensure optimal treatment, the European Association of Urology (EAU) guidelines categorise those tumours with the highest risk of recurrence and progression as "high-risk" NMIBC (HR-NMIBC) and these include tumours with high grade (G3), invasion into lamina propria (pT1), and carcinoma in situ (CIS) [3]. However, debate exists as to whether tumours are reliably categorised and whether the EORTC and CUETO tools are themselves consistently reliable [6-9]. Thus, the EAU guidelines advocate the search for molecular markers which may help to improve current risk assessment tools [3]. Better risk stratification may not only enable better risk prediction but may also translate into better outcomes owing to improved treatment selection, particularly when the recommendations for HR-NMIBC include intravesical BCG or radical cystectomy – markedly different treatments that can be associated with significant morbidity and differing impacts on HRQoL [3].

Epigenetics concerns the heritable regulation of gene expression independent of changes in genomic DNA sequence [10]. Not only important in embryogenesis and evolution, epigenetic dysregulation can lead to numerous pathologies including cancer [11]. DNA methylation is one of the most common mechanisms of epigenetic regulation essential for constitutional cellular homeostasis and its dysregulation is thought to be important in the pathogenesis of many cancers; methylation in

promoter regions can silence the expression of those genes [11]. DNA methylation can therefore be exploited as a biomarker to detect carcinogenesis and may also be used to target therapy [12]. In BC, methylation markers are reported as important in the detection of tumours and in predicting the risk of disease recurrence and progression [13-15]. The objective of this systematic review was to identify and evaluate methylation markers with utility in predicting clinical outcomes in HR-NMIBC; such an exercise could identify those markers worthy of further investigation and/or validation, whilst curtailing futile research on markers that have shown no or weak prognostic association. Additionally, by knowing which genes are likely silenced in BC by methylation, insights into the molecular basis of HR-NMIBC and its escape from current therapies may be obtained. With the ultimate aim of improving patient outcomes and with the knowledge that locus-specific DNA methylation assays represent mature technology ready for clinical applications [16], those methylation markers demonstrating the strongest evidence may subsequently be prospectively evaluated in clinical studies, either alone or as adjuncts to current risk calculators.

2. EVIDENCE ACQUISITION

2.1 Protocol

This review formed part of a wider survey of all prognostic biomarkers in the treatment of HR-NMIBC; therefore, the predetermined protocol was not focused on methylation studies alone. The review was undertaken according to standard systematic review methods with some adaptation to searching methodology due to the difficulty in locating some laboratory-based publications.

2.2 Search strategy

Initial scoping searches of published studies in PubMed, Embase, Health Technology Assessment Database, National Institute for Health Research (NIHR) database, American Society of Clinical Oncology (ASCO) conference proceedings and databases specific for systematic reviews, such as the

Cochrane Database of systematic reviews and the Prospective Register of Systematic Reviews (PROSPERO), confirmed the need for undertaking this systematic review (Appendix 1). Searches for primary publications related to the wider survey of all prognostic biomarkers. Searches were undertaken in Medline in-process citations and daily update (OvidSP) and EMBASE (OvisSP) bibliographic databases up to 1st of January 2018. To mitigate against limitations of indexing in this area, a combination of Medical Subject Headings (MeSH) and free text terms were employed (Appendix 1). Known publications in the field were utilised to refine the search terms and validate search results. The search strategy comprised of specific terms for four search strings – "bladder cancer", "high risk", "prognostic" and "biomarker". For BC, several terms such as "urothelial carcinoma", "carcinoma *in-situ*", and "transitional cell carcinoma" yielded appropriate results. For the 'prognostic' string, various prognostic terms were used, including "predictor of outcome", "predictor of progression", and "predictor of survival". To identify the high-risk group, terms included were "high-grade", "grade 3", "T1G3", and "G3" (Appendix 2). Biomarker studies are especially difficult to locate as they may not be explicitly named as such; therefore, general and specific biomarker terms including "biomarker," "protein," "DNA," "metabolite" and "methylation" were included in the search. The full search strategy in both databases is shown in Appendix 3. Endnote X7 was used to store and sort the searches. The four search strings were utilised again on 15th January 2019 in a PubMed search to update the review.

2.3 Eligibility and selection of relevant studies for inclusion

Initial screening of titles and abstracts was undertaken according to pre-specified inclusion and exclusion criteria (Appendix 4). Screening was undertaken independently by two pairs of investigators (AB & RTB, PMSG & JH). All studies that investigated patients with HR-NMIBC and reported prognostic data in relation to specific biomarkers were included; these studies investigated DNA methylation biomarkers measured in tumour tissue and/or circulating tumour DNA. HR-NMIBC patients included CIS, T1 and high-grade/grade 3 tumours as defined by EAU guidelines [3]. Previous

131 treatment was not an excluding factor, and no age restrictions were applied. All examples of HR-
132 NMIBC patients with sufficient prognostic information regarding a biomarker were considered.
133 Foreign language publications were screened using English abstracts and translations were obtained
134 where necessary and possible.
135
136 **2.4 Data extraction**
137 Studies that met the inclusion criteria were evaluated and reported in a Microsoft Excel
138 spreadsheet, with predefined data fields. Information collected included general study
139 characteristics: citation, study aim, number of patients, grade and stage of tumours, treatments,
140 targets studied, methylation analysis technique, and the gene(s) associated with prognosis. Details
141 of outcomes comprised of the Overall Survival (OS), Disease-Specific Survival (DSS), Disease-Specific
142 Mortality (DSM), Tumour Progression (TP) and Tumour Recurrence (TR).
143
144 **2.5 Method of evidence synthesis**
145 Due to the heterogeneity of the studies, a narrative synthesis was undertaken. Quality assessment
146 of the studies was undertaken utilising the Reporting Recommendations for Tumour Marker
147 Prognostic Studies (REMARK) as a template (Appendix 5) [17].
148
149
150 **3. EVIDENCE SYNTHESIS**
151 **3.1 Search outputs**
152 The original literature searches yielded 2343 publications after duplicates had been removed. Of
153 these, 1145 were potentially relevant to all biomarkers, with 52 of these relevant to DNA
154 methylation. The updated search in January 2019 added a further 64 potentially relevant
155 publications, 11 of which were relevant to DNA methylation. After scrutiny of full texts, 21 studies

met the inclusion criteria, as displayed in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) diagram (**Figure 1**) [18-38].

3.2 General Study Characteristics

The 21 studies were all retrospective cohort studies (**Table 1**). Of these, 11 studies focused on the prognostic significance of individual markers, whilst 10 studies evaluated a panel of markers. Across the studies, the requirements for patient inclusion comprised: histopathological confirmation of TCC, no previous history of malignant tumours, no chemotherapy or radiotherapy prior to treatment, sufficient sample availability and follow-up data. The number of patients evaluated per study ranged from 50 to 1239 patients, over a follow-up period of 2-235 months. The premise for most studies was based on previous findings implicating a link between frequent promoter hypermethylation of the chosen markers and BC; the studies selected in this review intended to validate this. Across all the studies, a targeted approach to analyse methylation was employed and this was most frequently measured in tumour specimens using MS-PCR (methylation specific polymerase chain reaction) assays with both relative and absolute methodologies. Across all the studies, the prognostic outcomes were defined by tumour recurrence (TR), tumour progression (TP), overall survival (OS), disease-specific survival (DSS) or disease-specific mortality (DSM). Most studies defined the prognostic endpoint by the overall survival time, and the survival analysis was determined from Kaplan-Meier survival curves and accompanying statistical tests. In general, the studies illustrated a relative risk between promoter hypermethylation and ensuing clinicopathological associations by hazard ratios (HRs) greater than 1.

Five of the studies explored protocadherin (*PCDH*) promoter methylation: *PCDH17, PCDH10* and *PCDH8* and their prognostic role as predictors of overall survival (OS) [18, 20-22]. Additionally, the promotor methylation of cadherin-13 (*CDH13*) and its prognostic role was explored in five studies [23-27]. Two studies evaluated the prognostic association between promoter hypermethylation of

Runt domain transcription factor 3 (*RUNX3*) and tumour recurrence (TR) or tumour progression (TP) [28, 29]. One study found a significant correlation between methylation of *myopodin* and OS [30]. Two studies found an association between RAS association family 1 (*RASSF1A)* methylation and TR or TP [31, 32]. Two independent studies evaluated the correlation *of Islet-1* (*ISL1)* and Homeobox 9 (*HOXA9)* genes' promoter methylation with TR and TP [33, 34]. Two studies, focusing on the promoter methylation of a panel of putative biomarkers, detected a significant correlation of methylation of *MLH1* (MutL homolog 1) and *TIMP-3* (tissue inhibitor of metalloproteinases-3) with OS and TR, respectively [35, 36]. One study, based on hierarchical clustering of genes with differential DSS, found that methylation status of *SOX1*, *PITX2*, *CSPG2* and *JAK3* were independent predictors of DSS on multivariate analysis [37]. One study investigated methylation of *GATA2, TBX2, TBX3* and *ZIC4* independently and in combination with point mutations in *FGFR3, PIK3CA, TERT* and *RAS* [38]. **Table 2** provides a summary of the prognostic methylation markers evaluated in this review.

3.3 *CDH13*

Three studies included in this review demonstrated a significant association between methylated *CDH13* versus unmethylated *CDH13* and worse outcomes for TR, TP, and OS [23-25]. Notably, Lin *et al.* investigated circulating tumour DNA (ctDNA) samples and indicated that methylated *CDH13* may even have significant potential as a non-invasive biomarker in HR-NMIBC [24]. Three studies evaluating the role of methylated *CDH13* in a panel of tumour suppressor and other genes (included in this review) did not identify *CDH13* as a significant prognostic marker [26, 27, 37]. In light of these contradictory data, further investigation is warranted.

3.4 *PCDHs*

Five studies identified an association between promoter methylation in *PCDHs* (*PCDH8, PCDH10* and *PCDH17*) and reduced OS in HR-NMIBC patients [18, 20-22]; across these studies, the HRs were

similar for all three *PCDH* subtypes [18, 20-22]. The most significant finding was the association between methylated *PCDH17* in serum samples and reduced OS, indicating the potential for *PCDH17* as a non-invasive prognostic marker [20]. Together, these findings support the independent prognostic role of methylated *PCDH8, PCDH10* and *PCDH17*.

3.5 *Myopodin*

This review identified a single study supporting the association between hypermethylation of *myopodin* and worse clinical outcomes for patients with T1G3 bladder cancer [30]. Notably, across the 25 markers identified in the literature, methylated *myopodin* had the highest HRs for TP (HR: 11.2) and reduced DSS (HR: 7.6) by multivariate analysis of T1G3 patients [30]. This single study also identified that *myopodin* methylated T1G3 tumours were more likely to recur compared to unmethylated tumours [30]. However, these findings are the first to indicate such an association; therefore, further replication studies are warranted prior to considering clinical development.

3.6 *RASSF1A*

Two studies demonstrated a prognostic role of methylated *RASSF1A* in HR-NMIBC patients [31, 32]. When investigating predominantly patients with carcinoma in situ (CIS), Dhawan *et al.* found that methylation occurs early in carcinogenesis, but was not uncommon in benign controls [31]. Perhaps counterintuitively, it was demonstrated that “superficial” recurrences occur less frequently in those with methylated *RASSF1A* [31]. However, the authors noted that gene expression studies had not been undertaken, and that low-density promoter methylation can occur without gene silencing and/or loss of function [31]. The second study, by Kim *et al.*, demonstrated that methylated *RASSF1A* was an independent prognostic factor for TP in 301 patients with HR-NMIBC [33]. However, in this systematic review, another study investigating methylated *RASSF1A* amongst a panel of potential biomarkers did not find a significant prognostic role for *RASSF1A* [35]. Therefore, further studies are required for validation.

3.7 *RUNX3*

Two studies significantly correlated methylated *RUNX3* status with clinical outcomes in BC [28, 29]. Yan *et al.* demonstrated that *RUNX3* methylation was associated with increased tumour grade, stage and number [28]. Additionally, *RUNX3* methylation status was an independent predictor of TP in NMIBC by univariate and multivariate analyses [28]. Furthermore, combining tumour grade and *RUNX3* methylation status revealed that patients with high grade (G3) tumours and *RUNX3* methylation had a significantly worse progression-free survival compared to patients with lower grade or unmethylated tumours (HR: 19.5) [28]. In the second study, Ha *et al*. investigated the prognostic potential of *RUNX3* methylation and *MGC17624* expression, and their findings indicated that a combination of unmethylated *RUNX3* and increased *MGC17624* expression correlated with good prognosis [29]. Kaplan-Meier estimates showed a statistically significant association of *RUNX3* methylation with TP (P=0.03), whilst reduced *MGC17624* mRNA expression was observed in patients with higher stage, higher grade and more progressive disease (P<0.05) [29].

3.8 *MLH1*

In the included study by Wojtczyk *et al.*, decreased *MLH1* mRNA expression was significantly correlated with worse OS in 50 patients with a median follow-up of 3 years (p=0.032) [36]. In particular, *MLH1* promoter methylation status was demonstrated to significantly correlate with poor OS (p=0.006) [36]. However, this is a single small study in which only 25 HR-NMIBC patients were studied. Another study also interrogated *MLH1* methylation amongst a panel of biomarkers and did not identify *MLH1* methylation status as predictive of clinical outcomes [35].

3.9 *HOXA9*, *ISL1* and *ALDH1A3*

Two included studies demonstrated a significant correlation of *HOXA9* and *ISL1* methylation status with clinical outcomes in HR-NMIBC [33, 34]. Kitchen *et al.* evaluated the targeted methylation of a panel of genes in a cohort of 51 patients with HR-NMIBC, and *HOXA9* and *ISL1* methylation status

was found to correlate significantly with clinical outcomes [33]. At one year following primary diagnosis, *HOXA9* and *ISL1* promoter methylation had an 84.2% and 87.5% positive predictive value, respectively, for TP and/or TR [33]. On logistical regression analyses, only *ISL1* methylation status was a significant independent predictor of TP and/or TR [33]. Additionally, *HOXA9* methylation status was significantly predictive of DSM [33]. The findings were concordant with a previous study, by Kim *et al.* demonstrating the association of *HOXA9*, *ISL1* and *ALDH1A3* methylation with prognosis in HR-NMIBC [34]. On univariate and multivariate Cox regression analyses, methylation status of the markers was significantly associated with recurrence (*HOXA9, ISL1* and *ALDH1A3*) and progression (*ISL1* and *ALDH1A3*) [34]. Nevertheless, neither *HOXA9* nor *ISL1* were independent predictors of DSS in the study by Lopez *et al.* [37].

3.10 *TIMP-3*

One study demonstrated a significant association between *TIMP-3* methylation status and recurrence-free survival in patients with HR-NMIBC [35]. Interestingly, this study by Freidrich *et al.* also included *MLH1* and *RASSF1A* but did not find methylation status of either as being significant predictors of clinical outcomes [35].

3.11 Others: multiple genes from screened panels

Several studies interrogated panels of methylated genes to investigate methylation status and clinical outcomes in HR-NMIBC (**Table 1**) [26, 27, 31, 33-38]. In particular, two studies analysed the methylation status of a panel of 25 TSGs using methylation-specific, multiplex, ligation-dependent probe amplification (MS-MLPA) [26, 27]. Notably, in both studies, *PAX6, RB1* and *PYCARD* methylation were identified as prognostic markers. Sacristan *et al.* evaluated the methylation of TSGs as predictors of recurrence, progression, DSS and OS [26]. Univariate and multivariate analyses revealed independent prognostic value of the methylation of these TSGs in each subgroup of NMIBC with recurrence as the clinical endpoint [26]. In both univariate and multivariate models, the most

commonly recurring pT1LG and pT1HG tumours demonstrated methylated *RB1* and *PYCARD*, respectively [26]. Furthermore, the presence of methylation in *VHL* and *THBS1* in pT1LG, and *PYCARD* in pT1HG tumours, indicated a 100% positive predictive value for recurrence, whereas *TP73, ESR1, PTEN, MGMT, PAX6* and *RB1* methylation provided 100% positive predictive values for progression in pT1HG tumours [26]. In addition, *TP73* and *PAX6* demonstrated a positive predictive value of 100% for DSS in the pT1HG cohort [26]. Moreover, Agundez *et al.* demonstrated that patients with methylated *PAX6* were most likely to have recurrent tumours, whilst tumours with unmethylated *MSH6, RB1, THBS1, PYCARD, TP73, ESR1,* and *GATA5* had high TP, and unmethylated *GATA5* was associated with shorter DSS [27]. However, none of these genes were independent predictors of outcome in multivariate or univariate analyses [27]; likewise, *ESR1* was not an independent predictor of DSS in the study by Lopez *et al*. [37]. Agundez *et al.* further analysed combinations of TSGs to enhance the prognostic significance of these markers [27]. Several marker combinations were found to have independent predictive values for progression by multivariate analysis, most significantly the combination of *THBS1* and *MSH6* (HR: 0.226; 95% CI: 0.0074-0.693; P=0.004) [27]. van Kessel *et al.* analysed the methylation status of *GATA2, TBX2, TBX3,* and *ZIC4* (independently and in combination with point mutations in *FGFR3, PIK3CA, TERT* and *RAS*) in 1239 primary and recurrent NMIBCs of all grades and stages derived from the UROMOL study [38]. In 333 HR-NMIBCs, *GATA2* methylation was associated with reduced time to TP (HR: 2.04; 95% CI: 1.01-4.10; P=0.046), and the combination of *GATA2* methylation and *FGFR3* mutation status segregated HR-NMIBCs into good, moderate, or poor subclasses with regard to TP (P<0.01) [38]. Together, these results suggest that methylated TSGs, especially in combination, may be useful in predicting TR, TP and DSS in HR-NMIBC patients. These findings are worthy of further evaluation and validation.

3.12 Study quality

We used the REMARK checklist to assess study quality [17]; most of the included studies could be considered as imperfect by these criteria (Figure S1). Specific recurrent weaknesses included:

- poor descriptions of patient selection, including the nature of patient recruitment and sampling (prospective, retrospective, consecutive, etc.), exclusion criteria, and treatments (re-TUR, intravesical therapy regimens, etc.);
- specifying whether assays were performed blind to outcomes or not;
- sample size calculations, the handling of missing data and dropouts, and biomarker cut-offs;
- the reporting of further investigations, such as internal validations and sensitivity analyses.

Furthermore, some studies appeared to demonstrate the use of the same patient cohort and biospecimens for multiple publications (example [20-23, 25, 28, 29, 32, 34]); this information was not transparent from the individual publications themselves.

4. DISCUSSION

Bladder cancer is a heterogeneous disease, consisting of multiple subtypes that display varying therapeutic responses and survival rates [39]. HR-NMIBCs are particularly challenging to manage due to their highly-recurrent nature and risk of progression to muscle-invasive disease [39]. In order to appropriately risk stratify and select optimal treatment, existing prognostication tools are limited and may consequently result in under- or over-treatment [40]. Molecular markers may better risk stratify patients by inferring more accurate prognostication than EAU risk groups alone [41], thus permitting clinicians to determine the best therapeutic strategy for individual patients [42]. Aberrant methylation is a common epigenetic abnormality in BC, with an established role in tumour initiation and progression [43]. Although several studies have reported significant results, the prognostic value of such markers requires validation ahead of clinical translation [42]. To select markers that merit further investigation, the current study provides a systematic review of the

published literature to date regarding prognostic methylation markers in HR-NMIBC and has identified several potentially promising prognostic markers (**Table 2**). Notwithstanding, in the setting of HR-NMIBC, deconvoluting tumour-specific biomolecular characteristics that are the intrinsic determinants of outcomes (prognostic biomarkers) from the multiple and complex tumour- and patient-specific characteristics that are determinants of BCG responses (predictive biomarkers) is challenging. It should be noted that only 3/21 studies gave detailed descriptions of the adjuvant intravesical therapies utilized [26, 27, 30], and no study incorporated the administration of intravesical BCG (either binary as yes/no, categorically as no/induction/induction plus maintenance, or continuously as the number of doses) as a factor for multivariate modelling. Furthermore, analysis of different CpG sites within the same promoter CpG island may lead to widely varying associations with gene transcription [44], and potentially widely varying associations with outcomes. For this reason, and where possible, we tried to incorporate the details of specific loci into the reporting of our findings (**Table 2**). However, where stated, it was notable that there was inconsistent terminology used for the descriptions of the loci analysed, making comparisons between studies of the same gene promoters challenging. The platform used to assess methylation also introduces variability between studies, potentially influencing findings. As demonstrated by the BLUEPRINT consortium, relative assays are generally less accurate and less concordant with each other than absolute assays although, despite lower quantitative accuracy, relative assays robustly distinguish methylated and unmethylated regions [16]. Notwithstanding, AmpliconBS and Pyroseq technologies are the recommended approaches for analyzing highly-fragmented and/or low amounts of input DNA [16], such that would be found in circulating tumour (ct)DNA or urinary cell-free (cf)DNA [45]; it was notable that neither approach was utilized in the three studies reviewed here that analysed serum-derived DNA [18, 20, 23], although the presence of ctDNA itself may indicate worse prognosis [46].

4.1 Promising prognostic methylation markers

On the basis of evidence in the literature proposing a biological rationale, a reported plausible prognostic role of these markers in BC and other human malignancies, and consistently significant associations with relevant clinical outcomes in HR-NMIBC between studies (confirmed by parameters of notable statistical size effects such as high HRs), we consider the most promising methylation markers for HR-NMIBC prognostication to be those associated with *CDH13, PCDHs, RUNX3, HOXA9* and *ISL1*, and *PAX6*.

Cadherins (CDHs) play an essential role in cell-cell adhesion in epithelial tissues and abnormal expression has been associated with increased invasiveness in BC by facilitating epithelial-to-mesenchymal transition (EMT) and the development of a cancer stem cell phenotype [47]. The human *CDH13* gene (chromosome 16q24) is a member of the cadherin superfamily and also plays an essential role in tumour suppression [48]. Aside from our findings in this review, a number of other studies also support the importance of *CDH13* in carcinogenesis [48-50]. Like other members of the cadherin superfamily, protocadherins (*PCDHs*) also have tumour suppressor functions, with aberrant methylation of their promoter regions leading to permanent gene repression and ensuing tumorigenesis in human BC [51].

RUNX3 belongs to the *RUNX* (Runt-related transcription factor) family of genes, encoding transcription factors which bind to DNA, partnering with cofactors to form complexes that regulate cellular growth, survival and differentiation [52, 53]. *RUNX3* was first reported as a tumour suppressor on account of its causal loss of expression in gastric carcinogenesis [54]. Subsequently, loss of *RUNX3* by promoter methylation has been demonstrated in various human malignancies including BC [55].

HOXA9 is a homeodomain-containing transcription factor which has an important role in hematopoietic stem cell expansion and is commonly dysregulated in acute leukaemias [56]. Although also implicated in other malignancies, *HOXA9* methylation had been demonstrated as potentially relevant in the early detection of BC in one previous report [57]. As with *HOXA9*, Islet-1 (*ISL1*) is also a homeodomain-containing transcription factor; it was initially cloned from rat pancreatic insulin-producing cells, where it binds the insulin gene enhancer [58]. *ISL1* has been implicated in a number of human cancers but reports of its role in BC are limited [33, 34].

PAX6, a member of the PAX family of transcription factors, is an evolutionarily highly conserved gene with important roles in the development of the eye and central nervous system [59, 60]. Methylation of *PAX6* has been demonstrated in tumour cell lines and human tumour tissues including BC [61]. However, the functional relationship between *PAX6* methylation, PAX6 expression and cancer progression is likely to be tissue-specific with studies describing both oncogenic and tumour suppressor effects. In the current review, two studies demonstrated significant associations between *PAX6* methylation and increased risks of TR [26, 27].

4.2 Strengths & limitations

A systematic method was used to search for studies to be included in this review using a robust search strategy to maximise identification of all relevant literature and to establish current understanding in the field, whilst also highlighting where further knowledge is required to guide future research. The review was conducted by two groups of independent investigators (PMSG, JH and AB, RTB) in order to ensure accuracy and avoid bias in study selection. Results from the included studies were recorded in a pre-defined data extraction table to clearly display the outcomes from the individual studies and to identify any discrepancies. The pre-determined inclusion and exclusion criteria were essential in avoiding bias whilst searching and scrutinising records.

Moreover, due to publication bias, articles reporting a positive result (e.g. with a potentially beneficial biomarker) are more likely to be published than negative studies, resulting in much research remaining unpublished and thereby making it challenging to identify all data for inclusion in this systematic review [62, 63]. Additionally, published articles may themselves also exclude non-significant results, leading to false representation of data [62]. Furthermore, a proportion of papers are not indexed in searchable databases, and so those findings may remain undiscoverable [63]. There was also noticeable heterogeneity in outcome reporting. Our pre-determined data extraction sheet was intended to minimise bias and to allow objective comparisons. However, data extraction to objectively clarify outcome measures and their relevant statistical associations was still problematic (**Table 2**). Some studies were able to present outcome measures with robust statistical analyses, such as multivariate analyses including HRs and accompanying confidence intervals and p-values [18, 20, 21, 24, 37, 38]. Other studies provided statistical significance based on Kaplan-Meier survival estimates [25, 26, 31, 32, 35]. Significant findings of methylation of genes, correlated to the odds of TR or TP, were also calculated and presented using Fisher's exact method for various combinations of genes [33]. Such discrepancies in statistical methodologies and reported endpoints may limit the direct comparison of results from the included studies. Furthermore, follow-up periods were variable. Studies with the largest sample sizes and longest durations of follow-up would generally be expected to produce more robust conclusions; alternatively, excluding smaller studies with significant findings would risk reducing the inclusivity of a systematic review.

NMIBC encompasses a heterogeneous disease of different stages, grades and varying prognoses such that sub-categorisation into different risk groups is clinically useful [3]. Higher grade and higher stage are established indicators of worse prognosis; thus, it was especially relevant for articles to separate the study populations to distinguish the prognostic effects of methylation from those associated with grade and stage. Some studies exclusively investigated HR-NMIBC patients [25-33]; other studies had mixed populations including HR-NMIBC patients [18, 20-23]. Studies utilising such

mixed populations required further scrutiny to ensure that significant results were also applicable to the HR-NMIBC subgroup. As already mentioned, separating the tumour-specific biomolecular characteristics that represent prognostic biomarkers from the complex interactions that are predictive of BCG responses will always be a challenge in this group of patients.

4.3 Recommendations for future research

This systematic review has effectively identified a number of methylation markers which show promising clinical potential (**Table 2**). Across the studies, the majority utilised targeted approaches to evaluate methylation and this may result in some prognostic markers remaining undiscovered, although several such studies had followed-on from unbiased genome-wide discovery phases [26, 27, 64]. At the time of undertaking, this systematic review is the first of its nature; therefore, further independent prognostic studies are required to validate the findings and overcome the limitations outlined above in order to generate more specific details of patient outcome in relation to methylation, and to exclude sources of bias such as variable durations of follow-up and poor descriptions of treatment following initial TURBT (re-TUR, intravesical BCG, etc.). The development of core outcome sets in BC (as developed for localised prostate cancer [65]) would also be of great benefit. Finally, future studies with clearly defined patient populations, using standardised techniques and statistical methods (and satisfying REMARK recommendations [17]) will potentially translate into the clinical adoption and uptake of accurate independent prognostic indicators to facilitate the management of HR-NMIBC.

5. CONCLUSION

This systematic review presents a comprehensive summary of the existing literature of prognostic DNA methylation markers in HR-NMIBC. The heterogeneity of the studies and discrepancies between results prevents the unequivocal endorsement of the selected markers for HR-NMIBC

prognostication. However, these findings have identified several promising markers that are worthy of further investigation: promoter methylation of *CDH13, PCDHs, RUNX3, HOXA9, ISL1* and *PAX6*.

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LEGENDS FOR TABLES & FIGURES

Table 1: General characteristics of the included studies. Pyroseq = Pyrosequencing; MS-PCR = Methylation specific PCR; MS-MLPA = Methylation-specific multiplex ligation–dependent probe amplification; MSRE-PCR = Methylation-sensitive restriction PCR.

Table 2: Overview of included studies and the data available for extraction. DSM = Disease Specific Mortality; DSS = Disease Specific Survival; OS = Overall Survival; TR = Tumour Recurrence; TP = Tumour Progression; HR = Hazard Ratio; CI = 95% confidence interval; OR = Odds Ratio; NA = Not Applicable.

Figure 1: PRISMA diagram.

Table 1: General characteristics of included studies. EnrichmentBS = Enrichment bisulphite sequencing; Pyroseq = Bisulphite pyrosequencing; MS-PCR = Methylation-specific PCR; qMS-PCR = Quantitative methylation-specific PCR; MS-MLPA = Methylation-specific multiplex ligation–dependent probe amplification; MSRE-PCR = Methylation-sensitive restriction PCR, MS-SNuPE = Methylation-sensitive single nucleotide primer extension. See Bock *et al* for specific details and comparisons of DNA methylation assays [16].

Study author [Ref]	**Study year**	**Total no. of patients**	**Age** (years)	**Gender** (Male %)	**Follow-up period** (months)	**Method** (absolute or relative)	**Sample**	**Gene(s) investigated**
van Kessel *et al.* [38]	2018	1239	70 (mean)	77.6	27.0 (median)	MS-SNuPE (absolute)	Tumour specimen	*GATA2, TBX2, TBX3, ZIC4* (plus point mutations in *FGFR3, PIK3CA, TERT, RAS*)
Lopez *et al.* [37]	2017	70	68.5 (mean)	84.3	3-120	EnrichmentBS (absolute)	Tumour specimen	*HOXA11, HOXA9, PENK, CYP1B1, EPHA5, JAK3, EYA4, TAL1, PITX2, CDH11, SOX1, NPY, GSTM2, CCNA1, APC, WT1, TWIST1, HS3ST2, GSTM1, ESR1, ATP10A, FZD9, CSPG2, BDNF, DCC, SOX17, NEFL, ISL1, IPF1, FLT3, CDH13, GATA6, TMEFF2*
Wojtczyk *et al*. [36]	2017	50	64.0 (median)	72.0	36.0 (median)	MS-PCR (*MLH1*) (relative) MSRE-PCR (*MLH3*, *MBD4*) (absolute)	Tumour specimen	*MBD4, MLH1, MLH3*

Kitchen *et al*. [33]	2015	51	-	-	2-67	Pyroseq (absolute)	Tumour specimen	*HOXA9, ISL1, NKX6-2, SPAG6, Z1C1* and ZNF154
Lin *et al*. [22]	2014	233	-	69.1	3-57	MS-PCR (relative)	Tumour specimen	*PCDH8*
Lin *et al*. [25]	2014	178	-	69.7	3-57	MS-PCR (relative)	Tumour specimen	*CDH13*
Luo *et al*. [20]	2014	151	68.0 (median)	70.9	6-57	MS-PCR (relative)	Serum	*PCDH17*
Sacristan *et al*. [26]	2014	251	-	84.9	3-235	MS-MLPA (absolute)	Tumour specimen	*STK11, MGMT-2, GATA5, RARB, CD44, PYCARD, WT1, CDH13, BRCA1, MSH6, GSTP1, TP53, PAX5A, IGSF4, BRCA2, ESR1, MGMT, ATM, TP73, PAX6, THBS1, PTEN, VHL, RB1-2, CDKN2A, RB1, CHFR*
Wang *et al*. [21]	2014	115	68.0 (median)	71.3	6-57	MS-PCR (relative)	Tumour specimen	*PCDH17*
Kim *et al*. [34]	2013	89	64.3 (mean)	81.2	6.1-183.3	Pyroseq (absolute)	Tumour specimen	*HOXA9, ISL1*, *ALDH1A3, EOMES*
Lin *et al*. [18]	2013	107	-	71.0	5-60	MS-PCR (relative)	Tumour specimen	*PCDH10*
Ha *et al*. [29]	2012	179	67.0 (median)	80.4	57.8 (median)	MS-PCR (relative)	Tumour specimen	*RUNX3* and *MGC17624*

Kim *et al*. [32]	2012	301	67.0 (median)	79.1	51.4 (median)	MS-PCR (relative)	Tumour specimen	*RASSF1A*
Lin *et al*. [19]	2012	117	69.0 (mean)	67.5	3-57	MS-PCR (relative)	Serum	*PCDH10*
Lin *et al*. [23]	2012	133	69.0 (median)	70.7	7-60	MS-PCR (relative)	Tumour specimen	*CDH13*
Yan *et al*. [28]	2012	186	67.0 (median)	77.4	51.4 (median)	MS-PCR (relative)	Tumour specimen	*RUNX3*
Lin *et al*. [24]	2011	127	-	69.3	6-57	MS-PCR (relative)	Serum	*CDH13*
Agundez *et al*. [27]	2011	91	-	90.1	90.0 (median)	MS-MLPA (absolute)	Tumour specimen	*TP73, MSH6, VHL, RARB, ESR1, CDKN2A, PAX5A, PTEN, MGMT, PAX6, WT1, CD44, GSTP1, ATM, IGSF4, CHFR, BRCA2, RB1,THBS1, PYCARD, CDH13, TP53, BRCA2, STK11, GATA5*
Alvarez-Múgica *et al*. [30]	2010	170	-	90.0	52.5 (median)	MS-PCR (relative)	Tumour specimen	Myopodin
Dhawan *et al*. [31]	2006	104	73.0 (median)	82.0	105.1 (median)	qMS-PCR (absolute)	Tumour specimen	*p16*, *p14*, *E-cadherin, RARβ2*, *RASSF1a*, and *GSTP1*

Freidrich *et al*. [35]	2005	105	68 (median)	73.0	3-48	MethyLight (relative)	Tumour specimen	*p14ARF, p16 CDKN2A, STAT-1, SOCS-1, DR-3, DR-6, PIG-7, BCL-2, H-TERT, BAX, EDNRB, DAPK, RASSF-1A, FADD, TMS-1, E cadherin, ICAM-1, TIMP-3, MLH-1, COX-2*

Table 2: Overview of included studies and the data available for extraction. DSM = Disease Specific Mortality; DSS = Disease Specific Survival; OS = Overall Survival; TR = Tumour Recurrence; TP = Tumour Progression; HR = Hazard Ratio; CI = 95% confidence interval; OR = Odds Ratio; NA = Not Applicable.

Gene(s) associated with prognosis in HR-NMIBC	Gene product & function	Study (author and reference)	Loci or probes utilised	No. of pts.	Treatment after initial TURBT	Results	Prognostic value
CDH13	Cadherin-13: cell-cell adhesion molecule, tumour suppressor.	Lin *et al.* [23]	Not stated	127	Re-TUR: Unspecified BCG: Unspecified Other: Unspecified	Multivariate analysis: **OS**: HR: 3.832 CI: 1.443-10.176, P<0.007	*CDH13* methylation was an independent predictor of reduced OS.
		Lin *et al*. [24]	Not stated	133	Re-TUR: Unspecified BCG: Unspecified Other: Unspecified	Multivariate analysis: **OS**: HR: 8.034 CI: 3.011-21.438, P<0.0001	*CDH13* methylation in serum-derived DNA was an independent predictor of reduced overall survival.
		Lin *et al*. [25]	Not stated	178	Re-TUR: Unspecified BCG: Unspecified Other: One cycle of intravesical mitomycin C for intermediate- & high-risk BCs	Multivariate analysis: **TR:** HR: 5.147 CI: 2.071-20.177, P=0.0043 **TP:** HR: 6.563 CI: 2.241-21.707, P=0.0016	Methylation of *CDH13* was a predictor of increased TR and TP.

PCDH10	Protocadherin-10: cell-cell adhesion molecule, tumour suppressor.	Lin *et al.* [19]	Not stated	117	Re-TUR: Unspecified BCG: Unspecified Other: Unspecified	Multivariate analysis: **OS:** HR: 3.719 CI: 1.328-10.426, P<0.0001	*PCDH10* methylation in serum-derived DNA was independently associated with a reduced overall survival.
		Lin *et al.* [18]	Not stated	107	Re-TUR: Unspecified BCG: Unspecified Other: Unspecified	Multivariate analysis: **OS:** HR: 3.164 CI: 1.152-8.694, P=0.0009	*PCDH10* methylation was an independent predictor of reduced OS.
PCDH17	Protocadherin-17: cell-cell adhesion molecule.	Wang *et al.* [21]	Not stated	115	Re-TUR: Unspecified BCG: Unspecified Other: Unspecified	Multivariate analysis: **OS:** HR: 3.725 CI: 1.578-9.557, P=0.0067	*PCDH17* methylation was associated with a reduced OS.
		Luo *et al.* [20]	Not stated	151	Re-TUR: Unspecified BCG: Unspecified Other: Unspecified	Multivariate analysis: **OS:** HR: 4.758 CI: 1.871-11.127, P<0.0001	*PCDH17* methylation in serum-derived DNA was associated with a reduced overall survival.
PCDH8	Protocadherin-8: cell adhesion molecule.	Lin et al. [22]	Not stated	233	Re-TUR: Unspecified BCG: Unspecified Other: Unspecified	Multivariate analysis: **TR:** HR: 4.739 CI: 1.872-12.053, P<0.0001 **TP:** HR: 2.523 CI: 1.654-7.431, P=0.036 **OS:** HR: 3.017	*PCDH8* methylation was an independent predictor of increased TR and TP, and reduced 5y OS.

						CI: 1.542-.251, P=0.0015	
Myopodin	Cytoskeleton protein.	Alvarez-Múgica *et al.* [30]	Not stated	170	Re-TUR: No BCG: 6w induction + 1y maintenance (n=108) Other: Weekly intravesical mitomycin C for 6w then every 2w for 6m	Multivariate analysis: **TR:** HR: 2.541 CI: 1.207-5.350, P=0.014 **TP:** HR: 11.227 CI: 1.530-82.361, P=0.017 **DSS:** HR: 7.552 CI: 1.019-55.970 P=0.048	*Myopodin* methylation was associated with increased TR, TP and reduced DSS.
RASSF1A	RAS association domain family 1 isoform A: tumour suppressor gene, roles in signal transduction from G-protein coupled receptors, cell adhesion, cell migration and apoptosis.	Kim *et al.* [32]	Not stated	301	Re-TUR: Yes, for high-grade BCs or when no detrusor in original specimen BCG: Unspecified Other: One cycle of adjuvant intravesical therapy for multiple, large or high-grade BCs	Multivariate analysis: **TR:** HR: 8.559 CI: 1.547-47.364, P=0.014	*RASSF1A* methylation was associated with reduced progression-free survival in recurrent NMIBC.
		Dhawan *et al*. [31]	Not stated	104	Re-TUR: Yes BCG: Yes (unspecified regimen) Other: N/A	Kaplan-Meier survival curve: **TR:** P=0.0038	*RASSF1A* methylation was associated with an increased recurrence-free survival.

RUNX3	Runt-related transcription factor 3: activation or suppression of DNA transcription, interaction with other transcription factors, tumour suppressor.	Yan *et al*. [28]	Not stated	186	Re-TUR: Yes, for high-grade BCs or when no detrusor in original specimen BCG: Unspecified Other: One cycle of adjuvant intravesical therapy for multiple, large or high-grade BCs	**TP:** Multivariate analysis: HR: 5.126 CI: 1.049-25.05, P=0.043	*RUNX3* methylation was associated with increased TP.
		Ha *et al*. [29]	Not stated	179	Re-TUR: Yes, for high-grade BCs or when no detrusor in original specimen BCG: Unspecified Other: One cycle of adjuvant intravesical therapy for multiple, large or high-grade BCs	Kaplan-Meier survival curve: **TP**: P=0.018	*RUNX3* promoter methylation was associated with increased TP.
MLH1	MutL homolog 1: DNA mismatch repair gene.	Wojtczyk *et al*. [36]	Not specified	25	Re-TUR: Unspecified BCG: Unspecified Other: Unspecified	Kaplan-Meier survival curve: **OS:** P=0.006	*MLH1* methylation was associated with reduced OS.

HOXA9* and *ISL1	Homeobox A9: DNA-binding transcription factor regulating gene expression, morphogenesis, and differentiation. Islet-1: transcription factor, regulation of insulin gene expression.	Kitchen *et al.* [33]	*HOXA9* chr7:27,205,000-27,205,200 *ISL1* chr5:50,679,000-50,678,800	51	Re-TUR: Unspecified BCG: Unspecified Other: Unspecified	**TR or TP:** Fisher's exact test: ***HOXA9:*** OR: 4.7 CI: 1.0-22.5, P=0.05 ***ISL:*** OR: 5.7 CI: 2.1-32.1, P=0.047 ***HOXA9 + ISL1:*** OR: 7.9 CI: 0.9-71.1, P=0.067 Independent specificity and NPV for **DSM:** *HOXA9*: 57.1% and 70.6%, respectively *ISL1*: 57.1% and 60.0%, respectively.	Frequent methylation of the genes was independently predictive for TR or TP within one-year of diagnosis in high-grade NMIBC. A combination of methylated *HOXA9* and *ISL1* was a more sensitive indicator of DSM.
HOXA9	As above.	Kim *et al*. [34]	chr7:27,170,287-27,173,690	89	Re-TUR: Yes, for high-grade BCs or when no detrusor in original specimen BCG: Unspecified Other: One cycle of adjuvant intravesical therapy intermediate- and high-risk NMIBC	Multivariate analysis: **TR:** HR: 1.87 CI: 1.14-3.47, P=0.032	*HOXA9* methylation was associated with increased TR.
ISL1	As above.	Kim *et al.* [34]	chr5:50,714,114-50,715,273	89	Re-TUR: Yes, for high-grade BCs or when no detrusor in original specimen	Multivariate analysis: **TR:** HR: 1.71 CI: 1.05-3.47, P=0.039	*ISL1* methylation was associated with increased TR and TP.

					BCG: Unspecified Other: One cycle of adjuvant intravesical therapy intermediate- and high-risk NMIBC	**TP:** HR: 3.30 CI: 1.05-12.92, P=0.041	
ALDH1A3	Aldehyde dehydrogenase 1 family member A3: aldehyde dehydrogenase enzyme.	Kim *et al*. [34]	chr15:99,236,455 -99,238,833	89	Re-TUR: Yes, for high-grade BCs or when no detrusor in original specimen BCG: Unspecified Other: One cycle of adjuvant intravesical therapy intermediate- and high-risk NMIBC	Multivariate analysis: **TR:** HR: 1.68 CI: 1.02-3.16, P=0.044 Multivariate analysis: **TP:** HR: 3.55 CI: 1.07-14.22, P=0.039	*ALDH1A3* methylation was associated with increased TR and TP.
TIMP-3	Tissue inhibitor of metalloproteinases -3: matrix metalloproteinase inhibitor.	Freidrich *et al*. [35]	Location relative to transcription start (bp): +1051/+1143	105	Re-TUR: Unspecified BCG: Unspecified Other: Adjuvant intravesical therapy administered to 91/105	Kaplan-Meier survival curve: **TR:** P=0.0036	*TIMP-3* methylation was correlated with increased recurrence-free survival.
PYCARD	PYD and CARD domain containing: activation of caspase, inflammatory and apoptotic signaling.	Sacristan *et al*. [26]	Probe: 02252-L01737	251	Re-TUR: No BCG: Induction & 6m maintenance for high-grade pT1 Other: Mitomycin C for	Multivariate analysis: **TR:** HR: 2.65 CI: 1.06-6.61, P=0.035	Methylated *PYCARD* was an independent predictor of TR in pT1HG tumours

					6m for low-grade pT1		
		Agundez *et al*. [27]	Probe: 02252-L01737	91	Re-TUR: No BCG: 6-wk course Other: N/A	Kaplan-Meier survival curve: **TP:** P=0.048	Methylated *PYCARD* was associated with reduced TP following BCG.
PAX6	Paired box 6: regulation of DNA transcription.	Sacristan *et al*. [26]	Probe: 03749-L03209	251	Re-TUR: No BCG: Induction & 6m maintenance for high-grade pT1 Other: Mitomycin C for 6m for low-grade pT1	Multivariate analysis: **TR:** HR: 2.23 CI: 1.01-4.9, P=0.044	Methylation increased prediction of TR in pT1LG on multivariate and univariate analysis.
		Agundez *et al*. [27]	Probe: 03749-L03209	91	Re-TUR: No BCG: 6-wk course Other: N/A	Kaplan-Meier survival curve: **TR:** P=0.025	Methylation of *PAX6* indicated increased TR following BCG.
RB1	Retinoblastoma transcriptional corepressor 1: negative regulator of the cell cycle, stabilization of heterochromatin, tumour suppressor.	Sacristan *et al.* [26]	Probes: 02734-L23112 04502-L02199	251	Re-TUR: No BCG: Induction & 6m maintenance for high-grade pT1 Other: Mitomycin C for 6m for low-grade pT1	Multivariate analysis: **TR:** HR: 3.51 CI:1.4-8.78, P=0.007	Methylation was associated with an increase in TR in pT1LG tumours.

		Agundez *et al.* [27]	Probes: 02734-L23112 04502-L02199	91	Re-TUR: No BCG: 6-wk course Other: N/A	Kaplan-Meier survival curve **TP:** P=0.042	Methylation was associated with reduced TP following BCG.
VHL	von Hippel-Lindau tumor suppressor: degradation of hypoxia-inducible-factor (HIF).	Sacristan *et al.* [26]	Probe: 03818-L03850	251	Re-TUR: No BCG: Induction & 6m maintenance for high-grade pT1 Other: Mitomycin C for 6m for low-grade pT1	Univariate analysis: **TR:** HR: 3.066 CI: 1.088-8.639, P=0.034	Methylation was associated with increased TR in pT1LG tumours on univariate analysis.
ATM	Ataxia telangiectasia mutated serine/threonine kinase: controller of cell cycle checkpoint signaling in response to DNA damage, genome stability.	Sacristan *et al.* [26]	Probes: 03023-L23862 02670-L02137	251	Re-TUR: No BCG: Induction & 6m maintenance for high-grade pT1 Other: Mitomycin C for 6m for low-grade pT1	Multivariate analysis: **TR:** HR: 3.03 CI: 1.4-6.5, P=0.009	Methylation was associated with increased TR in PT1LG tumours on multivariate analysis.
CHFR	Checkpoint with forkhead and ring finger domains: cell cycle regulation.	Sacristan *et al.* [26]	Probe: 18344-L23785	251	Re-TUR: No BCG: Induction & 6m maintenance for high-grade pT1 Other: Mitomycin C for 6m for low-grade pT1	Multivariate analysis: **TR:** HR: 2.43 CI: 1.06-5.54, P=0.035	Methylation was associated with increased TR in pT1LG tumours on multivariate analysis.

GATA2	GATA binding protein 2: transcription factor.	van Kessel *et al.* [38]	Probe: (5' to 3') ACAAACAAATTATACCTAAC	333	Re-TUR: Not stated BCG: Not stated Other: Not stated	Univariate analysis in HR-NMIBCs: **TP:** HR: 2.04 CI: 1.01-4.10, P=0.046	Methylation associated with increased TP in HR-NMIBCs on univariate analysis.
GATA5	GATA binding protein 5: transcription factor.	Agundez *et al.* [27]	Probe: 03752-L06199	91	Re-TUR: No BCG: 6-wk course Other: N/A	Kaplan-Meier survival curve: **TP:** P=0.019 **OS:** P=0.037	*GATA5* methylation was associated with reduced TP and improved DSS following BCG.
THBS1	Thrombospondin 1: mediator of cell-cell and cell-matrix interactions.	Agundez *et al.* [27]	Probe: 01678-L17140	91	Re-TUR: No BCG: 6-wk course Other: N/A	Kaplan-Meier survival curve: **TP:** P=0.041	Methylation was associated with reduced TP.
ESR1	Estrogen receptor 1: ligand-activated transcription factor.	Agundez *et al.* [27]	Probe: 02746-L02173	91	Re-TUR: No BCG: 6-wk course Other: N/A	Kaplan-Meier survival curve: **TP:** P=0.036	Methylation was associated with reduced TP.
TP73	Tumor protein p73: transcription factor involved in cellular responses to stress and development.	Agundez *et al.* [27]	Probe: 16004-L23287	91	Re-TUR: No BCG: 6-wk course Other: N/A	Kaplan-Meier survival curve: **TP:** P=0.048	Methylation was associated with reduced TP.
MSH6	MutS homolog 6: DNA mismatch repair.	Agundez *et al.* [27]	Probe: 01250-L00798	91	Re-TUR: No BCG: 6-wk course Other: N/A	Kaplan-Meier survival curve: **TP:** P=0.040	Methylation was associated with reduced TP.

SOX1, PITX2, CSPG2 & JAK3	SRY-box 1: transcription factor, regulation of cell fate. Paired like homeodomain 2: transcription factor regulating procollagen lysyl hydroxylase gene expression. Chondroitin sulfate proteoglycan 2: extracellular matrix, cell adhesion, proliferation, migration, angiogenesis, tissue morphogenesis and maintenance. Janus kinase 3: tyrosine kinase, cytokine receptor-mediated intracellular signal transduction.	Lopez *et al*. [37]	Probes: SOX1_P294_F PITX2_E24_R CSPG2_P82_R JAK3_P156_R	70	Re-TUR: Unspecified BCG: Unspecified Other: Intravesical chemotherapy or BCG according to standard protocols and risk stratification	Multivariate analysis **DSS:** ***SOX1:*** HR: 4.36 CI: 1.28-9.35 ***PITX2:*** HR: 4.17 CI: 1.46-11.90 ***CSPG2:*** HR: 5.35 CI: 1.75-16.10 ***JAK3:*** HR: 0.19 CI: 0.04-0.89 Kaplan-Meier survival curve: **DSS:** P<0.0001	Hierarchical clustering of 33 genes identified three clusters with differential DSS. Methylation status of *SOX1*, *PITX2*, *CSPG2* & *JAK3* were independent predictors of DSS on multivariate analysis. Hypermethylation of two of *SOX1*, *PITX2* or *CSPG2* compared to one or none are associated with worse DSS.

Figure 1: **PRISMA diagram.**

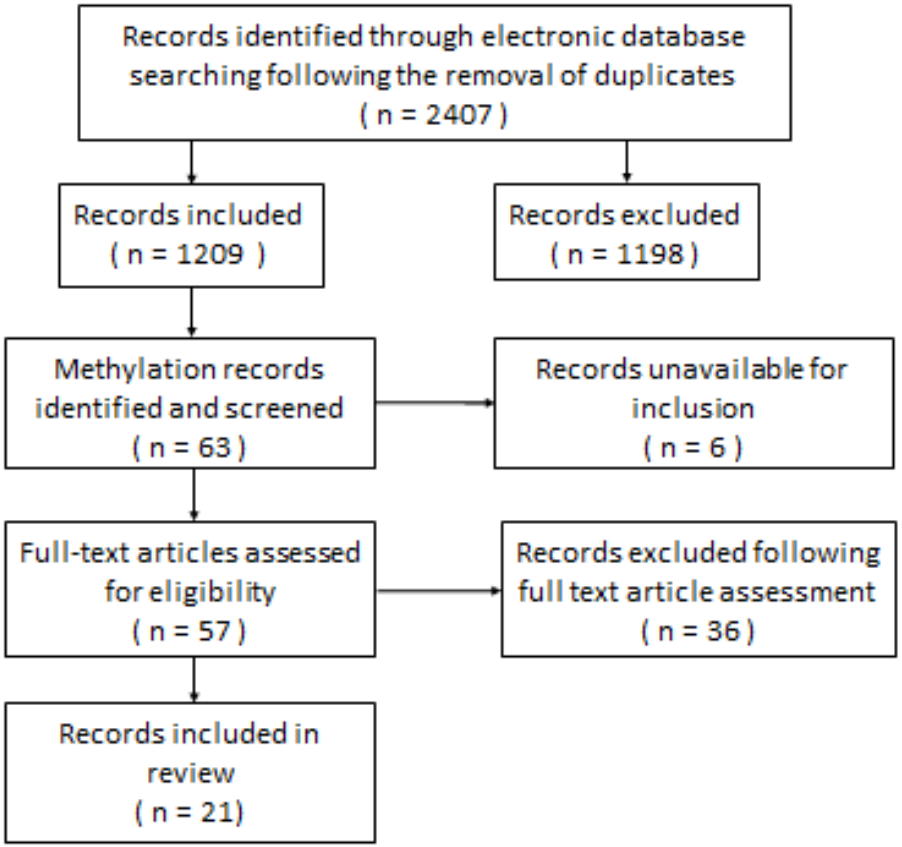
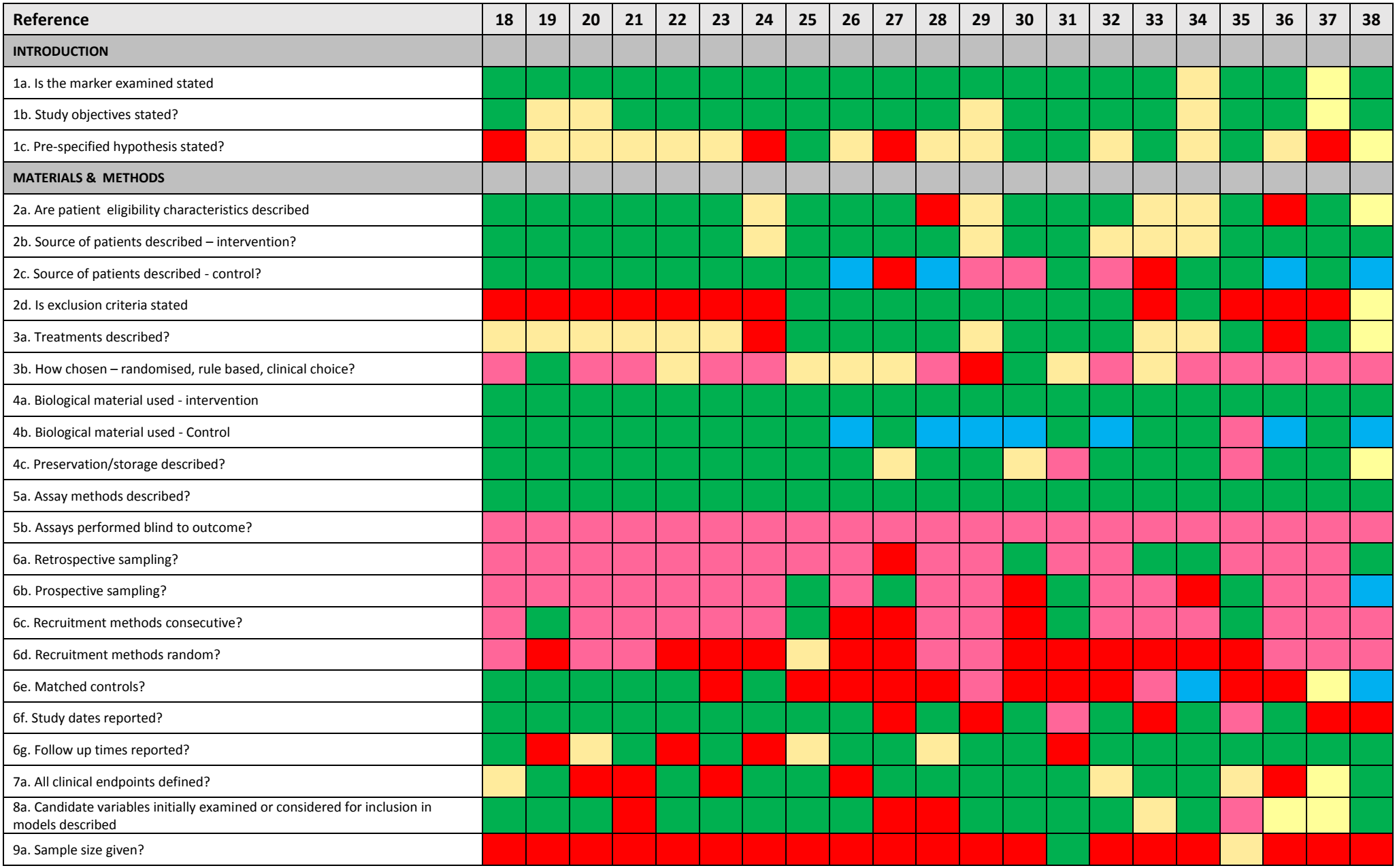
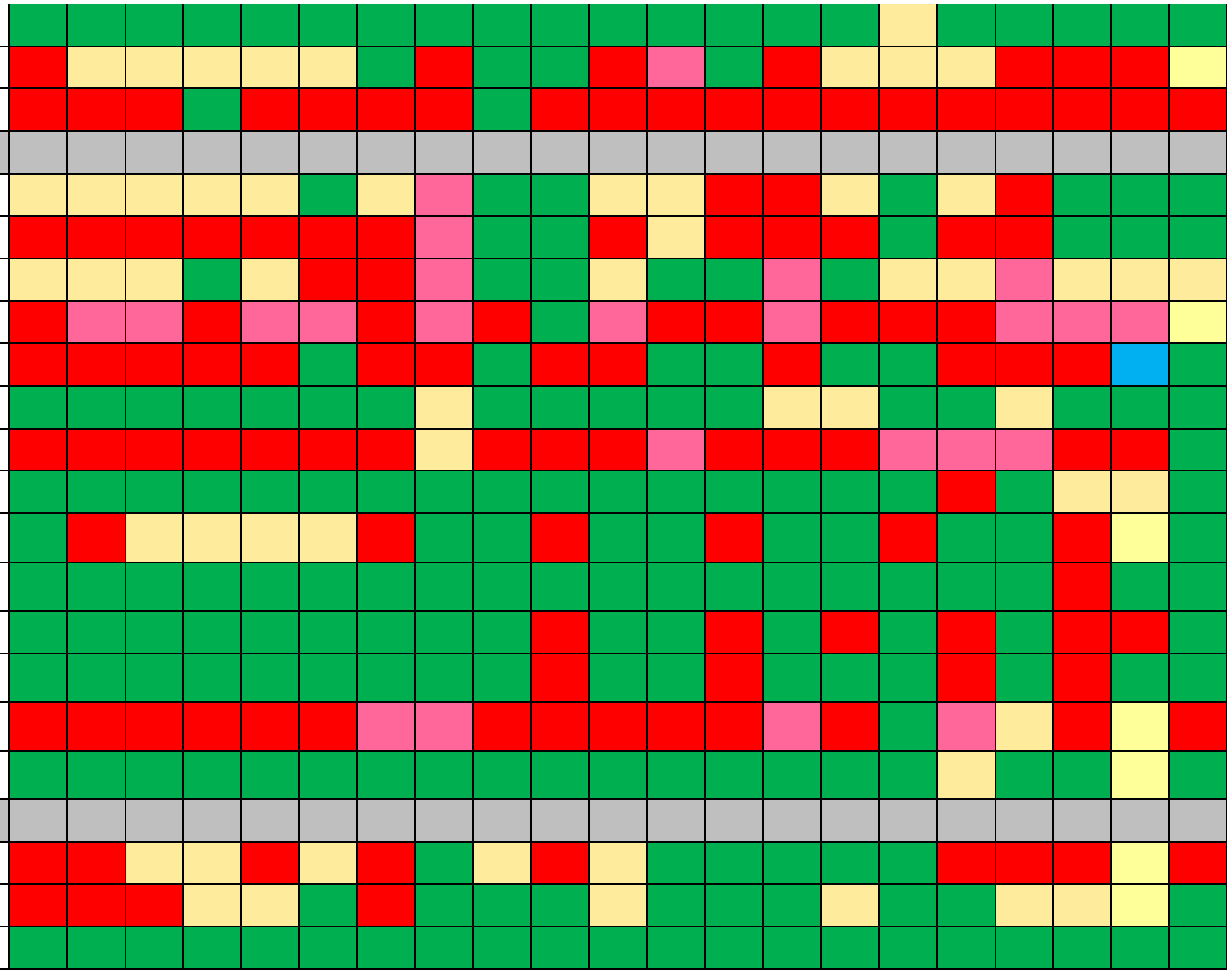


Figure S1: The REMARK array to demonstrate study quality. The REMARK checklist was divided into a total of 44 questions (Appendix 5), and each paper was assessed for each question with the possible responses of Yes (green), No (red), Partial (yellow), Unclear/Not Stated (pink), or not applicable (blue).



10a. Stats methods described?	
10b. Model building/assumptions described?	
10c. Missing data handling described?	
RESULTS	
11a. Marker values described?	
11b. Cut off points reported?	
12a. Flow of patients through the study reported?	
12b. Number of dropouts & reasons reported?	
12c. Subgroup analysis?	
13a. Demographic characteristics reported?	
13b. Missing values reported?	
14a. Show the relation of the marker to standard prognostic variables?	
15a. Present univariable analyses showing the relation between the marker & outcome, with estimated effect (e.g. HR & survival probability).	
16a. For key multivariable analyses, is the estimated effects reported - e.g. hazard ratio & confidence intervals for the marker	
16b. For final model are all variables reported	
17a. Provide estimated effects with CI from analysis where marker & standard prognostic variable are included	
18a. Are results from further investigations, such as checking assumptions, sensitivity analyses, & internal validation reported?	
19a. Are results interpreted in relation to the pre-specified hypotheses & other relevant studies.	
DISCUSSION	
19b. Are study limitations discussed?	
20a. Discuss implications for future research	
20b. & clinical value.	



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9

Appendix 1 - Search rationale and protocol

INTRODUCTION
Nature and context of the problem
- Bladder cancer is a heterogeneous disease, displaying multiple subtypes with varying therapeutic response and survival rates. - High-risk non-muscle invasive bladder cancer (HR-NMIBC) tumours are particularly challenging to manage and predict due to their highly-recurrent nature and increased risk of progression to muscle-invasive disease. - HR-NMIBC tumours are risk stratified by the EAU guidelines and this informs the current prognostications and selection of therapeutic interventions of the disease. The EAU guidelines recommend treatment with TURBT (transurethral resection of bladder tumour) together with adjuvant BCG instillations, and patients at high risk of tumour progression require radical cystectomy or radical radiotherapy. - Existing prognostications of the disease is limited and may result in insufficient or too aggressive a treatment being implemented.
Aim of review
To assess the existence and role of prognostic biomarkers in high risk non-muscle invasive bladder cancer (HR-NMIBC).
Rationale
Molecular markers to risk stratify patients and infer accurate prognostication of the disease would benefit clinicians in determining the best therapeutic strategy for individual patients. If there is supporting literature for the clinical usefulness of biomarkers, they need further investigation before going into clinical trial.
PICO
Population
All patients with HR-NMIBC - Majority patients will be primary HR-NMIBC - All terms for bladder cancer included in the population - Main limitation = the heterogeneity of the studied populations and the risk stratification terms used.
Intervention Description of drug, dose and duration.

N/A

Control
Description of drug, dose and duration

N/A

Outcomes

Progression can be quantified in different ways: progression rate (% of patients with marker that progress) / time to progression
Primary:
Overall survival (OS) – all cause mortality
Disease specific survival (DSS) – bladder cancer specific mortality
Tumour progression (TP) – progression to muscle invasive or metastatic disease
Tumour recurrence (TR) – recurrence of tumour on surveillance

Secondary:
Is it prognostic in high risk NMIBC

Study design

Cohort study
Case control study
– provide the primary source of data

Exclude reviews/systematic reviews and metanalysis – need primary evidence

SEARCH PLAN

Preliminary scoping searches - systematic/reviews, guidelines

Scoping searches to identify systematic reviews and health technology assessments on this topic will be undertaken in the following databases:
National Institute for Health and Care excellence
https://www.nice.org.uk/guidance
Cochrane Database of Systematic Reviews (CDSR):
http://www.cochranelibrary.com/cochrane-database-of-systematic-reviews/
National Institute for Health Research (NIHR): http://www.nihr.ac.uk/
Centre for Reviews and Dissemination (CRD):
http://www.crd.york.ac.uk/crdweb/
Health Technology Assessment Database (HTA):
https://www.journalslibrary.nihr.ac.uk/hta/#/
PUBMED:
https://www.ncbi.nlm.nih.gov/pubmed
Search: PROSPERO:
https://www.crd.york.ac.uk/PROSPERO/

Main review searches MEDLINE and EMBASE

The main aim of the search will be to systematically identify completed and ongoing studies

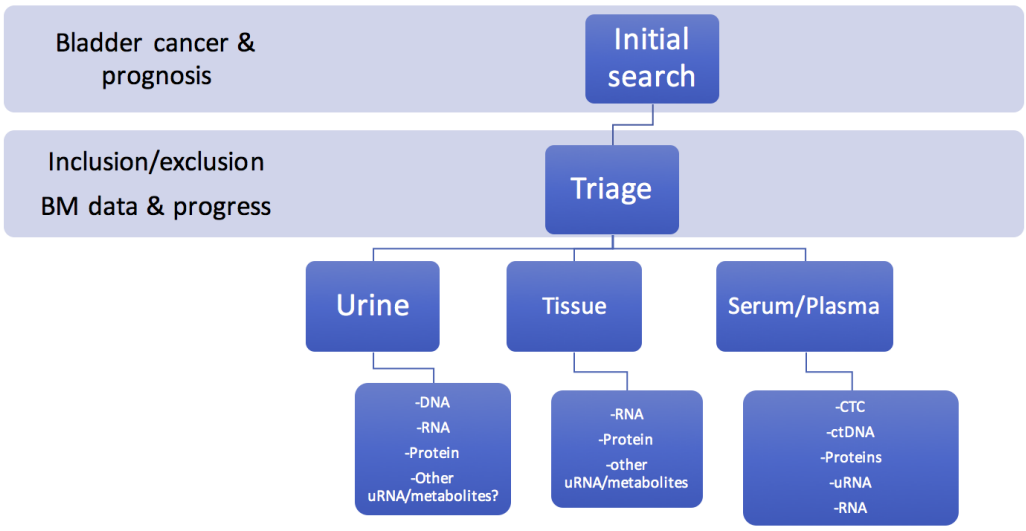
The following data sources will be searched:

- Bibliographic databases including: Cochrane Library, MEDLINE, EMBASE

No language or date restrictions will be applied initially - restrict to English after.

Example of search strategy

1. Urinary bladder Neoplasm (Mesh)
2. Urothelial cancer
3. Urothelial carcinoma
4. Urothelial carcinoma of the bladder
5. transitional cell carcinoma (TCC)
6. Urothelial Bladder cancer
7. UBC
8. Bladder cancer
9. 1 or 2 or 3 or 4 or 5
10. prognostic/prognosis
11. high-risk/high-grade/ superficial bladder cancer
12. 10 or 11
13. 9 and 12
14. Biomarker terms



Making inclusion/exclusion decisions.

Papers will be assessed for inclusion/exclusion, initially using the title and abstract. Disagreements will be resolved by discussion.
Full paper copies of relevant or potentially relevant references will be obtained for detailed examination.
Inclusion decisions will be made prior to detailed scrutiny of the results and study quality assessment.
Foreign language publications will be screened using English abstracts.

DATA HANDLING

Data extraction strategy
Data will be extracted using a pre-designed data extraction form. Where information is missing, authors will be contacted but within the resources and timeframe of the project. Data from studies with multiple publications will be extracted and reported as a single study, in case of discrepancies the most recent publication will be utilized.
Quality assessment strategy
Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK) to be utilized as a template. Results to be tabulated and described.
Methods of analysis
A descriptive analysis of included studies will be undertaken and relevant evidence will be categorised and summarised in tables (excel and word). Identified research evidence will be appropriately interpreted according to the assessment of methodological strengths and weaknesses and the possibility of potential biases → statistical analysis will be achieved by combining multiple publications of the same marker if the sample allows. The following subgroup analyses will be undertaken: HR-NMIBC bladder patients according to the EAU guidelines and risk stratifications.

Data extraction/ results
- No. of patients - Outcome measure (define) – recurrence, progression, metastasis and cancer specific mortality? - Statistically significant – HR, 95% CI, OR, P-value - Identified marker - Genes studies - Prognostic outcomes: tumour recurrence, tumour progression, disease-specific survival, disease specific mortality, etc. - Targeted or genome-wide approach

Appendix 2: Search strategy with the search strings

Search Strategy	Terms
Bladder cancer	bladder cancer OR transitional cell carcinoma OR urothelial carcinoma OR superficial bladder cancer or high-grade non-muscle invasive bladder cancer OR non-muscle invasive bladder cancer OR urothelial bladder cancer.
AND	
High-risk	high risk OR high-risk OR high-grade OR high grade OR grade 3 OR G3 OR T1 OR pT1 OR G3T1 OR pT1 G3 OR pT1G3 OR G3pT1 OR G3T1 or T1 G3 or T1G3 OR G3 pT1.
AND	
Prognostic	prognosis OR prognostic OR predictor adj survival OR predictor adj outcome OR predictor adj progression.
AND	
Biomarker	Biomarker OR protein OR nucleic acid OR methylation OR DNA methylation Or nuclear matrix protein OR nuclear matrix associated proteins OR NMP22 OR circulating tumour cell OR survivin OR Ki-67 Antigen OR Ki67 OR Receptor, fibroblast growth factor, type 2 OR FGFR3 OR phosphatidylinositol 3-kinase OR PIK3CA OR HRAS or cyclin E OR CCNE1 OR mutation OR TERT OR tumour suppressor protein, p53 OR cyclin-dependent kinase inhibitor p16 OR cyclin-dependent kinase inhibitor p27 OR retinoblastoma gene OR RNA OR mRNA OR miRNA metabolite OR tumour suppressor protein OR p21 OR gene Or gene expression OR cytokeratins OR BLCA OR telomerase OR circulating tumour cell OR ctDNA OR circulating free DNA OR cfDNA OR cell free DNA.

Table A: The search strategy used in the MEDLINE and EMBASE searches. The search included the possible terminologies used for the four categories that formed the search strings of the research question: bladder cancer, high-risk, prognostic and biomarker terms.

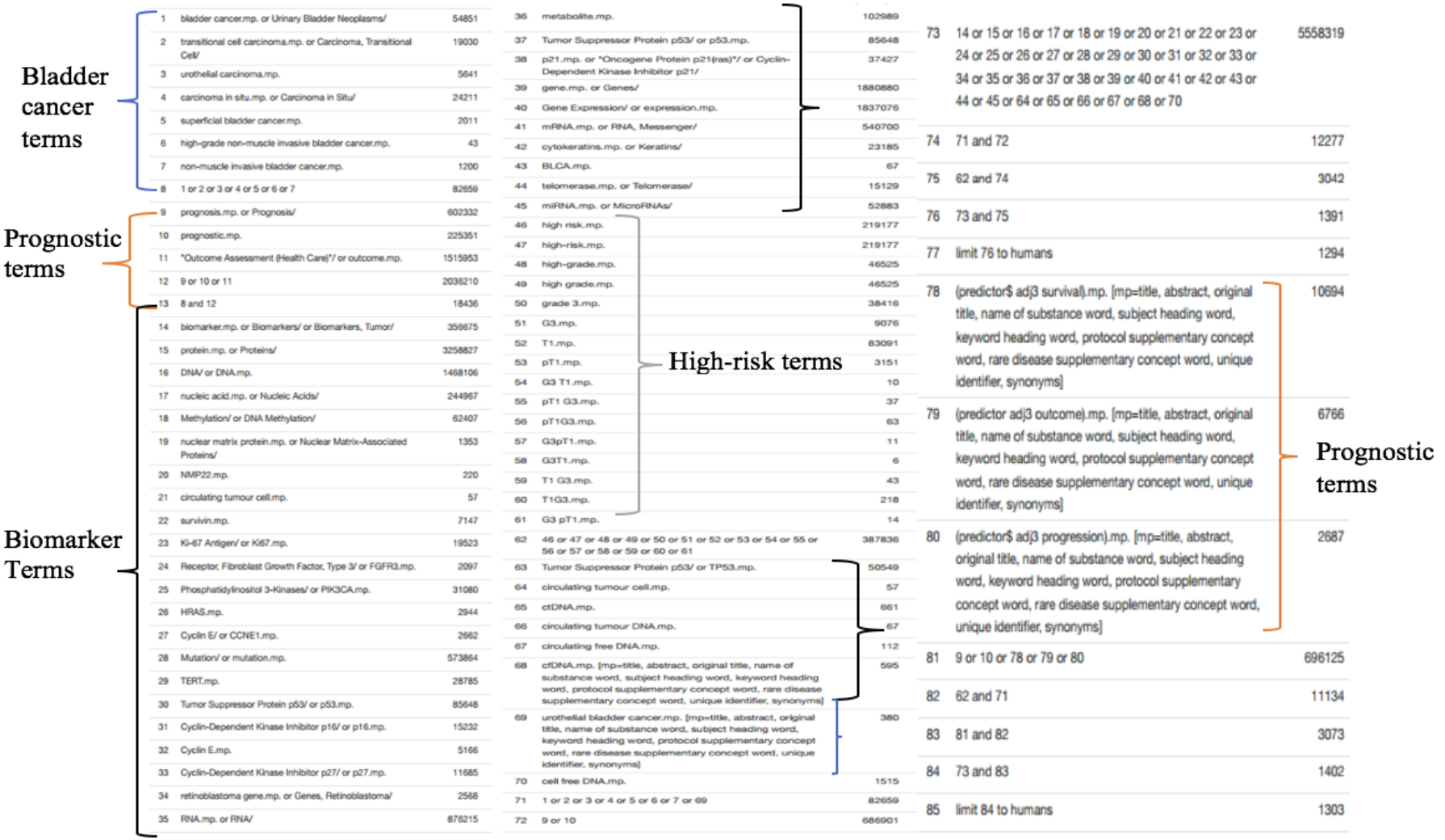
Appendix 3: The full search strategy

The search in the MEDLINE database

1	bladder cancer.mp. or Urinary Bladder Neoplasms/	54851
2	transitional cell carcinoma.mp. or Carcinoma, Transitional Cell/	19030
3	urothelial carcinoma.mp.	5641
4	carcinoma in situ.mp. or Carcinoma in Situ/	24211
5	superficial bladder cancer.mp.	2011
6	high-grade non-muscle invasive bladder cancer.mp.	43
7	non-muscle invasive bladder cancer.mp.	1200
8	1 or 2 or 3 or 4 or 5 or 6 or 7	82659
9	prognosis.mp. or Prognosis/	602332
10	prognostic.mp.	225351
11	*Outcome Assessment (Health Care)*/ or outcome.mp.	1515953
12	9 or 10 or 11	2036210
13	8 and 12	18436
14	biomarker.mp. or Biomarkers/ or Biomarkers, Tumor/	356675
15	protein.mp. or Proteins/	3258827
16	DNA/ or DNA.mp.	1468106
17	nucleic acid.mp. or Nucleic Acids/	244967
18	Methylation/ or DNA Methylation/	62407
19	nuclear matrix protein.mp. or Nuclear Matrix-Associated Proteins/	1353
20	NMP22.mp.	220
21	circulating tumour cell.mp.	57
22	survivin.mp.	7147
23	Ki-67 Antigen/ or Ki67.mp.	19523
24	Receptor, Fibroblast Growth Factor, Type 3/ or FGFR3.mp.	2097
25	Phosphatidylinositol 3-Kinases/ or PIK3CA.mp.	31080
26	HRAS.mp.	2944
27	Cyclin E/ or CCNE1.mp.	2662
28	Mutation/ or mutation.mp.	573864
29	TERT.mp.	28785
30	Tumor Suppressor Protein p53/ or p53.mp.	85648
31	Cyclin-Dependent Kinase Inhibitor p16/ or p16.mp.	15232
32	Cyclin E.mp.	5166
33	Cyclin-Dependent Kinase Inhibitor p27/ or p27.mp.	11685
34	retinoblastoma gene.mp. or Genes, Retinoblastoma/	2568
35	RNA.mp. or RNA/	876215
36	metabolite.mp.	102989
37	Tumor Suppressor Protein p53/ or p53.mp.	85648
38	p21.mp. or *Oncogene Protein p21(ras)*/ or Cyclin-Dependent Kinase Inhibitor p21/	37427
39	gene.mp. or Genes/	1880880
40	Gene Expression/ or expression.mp.	1837076
41	mRNA.mp. or RNA, Messenger/	540700
42	cytokeratins.mp. or Keratins/	23185
43	BLCA.mp.	67
44	telomerase.mp. or Telomerase/	15129
45	miRNA.mp. or MicroRNAs/	52883
46	high risk.mp.	219177
47	high-risk.mp.	219177
48	high-grade.mp.	46525
49	high grade.mp.	46525
50	grade 3.mp.	38416
51	G3.mp.	9076
52	T1.mp.	83091
53	pT1.mp.	3151
54	G3 T1.mp.	10
55	pT1 G3.mp.	37
56	pT1G3.mp.	63
57	G3pT1.mp.	11
58	G3T1.mp.	6
59	T1 G3.mp.	43
60	T1G3.mp.	218
61	G3 pT1.mp.	14
62	46 or 47 or 48 or 49 or 50 or 51 or 52 or 53 or 54 or 55 or 56 or 57 or 58 or 59 or 60 or 61	387836
63	Tumor Suppressor Protein p53/ or TP53.mp.	50549
64	circulating tumour cell.mp.	57
65	ctDNA.mp.	661
66	circulating tumour DNA.mp.	67
67	circulating free DNA.mp.	112
68	cfDNA.mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	595
69	urothelial bladder cancer.mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	380
70	cell free DNA.mp.	1515
71	1 or 2 or 3 or 4 or 5 or 6 or 7 or 69	82659
72	9 or 10	686901

73	14 or 15 or 16 or 17 or 18 or 19 or 20 or 21 or 22 or 23 or 24 or 25 or 26 or 27 or 28 or 29 or 30 or 31 or 32 or 33 or 34 or 35 or 36 or 37 or 38 or 39 or 40 or 41 or 42 or 43 or 44 or 45 or 64 or 65 or 66 or 67 or 68 or 70	5558319
74	71 and 72	12277
75	62 and 74	3042
76	73 and 75	1391
77	limit 76 to humans	1294
78	(predictor$ adj3 survival).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	10694
79	(predictor adj3 outcome).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	6766
80	(predictor$ adj3 progression).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	2687
81	9 or 10 or 78 or 79 or 80	696125
82	62 and 71	11134
83	81 and 82	3073
84	73 and 83	1402
85	limit 84 to humans	1303

An annotated full search in MEDLINE:



The search in the EMBASE database

1	bladder cancer.mp. or Urinary Bladder Neoplasms/	59246
2	Carcinoma, Transitional Cell/ or urothelial carcinoma of the bladder.mp. or Urinary Bladder Neoplasms/	20206
3	urothelial carcinoma.mp.	9284
4	non-muscle invasive bladder cancer.mp. or non muscle invasive bladder cancer/	2932
5	superficial bladder cancer.mp.	2521
6	high-grade non-muscle invasive bladder cancer.mp.	84
7	prognosis/ or cancer prognosis/ or prognosis.mp.	735810
8	prognostic.mp.	332297
9	carcinoma in situ.mp. or carcinoma in situ/	41569
10	urothelial bladder cancer.mp.	673
11	1 or 2 or 3 or 4 or 5 or 6 or 9 or 10	108169
12	outcome.mp. or outcome assessment/	1935848
13	7 or 8 or 12	2595312
14	11 and 13	24996
15	biomarker.mp. or biological marker/	272049
16	predictor.mp.	226885
17	protein.mp. or protein/	4540592
18	DNA/ or DNA.mp.	1720748
19	nucleic acids.mp. or nucleic acid/	49713
20	DNA methylation/ or methylation/ or methylation.mp.	124779
21	nuclear matrix protein 22/ or NMP22.mp.	588
22	circulating tumour cell.mp. or circulating tumor cell/	6844
23	survivin.mp. or survivin/	12156
24	Ki 67 antigen/ or Ki67.mp.	39977
25	FGFR3.mp. or fibroblast growth factor receptor 3/	4123
26	phosphatidylinositol 3 kinase/ or PIK3CA.mp.	60553
27	HRAS.mp.	2280
28	cyclin E/ or CCNE1.mp.	7453
29	mutation/ or mutation.mp.	798352
30	TERT.mp.	51860
31	metabolite/ or metabolite.mp.	332109
32	p53.mp. or protein p53/	122772
33	protein p16/ or p16.mp.	17524
34	protein p27/ or p27.mp.	13266
35	retinoblastoma gene.mp. or tumor suppressor gene/	62295
36	p21.mp. or p21 activated kinase/ or protein p21/	38834
37	RNA.mp. or RNA/	1190057
38	gene.mp. or gene/	2866612
39	expression.mp. or gene expression/	2544221
40	circulating tumour cell.mp. or circulating tumor cell/	6844
41	mRNA.mp. or messenger RNA/	619303
42	cytokeratin/ or cytokeratins.mp.	19380
43	BLCA.mp.	101
44	telomerase.mp. or telomerase/	20944
45	miRNA.mp. or microRNA/	89140
46	high risk population/ or high risk.mp.	450180
47	high-risk.mp.	450180
48	high-grade.mp.	69992
49	high grade.mp.	69992
50	grade 3.mp.	68389
51	G3.mp.	19183

Appendix 4: Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria	Reason
Tumours staged as T1, G3, CIS, Multiple, recurrence and large: TaG1G2 [**3**]	Low risk tumours staged as Ta, G1, LG, no CIS, G2Ta, LGTa [**3**]	Only HR-NMIBC patients are relevant to the research question. According to the EAU guidelines those included are staged according to high-risk NMIBC [**3**]
Biomarker/molecular marker data	No biomarker/molecular marker data	Reported biomarker or molecular marker data in relation to prognosis is required for the understanding of the marker's value to address the research topic.
Survival outcome/recurrence/progression data, given by HR, CI and P-value	No survival outcome/recurrence/progression data, given by HR, CI and P-value	Prognostic data such as survival outcomes linked to the marker is essential to understanding the prognostic value of the marker.
Patient sample >20	Patient sample <20	An adequate sample size ensures reliable and valid interpretations can be drawn from the patient population.
Primary research	Secondary or tertiary research	Using primary data can avoid potential bias that reviews may hold due to the tendency to report on markers that have been the most extensively studied.

Table B: The inclusion and exclusion criteria.

Appendix 5: REMARK Table

Introduction
1a. Is the marker examined stated
1b. Study objectives stated?
1c. Pre-specified hypothesis stated?
Materials and Methods
Patients
2a. Are patient eligibility characteristics described
2b. Source of patients described – intervention?
2c. Source of patients described - control?
2d. Is exclusion criteria stated?
3a. Treatments described?
3b. How chosen – randomised, rule based, clinician choice?
Specimen characteristics
4a. Biological material used - intervention
4b. Biological material used - Control
4c. Preservation/storage described?
Assay methods
5a. Assay methods described?
5b. Assays performed blind to outcome?
Study design
6a. Retrospective sampling?
6b. Prospective sampling?
6c. Recruitment methods consecutive?
6d. Recruitment methods random?
6e. Matched controls?
6f. Study dates reported?
6g. Follow up times reported?
7a. All clinical endpoints defined?
8a. Candidate variables initially examined or considered for inclusion in models described
9a. Sample size given?

Statistical analysis methods
10a. Stats methods described?
10b. Model building/assumptions described?
10c. Missing data handling described?
11a. Marker values described?
11b. Cut off points reported?
Results
Data
12a. Flow of patients through the study reported?
12b. Number of dropouts and reasons reported?
12c. Subgroup analysis?
13a. Demographic characteristics reported?
13b. Missing values reported?
Analysis and presentation
14a. Show the relation of the marker to standard prognostic variables?
15a. Present univariable analyses showing the relation between the marker and outcome, with the estimated effect (eg, hazard ratio and survival probability).
16a. For key multivariable analyses, is the estimated effects reported - e.g. hazard ratio and confidence intervals for the marker
16b. For final model are all variables reported
17a. Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variable are included, regardless of their statistical significance
18a. Are results from further investigations, such as checking assumptions, sensitivity analyses, and internal validation reported?
Discussion
19a. Are results interpreted in relation to the pre-specified hypotheses and other relevant studies.
19b. Are study limitations discussed?
20a. Discuss implications for future research
20b. and clinical value.

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