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Review – Kidney Cancer

Diagnostic DNA Methylation Biomarkers for Renal Cell Carcinoma: A Systematic Review

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Abstract

Context: The 5-yr survival of early-stage renal cell carcinoma (RCC) is approximately 93%, but once metastasised, the 5-yr survival plummets to 12%, indicating that early RCC detection is crucial to improvement in survival. DNA methylation biomarkers have been suggested to be of potential diagnostic value; however, their current state of clinical translation is unclear and a comprehensive overview is lacking.

Objective: To systematically review and summarise all literature regarding diagnostic DNA methylation biomarkers for RCC.

Evidence acquisition: We performed a systematic literature review of PubMed, EMBASE, Medline, and Google Scholar up to January 2019, according to the Preferred Reporting Items for Systematic Review and Meta-Analysis of Diagnostic Test Accuracy Studies (PRISMA-DTA) guidelines. Included studies were scored according to the Standards for Reporting of Diagnostic Accuracy Studies (STARD) criteria. Forest plots were generated to summarise diagnostic performance of all biomarkers. Level of evidence (LoE) and potential risk of bias were determined for all included studies.

Evidence synthesis: After selection, 19 articles reporting on 44 diagnostic DNA methylation biomarkers and 11 multimarker panels were included; however, only 15 biomarkers were independently validated. STARD scores varied from 4 to 13 out of 23 points, with a median of 10 points. Large variation in subgroups, methods, and primer locations was observed. None of the reported biomarkers exceeded LoE III, and the majority of studies reported inadequately.

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Conclusions: None of the reported biomarkers exceeded LoE III, indicating their limited clinical utility. Moreover, study reproducibility and further development of these RCC biomarkers are greatly hampered by inadequate reporting.
Patient summary: In this report, we reviewed whether specific biomarkers could be used to diagnose the most common form of kidney cancer. We conclude that due to limited evidence and reporting inconsistencies, none of these biomarkers can be used in clinical practice, and further development towards clinical use is hindered.
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1. Introduction

Worldwide, 400 000 people were diagnosed with renal cell carcinoma (RCC) and 175 000 people died of this disease in 2018 [1]. The significant health burden of RCC is mainly caused by the high number of patients (up to 17%) who present advanced disease at the time of diagnosis [2,3]. This is attributed to the typical lack of symptoms of the primary RCC, leading to a substantial number of metastasised RCC cases that could have been prevented if diagnosed earlier. Currently, the majority of patients are diagnosed after a coincidental finding (incidentaloma) during unrelated imaging procedures [4,5]. While 5-yr survival rates of early-stage RCC are around 93%, patients presenting with metastasised RCC have poor 5-yr survival rates, around 12% [6]. These numbers indicate the great importance of accurately diagnosing RCC at an early stage. As the current diagnostic RCC imaging techniques leave room for improvement, several studies have focused on molecular techniques instead [7,8]. The possibility to diagnose RCC using a noninvasive liquid-biopsy–based molecular test, in addition to imaging, could not only enhance early diagnosis, but also facilitate differentiation of benign and malignant masses, proven to be challenging in case a small renal mass ($\leq$4 cm) is discovered [9–11].

Recently, within the TRACERx Renal study, seven evolutionary subtypes were identified for the most common RCC subtype: clear cell RCC (ccRCC), for which the most prevalent abnormality was found to be the simultaneous loss of 3p and 5q gain (36% of ccRCC patients) [12,13]. The well-known *VHL*, *PBRM1*, *BAP1*, and *SETD2* genes are the most frequently mutated (60–70%, 40%, 10%, and 10%, respectively) and subsequently inactivated genes in ccRCC as a result of these chromosomal aberrations [13]. For the other RCC subtypes, however, genetic mutations such as mutations in *MET* or *FH* in papillary RCC (pRCC), and mutations of *PTEN* or *FLCN* in chromophobe RCC (chRCC) are less frequent [14–16]. Compared with genetic alterations, DNA hypermethylation is more pronounced and frequently found in all RCC subtypes, and involved in several RCC-related pathways such as angiogenesis [14,15]. As DNA methylation is considered a common, early, and stable event in tumorigenesis that is easily detectable in small amounts of DNA, these alterations could be interesting cancer biomarkers [17]. This is illustrated by the successful implementation of seven DNA methylation biomarkers in four clinical diagnostic tests for prostate, colorectal, and lung cancer [18].

However, despite their potential, no diagnostic RCC DNA methylation marker has reached the clinic yet. In addition, there is currently no overview showing which markers can be considered as potential diagnostic RCC biomarkers, and for which further validation or development is desirable. We have systematically reviewed the literature on diagnostic DNA methylation biomarkers in RCC to provide this overview and summarise current evidence for these biomarkers.

2. Evidence acquisition

Preferred Reporting Items for Systematic Review and Meta-Analysis of Diagnostic Test Accuracy Studies (PRISMA-DTA) guidelines were applied in the process of writing this systematic review [19].

2.1. Search strategy, eligibility criteria, and study selection

Electronic literature searches (up to January 2019) of PubMed, EMBASE, Medline, and Google Scholar were conducted (Supplementary Table 1). Articles eligible for this systematic review were all original articles on diagnostic DNA methylation biomarkers in RCC. Other inclusion criteria were the following: English language, specific genes being evaluated, and biomarker potential being expressed in at least one measure of diagnostic value. Studies were excluded when reporting on global methylation analysis, hereditary RCC, transitional cell carcinoma, Wilms' tumours and renal sarcomas. As this review focuses specifically on DNA methylation, studies reporting on micro-RNA methylation were excluded. After initial screening, six additional articles were included through scanning reference lists of the full-text assessed articles. Ultimately, 19 articles were included in this systematic review (Fig. 1).

2.2. Data extraction

All data were extracted by two independent authors (K.L. and K.S.) using a standardised data extraction sheet. In addition, articles were assessed for reporting quality using the Standards for Reporting of Diagnostic Accuracy Studies (STARD) 2015 criteria [20], which consider 34 items for good reporting of diagnostic accuracy studies. Each of these items was awarded 1 point if the item was fully reported, 0.5 point if part of the item was reported, and 0 points if an item was not reported. Each item of the STARD criteria not applicable to biomarker research was excluded. Based on the adapted STARD criteria (Supplementary Table 2), the maximum

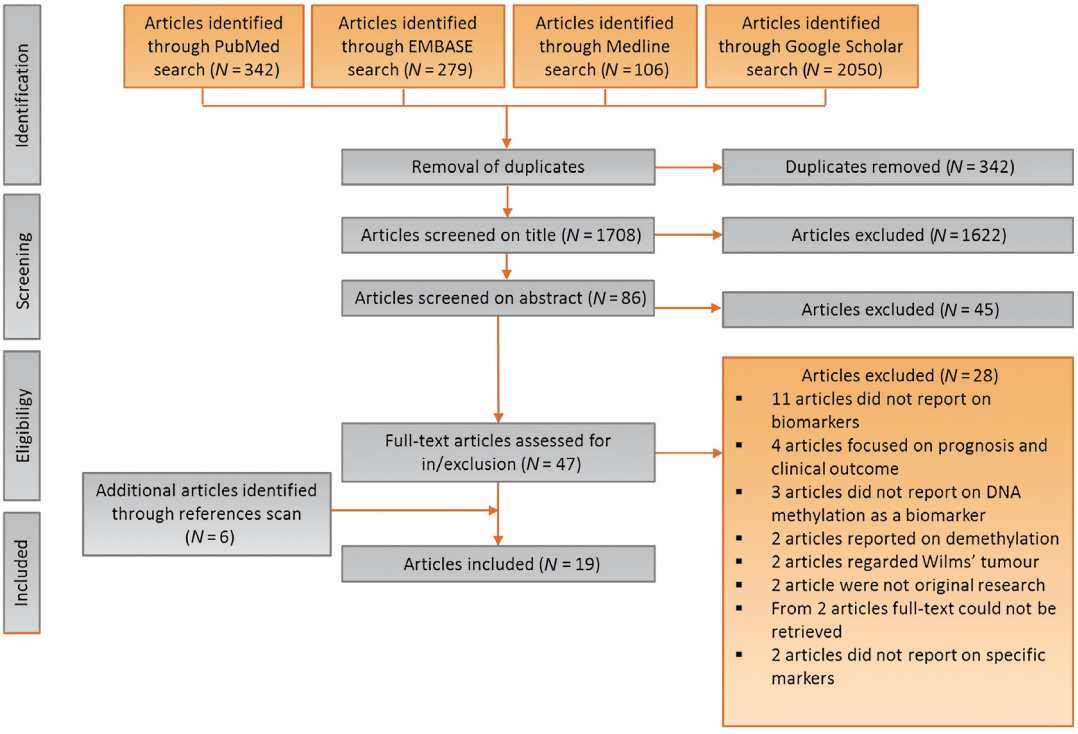

Fig. 1 – PRISMA flow diagram visualising the study selection process. PRISMA = Preferred Reporting Items for Systematic Reviews and Meta-Analyses.

reporting score was 23 points. Mutual consensus was reached whenever interobserver variation occurred. The risk of potential bias across or within studies was analysed per study using the STARD scores (Supplementary Table 3). In case a study scored ≥0.5 points per item for STARD items 5–9, the potential risk of selection bias was low. Whenever this criterion was not met, the potential risk of selection bias was increased. Measurement bias regarding the assay method and outcome assessment was measured similarly, using STARD items 10a, 12a, and 13a for the assay method and STARD items 14, 21a, and 24 for outcome assessment. Other variable assessment measurement bias was based on STARD item 20. In case of a full score (score = 1), measurement bias risk was low. Whenever this item was partially reported or not reported, the potential measurement bias risk was increased (Supplementary Table 4).

To obtain a summary of current evidence on diagnostic DNA methylation biomarkers in RCC, the level of evidence (LoE) for each biomarker was determined according to two established reference schemes [21,22]. Five LoE categories represent the current evidence for clinical utility of a diagnostic biomarker, with LoE I representing the highest evidence and LoE V representing the poorest evidence for clinical utility.

2.3. Forest plots

Forest plots were created to summarise diagnostic performance of all studied biomarkers. Sensitivity, specificity, and 95% confidence intervals were reported where available. If sensitivity and specificity were not reported, these measures were calculated from the percentage of DNA methylation. In addition, forest plots depict the DNA methylation detection method, specimen type, LoE, genomic location of primers, TNM stage, and Fuhrman grade.

3. Evidence synthesis

3.1. Study characteristics

Nineteen articles (published between 2003 and 2017) were included in this systematic review using a standardised selection procedure (Fig. 1). Four (21%) studies described a single biomarker, whereas 15 (79%) reported on multiple markers. A total of 44 individual biomarkers were studied, and sample sizes ranged from 21 to 196 patients. Twelve (63%) studies analysed tissue only, three (16%) studies investigated blood, three (16%) studies analysed both tissue and urine, and one study (5%) included tissue, urine, and blood. Twelve (63%) studies investigated several RCC subtypes, three (16%) studies focused solely on ccRCC patients, one (5%) focused on pRCC patients, and three (16%) studies did not specify RCC subtype. Study characteristics are summarised in Table 1.

3.2. STARD reporting assessment and potential bias

The STARD criteria were first introduced in 2003 and updated in 2015, striving towards improving the reporting quality of diagnostic accuracy studies [20,23]. STARD scores varied from 4 to 13 out of a maximum of 23 points, with a median of 10 points (Supplementary Table 3). Only items 3 and 4 were partially or completely described in every study, whereas items 13a, 18, and 19 were not reported in

Table 1 – Characteristics of the 19 studies included in this systematic review

Diagnostic DNA methylation biomarker studies									
First author (year) [ref]	Study characteristics				Evaluation of DNA methylation				STARD score
	Sample size	Specimen	Preservation method	Tumour type	Method	Biomarker studied	Sensitivity (%) [a]	Specificity (%)	
Ahmad (2012) [28]	196	PTT	Fresh frozen	ccRCC, pRCC, chRCC, TCRCC	MSP	*APAF1*	63.8	87.8	13
						DAPK1	41.3	85.2	
						SPARC	12.2	91.8	
Battagli (2003) [5]	50	PTT	NR	ccRCC, pRCC, chRCC, RCC unclassified, oncocytomas, collecting duct, TCC renal pelvis	MSP	*VHL*	PTT 12, urine 12	PTT NR, urine 100	12.5
		Urine				*CDKN2A (p16)*	PTT 10, urine 8	PTT NR, urine 100	
						CDKN2A (p14)	PTT 18, urine 18	PTT NR, urine 100	
						APC	PTT 18, urine 16	PTT NR, urine 100	
						RASSF1A	PTT 52, urine 50	PTT NR, urine 100	
						TIMP3	PTT 60, urine 52	PTT NR, urine 100	
						Panel of *VHL, CDKN2A (p16), CDKN2A (p14), APC, RASSF1A, TIMP3*	PTT 100, urine 88	PTT NR, urine 100	
Christoph (2008) [29]	85	PTT	Fresh frozen	ccRCC	qMSP	*APAF1*	89	85	10.5
						CASP8	0	100	
						DAPK1	66	95	
						IGFBP3	4	100	
Costa (2007) [24]	85	PTT	Fresh frozen	ccRCC, pRCC, chRCC, oncocytomas	qMSP	*APC*	15	91.9	15
						ARH1	100	0	
						CDH1	67	12.9	
						CTNNB1	0	100	
						SFN	100	0	
						CDKN2A (p14)	10.6	72.6	
						CDKN2A (p16)	0	100	
						RASSF1A	80	0	
						GSTP1	5.9	100	
						MDR1	85.9	3.2	
						MTHFR	100	0	
						PTGS2	94.1	0	
						TIMP3	15.3	75.8	
						ESR1	69.4	22.6	
						ESR2	50.6	56.5	
						FHIT	51.8	30.7	
						MGMT	1.1	88.7	
						RARb2	1.1	100	
Costa (2011) [30]	33	PTT	Fresh frozen	PTT: ccRCC, pRCC, chRCC, oncocytomas	qMSP	*TCF21*	PTT 61, urine NR	PTT NR, urine NR	12.5
	50	Urine	Centrifugation, −80 °C storage			*PCDH17*	PTT 61, urine NR	PTT NR, urine NR	
				Urine: general RCC		Panel of *TCF21, PCDH17*	PTT 67 [b], urine 32 [b]	PTT 100 [b], urine 100 [b]	

Table 1 (*Continued*)

Diagnostic DNA methylation biomarker studies									
First author (year) [ref]	Study characteristics				Evaluation of DNA methylation				STARD score
	Sample size	Specimen	Preservation method	Tumour type	Method	Biomarker studied	Sensitivity (%) [a]	Specificity (%)	
Dalgin (2008) [31]	38	PTT	Fresh frozen	ccRCC	MALDI-TOF MS	*sFRP1*	NR	NR	5
						SCNN1B	NR	NR	
						SYT6	NR	NR	
						TFAP2A	NR	NR	
						DACH1	NR	NR	
						MT1G_001	NR	NR	
de Martino (2012) [38]	157	Blood	Centrifugation, −80 °C storage	ccRCC, pRCC, chRCC	Restriction endonuclease qPCR	*RASSF1A*	45.9 [b]	93 [b]	14
						VHL	50.3 [b]	90.7 [b]	
						PTGS2	38.2 [b]	65.1 [b]	
						CDKN2A(p16)	46.5 [b]	55.8 [b]	
Dulaimi (2004) [25]	100	PTT	Imbedded in OCT	ccRCC, pRCC, chRCC, collecting duct, RCC unclassified, oncocytomas, TCC renal pelvis, Wilms' tumour	MSP	*VHL*	8	100	10
						RASSF1A	45	100	
						CDKN2A (p16)	10	100	
						CDKN2A (p14)	17	100	
						APC	14	100	
						MGMT	7	100	
						GSTP1	12	100	
						RARb2	12	100	
						CDH1	11	100	
						TIMP3	58	100	
Ellinger (2011) [27]	32	PTT	FFPE	pRCC	qMSP	*APC*	3.1	93.3	13.5
						CDH1	15.6	100	
						GSTP1	21.9	93.9	
						RASSF1A	87.5 [b]	73.3 [b]	
						TIMP3	6.3	100	
Ge (2015) [32]	50	PTT	NR	RCC	MSP, BGS	*RIZ1*	30	93	9.5
Hauser (2013) [39]	35	Blood	NR	ccRCC, pRCC, chRCC	Restriction endonuclease qPCR	*APC*	54.3 [b]	90.7 [b]	13.5
						GSTP1	17.1 [b]	98.1 [b]	
						CDKN2A (p14)	14.3 [b]	100 [b]	
						CDKN2A (p16)	25.7 [b]	83 [b]	
						RASSF1A	22.9 [b]	98.2 [b]	
						TIMP3	57 [b]	61 [b]	
						PTGS2	22.9 [b]	96.3 [b]	
						RARb2	40 [b]	85.2 [b]	
						Panel *APC* or *GSTP1*	57.1 [b]	88.9 [b]	
						Panel *APC* or *PTGS2*	60 [b]	87 [b]	
						Panel *APC* or *RARb2*	74.3 [b]	77.8 [b]	
						Panel *PTSG2* or *GSTP1*	62.9 [b]	87 [b]	

Table 1 (*Continued*)

Diagnostic DNA methylation biomarker studies									
First author (year) [ref]	Study characteristics				Evaluation of DNA methylation				STARD score
	Sample size	Specimen	Preservation method	Tumour type	Method	Biomarker studied	Sensitivity (%) [a]	Specificity (%)	
Hoque (2004) [40]	18	Blood	NR	ccRCC, pRCC, chRCC, collecting duct	qMSP	*APC*	Blood 6 [b], urine 38 [b]	Blood 97 [b], urine 96 [b]	11
	26	Urine				*CDKN2A (p14)*	Blood 6 [b], urine 31 [b]	Blood 97 [b], urine 100 [b]	
						CDH1	Blood 33 [b], urine 38 [b]	Blood 93 [b], urine 95 [b]	
						GSTP1	Blood 6 [b], urine 15 [b]	Blood 100 [b], urine 100 [b]	
						MGMT	Blood 0 [b], urine 8 [b]	Blood 97 [b], urine 100 [b]	
						CDKN2A (p16)	Blood 22 [b], urine 35 [b]	Blood 100 [b], urine 100 [b]	
						RARb2	Blood 6 [b], urine 31 [b]	Blood 100 [b], urine 91 [b]	
						RASSF1A	Blood 11 [b], urine 65 [b]	Blood 97 [b], urine 89 [b]	
						TIMP3	Blood 17 [b], urine 46 [b]	Blood 100 [b], urine 91 [b]	
Onay (2009) [26]	21	PTT	FFPE	RCC	MSP	*RASSF1A*	52	62	8
						CDH1	19	86	
						TIMP3	10	90	
						APC	5	95	
						MGMT	14	86	
						CDKN2A (p16)	57	48	
						RARb2	5	86	
Pires-Luís (2015) [34]	120	PTT	Fresh frozen	ccRCC, pRCC, chRCC, oncocytomas	qMSP	*MST1R*	NR	86.7 [c]	12.5
Pires-Luís (2017) [33]	120	PTT	Fresh frozen	ccRCC, pRCC, chRCC, oncocytomas	qMSP	*HOXA9*	73	89	13
						OXR1	87	100	
						Panel *OXR1*, *MST1R*	98	100	
Skrypkina (2016) [41]	27	Blood	Centrifugation, −70 °C storage	ccRCC, pRCC/ccRCC mix, sarcoma-like, cancer of the renal pelvis	qMSP	*LRRC3B*	74 [b]	66.7 [b]	11.5
						APC	51.9 [b]	93.3 [b]	
						FHIT	55.6 [b]	100 [b]	
						RASSF1A	62.9 [b]	93.3 [b]	
						VHL	0 [b]	100 [b]	
						ITGA9	0 [b]	100 [b]	
						Panel *RASSF1A* or *FHIT* or *APC*	92.3 [b]	86.7 [b]	
						Panel *RAFFF1A* or *FHIT*	77.8 [b]	93.3 [b]	
						Panel *RASSF1A* or *APC*	77.8 [b]	93.3 [b]	
Urakami (2006) [35]	62	PTT	FFPE	ccRCC, granular cell RCC, ccRCC/granular cell RCC mix	MSP	*sFRP1*	27.3	100	12.5
						sFRP2	48.5	100	
						sFRP4	24.2	100	
						sFRP5	45.5	100	
						Wif1	27.3	100	
						Dkk3	27.3	100	
Xin (2016) [36]	55	PTT	Fresh frozen	RCC	Pyrosequencing	*TCF21*	PTT 89 [b], urine 79 [b]	PTT 61.9 [b], urine 100 [b]	10.5
		Urine	Centrifugation, −80 °C storage						
Xu (2015) [37]	101	PTT	Fresh frozen	ccRCC	MSP, BGS	*ADAMTS18*	43.6	85	8

BGS = bisulphite genomic sequencing; ccRCC = clear cell renal cell carcinoma; chRCC = chromophobe renal cell carcinoma; FFPE = formalin-fixed, paraffin-embedded; MALDI-TOF MS = matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry; MSP = methylation-specific polymerase chain reaction; NR = not reported; OCT = optimum cold temperature medium; pRCC = papillary renal cell carcinoma; PTT = primary tumour tissue; qMSP = quantitative methylation-specific polymerase chain reaction; qPCR = quantitative polymerase chain reaction; RCC = renal cell carcinoma; ref = reference; STARD = Standards for Reporting Diagnostic Accuracy Studies; TCC = transitional cell carcinoma; TCRCC = transitional cell renal cell carcinoma.

[a] Sensitivity and specificity calculated from percentage of methylated samples.

[b] Actual sensitivity and specificity.

[c] Compared with oncocytomas.

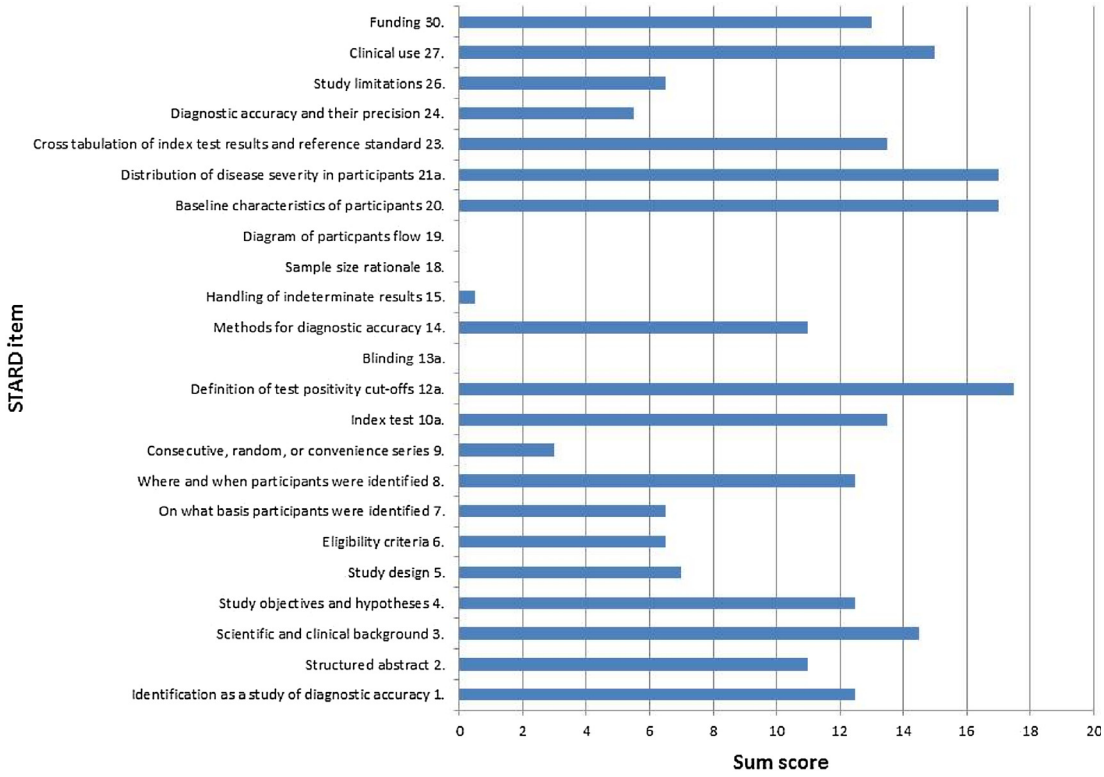

Fig. 2 – Sum score of all articles per STARD item. STARD = Standards for Reporting of Diagnostic Accuracy Studies.

any study (Fig. 2). None of the included studies obtained the maximum quality score. The risk of potential selection and measurement bias in the included studies is summarised in Supplementary Table 4, showing that most studies suffer from selection and measurement bias.

3.3. *Study findings*

Fifteen DNA methylation biomarkers were studied in at least two independent study populations (Fig. 3). Results of biomarkers without independent validation are shown in Supplementary Fig. 1.

Overall, large methodological differences were observed between studies, including differences in DNA methylation detection techniques, study population, and subgroup analyses. Most studies ($n = 15$; 79%) measured DNA methylation using methylation-specific polymerase chain reaction (MSP) or quantitative MSP. We observed similar sensitivities and specificities of biomarkers studied by different research groups, when measured in the exact same genomic region, even if different laboratory techniques were used (Fig. 3). This trend was observed in general RCC tissue samples for *APC* [5,24–26], *CDKN2A (p16)* [5,24,25], *MGMT* [25,26], *RARb2* [24,26], *RASSF1A* [5,25,26] (another genomic location of *RASSF1A* showed similar results for pRCC [24,27]), *TIMP3* [5,25], and *VHL* [5,25].

3.3.1. *Findings in tissues*

The majority of included studies ($n = 15$; 79%) investigated tissue (Table 1) [5,24–37]. Thirteen (68%) studies exclusively investigated tissue [24–35,37] and two (11%) studies examined both tissue and urine [5,36] (Table 1). Fourteen single tissue markers were independently studied in at least two populations. The remaining 27 single tissue markers and four tissue multimarker panels were reported only once, without validation. Among the independently validated tissue markers, the highest single-marker sensitivity was reported for *PTGS2* (94.1% and 96.1% for general RCC and ccRCC, respectively), however with 0% specificity [24]. For pRCC, the highest sensitivity in tissue was reported for *RASSF1A* (100%; 0% specificity); in chRCC tissues, 100% sensitivity and 100% specificity was reached for *TIMP3* [24,25]. For the other markers, a wide range of sensitivities and specificities were observed by different research groups, for example, for *RASSF1A*, sensitivities of 45–80% and specificities of 0–100% were reported in RCC overall (Fig. 3) [5,24–26].

Among the tissue markers that were not validated in independent populations or studies, *HOXA9* (73% sensitivity; 89% specificity) and *OXR1* (87% sensitivity; 100% specificity) appeared to be the most promising individual biomarkers [33]. However, as independent validation of these markers is lacking, these results should be interpreted with caution. Similarly, a panel utilising *OXR1* and *MST1R* appeared to be the best performing multimarker panel for general RCC tissue samples with 98% sensitivity and 100% specificity; yet this panel also requires validation and assessment in liquid biopsies before any statement regarding diagnostic potential can be made [33].

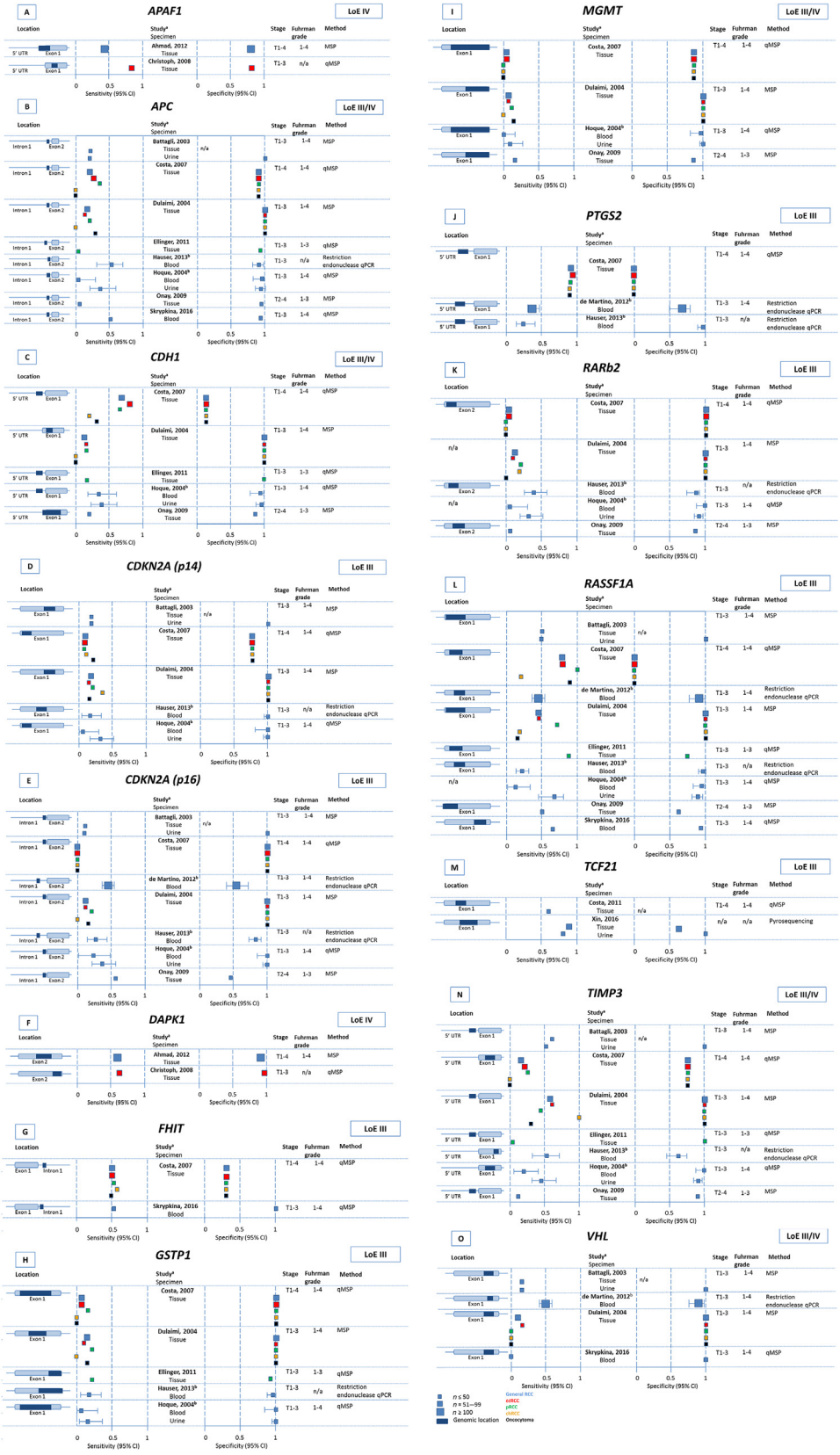

Fig. 3 – Forest plots of diagnostic RCC methylation markers that are independently validated in at least two studies. Forest plots of genomic location, sensitivity, and specificity associated with RCC diagnosis for (A) *APAF1*, (B) *APC*, (C) *CDH1*, (D) *CDKN2A (p14)*, (E) *CDKN2A (p16)*, (F) *DAPK1*, (G) *FHIT*, (H) *GSTP1*, (I) *MGMT*, (J) *PTGS2*, (K) *RARb2*, (L) *RASSF1A*, (M) *TCF21*, (N) *TIMP3*, and (O) *VHL* and corresponding LoE. ccRCC = clear cell renal cell carcinoma; chRCC = chromophobe renal cell carcinoma; CI = confidence interval; LoE = level of evidence; MSP = methylation specific polymerase chain reaction; n/a = not available; pRCC = papillary renal cell carcinoma; qMSP = quantitative methylation specific polymerase chain reaction; qPCR = quantitative polymerase chain reaction; RCC = renal cell carcinoma. [a] Sensitivities and specificities calculated from percentage of methylated samples. [b] Actual sensitivities and specificities.

3.3.2. Findings in liquid biopsies

Less invasive sample types such as blood or urine were investigated in six studies [5,36,38–41], with three (16%) studies reporting on blood [38,39,41], one (5%) describing both blood and urine [40], and two (11%) reporting on urine in addition to tissue [5,36]. Not every marker was independently validated in the same specimen type; five single urine biomarkers and nine single blood biomarkers were independently studied. The remaining six single urine biomarkers, two urine multimarker panels, 11 single blood biomarkers, and seven blood multimarker panels were reported only once, without validation. In general, reported sensitivities for most markers were lower for liquid biopsies than for tissue samples, with the highest reported single-marker sensitivity for *TCF21* (79%) in urine (specificity 100%; Fig. 3) [36]. Other single-marker sensitivities were low, ranging from 0% for *VHL* or *MGMT* methylation in blood (specificities 100% and 97%, respectively) to 65% for *RASSF1A* in urine (specificity 89%). Moreover, sensitivities for the same marker greatly varied: for example, sensitivities for *RASSF1A* ranged from 11% to 62.9% in blood (specificities of 93–98%) [38–41] and from 50% to 65% in urine (specificities 89–100%) [5,40]; sensitivities for *TIMP3* in blood ranged from 17% to 57% (specificities 61–100%) [39,40] and from 46% to 52% in urine (specificities 91–100%) [5,40]. Importantly, several genomic locations for the same biomarkers were investigated across these studies.

DNA methylation analysis in urine was always measured in addition to either tissue [5,36] or blood [40], but results did not always correspond. Battagli et al [5] were able to detect similar results regarding DNA methylation in *APC*, *CDKN2A (p14)*, *CDKN2A (p16)*, *RASSF1A*, *TIMP3*, and *VHL* between tissue and urine. Xin et al [36], however, reported 89% sensitivity and 62% specificity in tissue, and 79% sensitivity and 100% specificity in urine. Hoque et al [40] investigated blood and urine, but did not find any overlapping results between both specimen types. DNA methylation measured in urine always outperformed the same analysis in blood for *CDH1*, *APC*, *CDKN2A (p14)*, *CDKN2A (p16)*, *GSTP1*, *MGMT*, *RARb2*, *RASSF1A*, and *TIMP3*. The most promising independently validated individual marker was *RASSF1A* in both urine (sensitivities 50–65%; specificities 89–100%) and blood (sensitivities 11–63%; specificities 93–98%; Fig. 3). *LRRC3B* (74% sensitivity, 66.7% specificity) [41] appeared to be the most promising individual marker, but independent validation is lacking (Supplementary Fig. 1). Although several multimarker panels studied by Hauser et al [39], Battagli et al [5], and Skrypkina et al [41] showed promising results in liquid biopsies (sensitivities 74.3–92.3%; specificities 77.8–100%; Supplementary Fig. 1), independent validation studies are not available.

3.3.3. Biomarker selection procedure

Most studies (84%) based their biomarker selection procedure on literature reporting on methylated biomarkers in several cancer types (Fig. 4), whereas only three studies (16%) [30,33,34] based their biomarker selection on RCC microarray and expression data, thereby focusing on identification of RCC-specific candidate biomarkers. Although not validated yet, the latter studies reported relatively high sensitivities (61–100%) compared with studies examining nonspecific RCC markers.

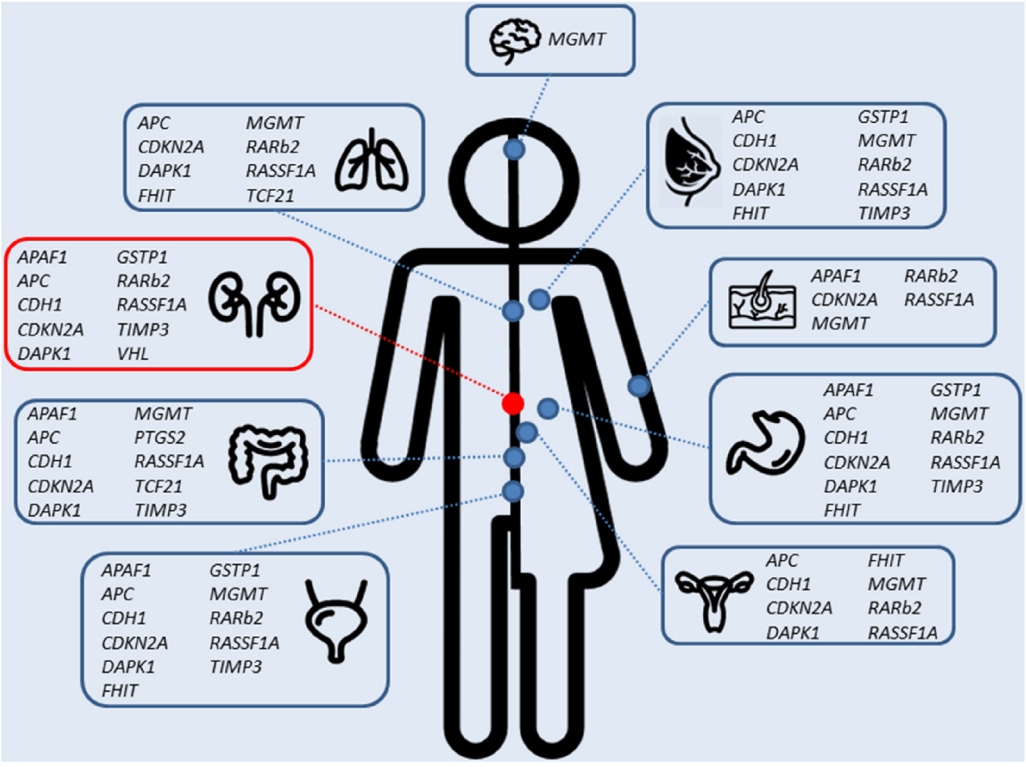

Fig. 4 – Independently validated markers known to be involved or methylated in several human cancers.

3.3.4. Level of evidence

Finally, we estimated the current LoE of the included biomarkers. The current LoE is III for 18 biomarkers, nine biomarkers currently have LoE III/IV, and 17 biomarkers have LoE IV. None of the 44 individual biomarkers reached the desired LoE I or II for clinical implementation.

3.4. Discussion

The purpose of this systematic review was to provide a comprehensive overview of all diagnostic DNA methylation biomarkers for RCC. To limit potential publication bias, studies were carefully identified and included by two independent researchers [42].

Although 44 individual diagnostic DNA methylation biomarkers have been published, only 15 were investigated in an independent study or population. Generally, sensitivities for the independently validated biomarkers in both tissue and liquid biopsies were low, indicating that these biomarkers do not have a clinical value. Among these 44 biomarkers, only a few individual markers (*LRRC3B* [41] and *TCF21* [36]) or multimarker panels (investigated by Battagli et al [5], Hauser et al [39], and Skrypkina et al [41]) showed sensitivities $>$ 70% in liquid biopsies, thereby making them potentially promising diagnostic biomarkers. However, most of these markers or panels have been studied in small populations and have not been validated independently yet. Among the 15 independently validated markers, none showed sensitivities high enough to merit further validation in future studies. Moreover, as none of the markers described in this review exceed LoE III, these markers cannot be considered for use in clinical practice yet.

Previous studies postulate various reasons for the hampered translation of biomarkers into clinical practice, including lack of validation, lack of standardisation, and other methodological problems such as identifying the most clinically relevant genomic location of an assay [18,43–46]. Many of these problems were also identified here, thereby impeding the head-to-head comparison of different studies assessing the same biomarker, and making it impossible to perform meta-analyses. Here, we address the influence of the biomarker selection, patient selection, and research methodologies, as these problems were consistently identified among included studies.

The majority of included studies examined the diagnostic value of tumour suppressor genes known to be methylated in several cancer types (Fig. 4). Although their importance in cancer has been established, these genes are not RCC specific and may therefore not be appropriate for RCC diagnosis. It would be more appropriate to study potential biomarkers derived from subtype-specific driver events in a systematic manner, for example, by using publicly available databases such as The Cancer Genome Atlas [18]. Although not independently validated yet, the empirically identified biomarkers in this review indeed showed better performance.

To improve current RCC diagnosis, noninvasive evaluation of biomarkers in liquid biopsies is preferred. Nevertheless, most markers in this review were studied in tissue samples, without assessing the marker performance in liquid biopsies. A tissue-based diagnostic test will have no additional clinical value as pathological evaluation can already accurately diagnose RCC. Most studies examined heterogeneous patient populations including all TNM stages and varying Fuhrman grades. Although it is important for diagnostic biomarkers to be measurable in early stages and all grades, the inclusion of a large number of highly staged and/or graded tumours may distort the performance of a specific biomarker, as these tumour characteristics are associated with invasion and metastasis [47]. Moreover, not every study reported which RCC subtypes were included in their analyses, even though these subtypes originate from distinct biological pathways [12]. Analysis of all patients in one group may conceal the diagnostic potential of a biomarker, as a specific biomarker may be methylated in one subtype but not in another. This problem might be solved by selecting the best performing biomarker per subtype and combining these in a multimarker panel. In general, multimarker panels outperform single markers as these panels better reflect the inter- and intratumour heterogeneity in cancer [48]. Consistently, multimarker panels in this systematic review outperformed single markers.

Contradictory results between studies could also have been caused by diverse research methodologies such as sample selection and handling, DNA methylation detection methods, and genomic location of the assay [18,44,46,49]. Although not described for RCC, the phenomenon of DNA hypermethylation in normal appearing tissue surrounding the tumour has been described for prostate, colorectal, and breast cancer [50], suggesting that this normal appearing tissue is not an appropriate control. Nevertheless, histologically normal appearing tissue adjacent to the tumour or oncocytomas is frequently selected as control tissue. In agreement with our previous publications [18,44,46], we here observed that the use of different genomic locations for one biomarker can impact study outcome, and hamper interindividual study comparison and further biomarker validation.

At this moment, none of the studied biomarkers exceed LoE III, indicating their limited clinical utility. Results of this systematic review show that after initial publication of a potential biomarker, subsequent studies do not substantially add to the LoE. To improve the LoE, prospective cohort studies and/or meta-analyses including sufficient cases are required. However, most researchers do not evaluate upfront which study design is needed to ensure that their results contribute to the development of a sufficient LoE. Further, to facilitate individual study comparisons, more standardised methodology and reporting should be applied. Despite the introduction of the STARD criteria [20,23], notable variation in reporting is observed, indicating that the STARD criteria are not fully applied. Full adherence to the STARD criteria is difficult, as these were not specifically developed for diagnostic biomarker research. As a measure of reporting quality, a STARD score is not interchangeable with the study quality itself; a low STARD score does not mean that the studied biomarker should be discarded, but it can hamper a study's reproducibility, thereby hindering clinical translation. Increased awareness and STARD criteria

adapted to diagnostic biomarker studies are urgently needed.

4. Conclusions

DNA methylation biomarkers may facilitate RCC diagnosis in patients presenting with unidentifiable renal masses and screening of people at a high risk of developing RCC. In conclusion, in order to work towards clinically useful diagnostic RCC biomarkers, we need an empirical biomarker identification and selection procedure, further validation in large prospective cohorts and meta-analyses, more standardised research methodology, and reporting guidelines applicable to diagnostic biomarker research.

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Study concept and design: Lommen, Smits.
Acquisition of data: Lommen, Smits.
Analysis and interpretation of data: Lommen, Vaes, Aarts, van Roermund, Schouten, Oosterwijk, Melotte, Tjan-Heijnen, van Engeland, Smits.
Drafting of the manuscript: Lommen, Vaes, Smits.
Critical revision of the manuscript for important intellectual content: Lommen, Vaes, Aarts, van Roermund, Schouten, Oosterwijk, Melotte, Tjan-Heijnen, van Engeland, Smits.
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Appendix A. Supplementary data

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