Translation of DNA methylation markers for the early detection of renal cell cancer

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RAISING THE ODDS

KIM LOMMEN

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ISBN 978-94-6419-581-1
Printed by Ipskamp Printing | proefschriften.net
Layout and design: W. Aalberts, persoonlijkproefschrift.nl

The research presented in this thesis was performed within GROW, School for Oncology and Reproduction, Department of Pathology, Maastricht University.
This research was financially supported by Kankeronderzoekfonds Limburg as part of Health Foundation Limburg (2018-03/KOFL) and the Van Koeverden-Van Wijk Foundation.

Translation of DNA methylation markers for the early detection of renal cell cancer:

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PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Universiteit Maastricht,

op gezag van de Rector Magnificus, Prof. dr. Pamela Habibović

volgens het besluit van het College van Decanen,

in het openbaar te verdedigen

op maandag 17 oktober 2022 om 16.00 uur

door

Kim Wilhelmina Jacoba Martina Petronella Lommen

Geboren op 23 februari 1994 te Helden

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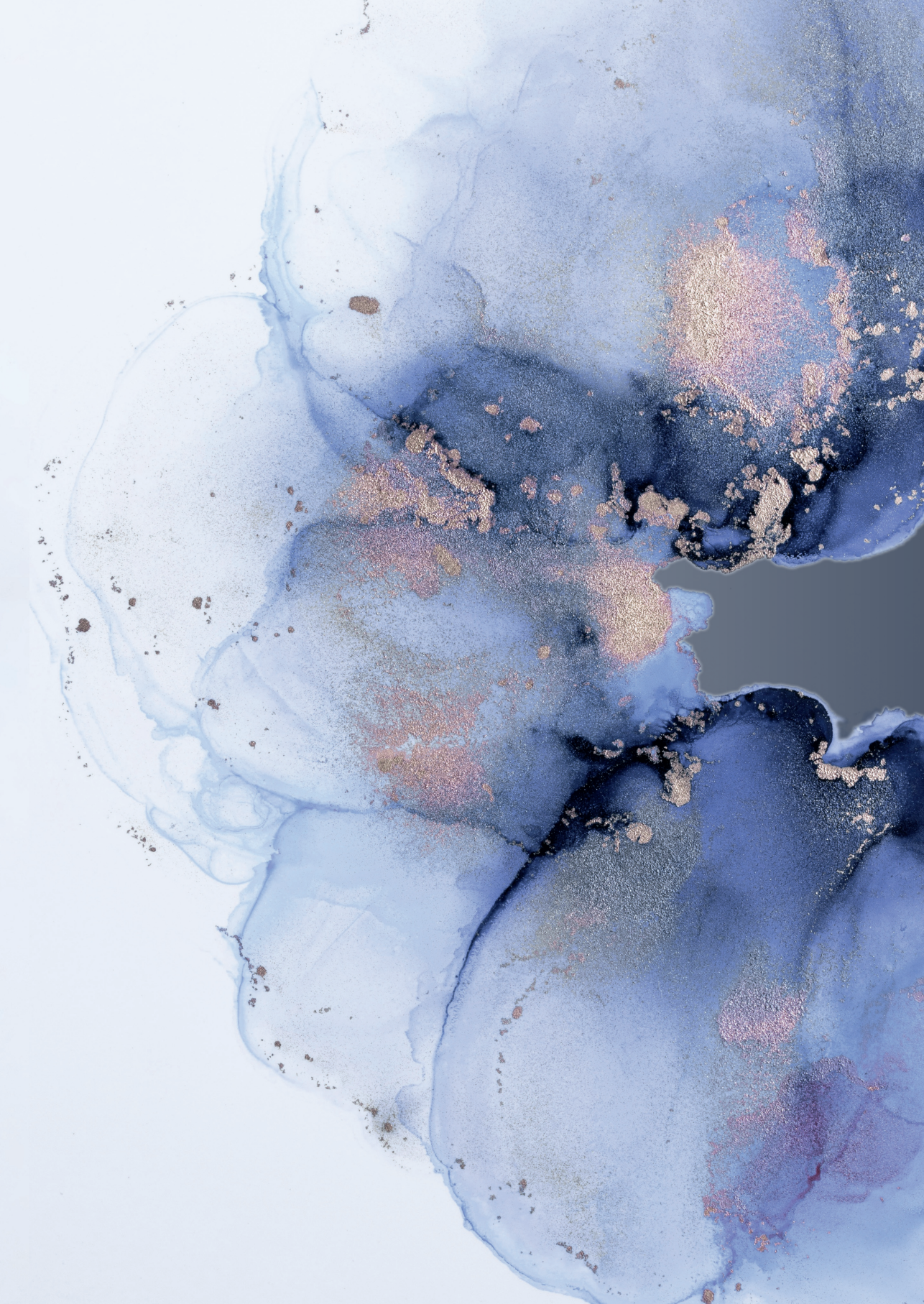
TABLE OF CONTENTS



CHAPTER 1

GENERAL INTRODUCTION

RENAL CELL CARCINOMA

In 2020, approximately 430.000 new cases of kidney cancer were reported globally, representing 2.2% of all cancers diagnosed[1]. In that same year, an estimated 180.000 deaths could be attributed to kidney cancer[1, 2].

Renal cell carcinoma (RCC) is the most prevalent kidney cancer, responsible for 90-95% of all cases, and the incidence rates have been rising with 2% per year over the past two decades[2, 3]. Although a shift towards detection of smaller masses can be observed, 25-40% of RCC patients still present with locally advanced disease or distant metastases at time of diagnosis[2, 3]. These late stages often represent an incurable stage of the disease[4, 5]. The 5-year survival rate of RCC confined to the kidney is 93%, whereas the 5-year survival of distant metastasized RCC is only 13%[6]. Major risk factors for RCC include modifiable risk factors such as obesity, smoking and hypertension[7, 8]. Among the non-modifiable risk factors, age and sex are the most important factors, as RCC incidence peaks at approximately 75 years and men are twice as likely to develop RCC compared to women[1, 9, 10]. Pre-existing (chronic) kidney diseases including kidney stones and kidney injury, and the use of several analgesics are also associated with an increased risk of developing RCC. The vast majority of RCCs is sporadic, but a small proportion, 2-5%, are hereditary RCCs[7, 8, 11, 12].

DIAGNOSIS OF RENAL CELL CARCINOMA

Early diagnosis has become a main focus in cancer research over the past decades, as it often allows curative treatment, corresponding to a favorable prognosis and low disease burden[13]. However, a primary RCC tumor does not often cause symptoms, thereby complicating the early diagnosis of this disease. Whenever symptoms occur, these mostly include a palpable mass, flank pain and hematuria; also referred to as the classic triad[14]. Because of the increasing use of cross-sectional imaging techniques, renal masses are also frequently detected coincidently during unrelated procedures; these masses are called incidentalomas[4, 8]. Computed tomography (CT) scans, magnetic resonance imaging (MRI) scans, positron emission tomography (PET) scans and ultrasounds are used to diagnose RCC[15]. A drawback of these imaging techniques is that for small renal masses (SRM) (<4 centimeters in diameter), they cannot always clearly distinguish whether the SRM is benign or malignant. Only an estimated 50-70% of all SRMs can be accurately categorized as benign or malignant based on imaging[3, 16-19]. Recently, several radiological imaging characterizations, composite models, nomograms and deep learning strategies to distinguish benign and malignant SRMs have been published, but these have not been able to improve discrimination rates yet[18-20]. In some countries, needle biopsies are

used to decide upon (partial) nephrectomy in case imaging cannot confirm or rule out a malignant tumor[21, 22]. However, the use of needle biopsies to molecularly characterize a tumor in the kidney is debatable[23, 24]. Because of intratumor heterogeneity, needle biopsies are not always representative of the tumor. As a result, up to 20% of needle biopsies cannot be used to diagnose, and in another 10%, they provide a false diagnosis in terms of subtype, stage or grade[18]. Both intratumor heterogeneity and the risk of tumor seeding therefore drive the lack of worldwide consensus about the clinical use and value of biopsies in diagnosing SRMs[20]. The increasing amount of incidentalomas and the challenge to diagnose these masses accurately based on imaging and biopsies emphasize the room for improvement in diagnosing early-stage RCC.

LOCALIZED RENAL CELL CARCINOMA

Localized renal tumors are treated by either partial or radical nephrectomy (laparoscopic or open)[25, 26]. According to the European Association of Urology (EAU) Guidelines on RCC, nephron sparing partial nephrectomy is the standard procedure for tumors up to 7 centimeters[14], and radical nephrectomy is performed when a RCC >7 centimeters is present. Despite the increased detection rates of small and early stage renal tumors, mortality rates have not decreased over the last decade, indicating that resecting SRMs might not be beneficial in all patients[1, 27-29]. SRMs have low growth rates of approximately 1-3 mm per year and rarely metastasize (1-3%)[30]. Therefore, to avoid overtreatment, preserve kidney function, and limit surgery risks, an active surveillance policy could be decided upon for SRMs, also considering patient characteristics like age, comorbidities and physical state[30, 31]. A recent prospective cohort study in which SRM patients were given the choice for either surgical resection (47%) or active surveillance (53%) indicates that active surveillance might decrease overall survival compared to surgical resection (66% vs. 85-90%)[31, 32]. Additionally, a statistically significant decreased quality of life in active surveillance compared to partial nephrectomy was measured, but that difference was attributed to the variation in physical state of patients in both groups[32]. In contrast, another prospective study concluded that there is no difference in quality of life between active surveillance and surgical intervention[33].

After surgical resection, RCC patients are classified by stage using the widely used tumor node metastasis (TNM) classification system, to guide clinicians in optimal treatment decisions. This system considers three components: primary tumor size and degree of invasion in neighboring tissues (T), metastases in regional lymph nodes (N), and distant metastases (M)[25]. This information is combined with histopathological features of the tumor, which indicate aggressiveness of the tumor[34, 35]. Until 2016, Fuhrman was the standard grading system for RCC, but it has been replaced by the WHO/ISUP system.

The WHO/ISUP grading system is based on nucleolar prominence rather than nucleolar size that was used in the Fuhrman grading system[36]. However, individual patients can have varying outcomes despite similar TNM stage and WHO/ISUP grade. Therefore, additional classification systems like the stage, size, grade, and necrosis (SSIGN) score that includes tumor necrosis[37] and the UCLA Integrated Staging System (UISS)[38] that includes performance status have been introduced. Even though these prognostic models are considered to be good indicators, they cannot predict patient outcome with the level of accuracy desired[39].

METASTATIC RENAL CELL CARCINOMA

In contrast to the often curative treatment regime of localized RCC, metastatic RCC (mRCC) has proven difficult to treat as it is highly resistant to both chemotherapy and radiation therapy[14]. The response rate of mRCC to chemotherapy alone is only 5-10%; this chemotherapy resistance may be related to expression of the multidrug resistance transporter in the proximal tubule cells, from which the majority of RCCs arise[40, 41]. RCC is considered insensitive to conventional radiation therapy, as it requires a relatively high dose to kill the RCC cells, while surrounding tissues like the jejunum, duodenum, and colon are highly susceptible to radiation toxicity[42-44]. Therefore, it is rarely used in a curative setting; however, it can be used as a palliative treatment to relieve symptoms at the sites of metastases[45]. Recent advances in radiotherapy options and refinements, such as stereotactic radiotherapy and proton therapy, has regained the interest in using radiation therapy either alone or in combination treatments[44, 46-49]. Until recently, first- and second-line systemic treatment for mRCC included targeted treatment with cytokines like INF-α, angiogenesis inhibitors like sorafenib and sunitinib and mTOR inhibitors temsirolimus and everolimus combined with sunitinib[14, 25, 26, 50]. Recent advances in the field of immunotherapy have resulted in the availability of amongst others bevacizumab and the combination of nivolumab and ipilimumab as first-line treatment for mRCC[14, 51, 52]. As many immune- and combination therapies are currently in a clinical trial setting, new treatment options for mRCC are expected in the near future[14, 50, 52]. The major expansion in targeted treatment and immunotherapy options for RCC has resulted in the necessity for tools that can select patients that will benefit from certain treatments. In order to work towards such a personalized approach, molecular features of the disease could provide information that is lacking in current models, and act as biomarkers for diagnosis, prognosis and treatment response prediction.

GENETICS AND EPIGENETICS OF RENAL CELL CARCINOMA

Even though most RCCs are diagnosed and treated in a similar way, RCC is a collective name for several subtypes which all originate from different parts of the nephron and therefore present with distinct (epi)genetic, molecular, histological and clinical characteristics[9].

Clear cell RCC (ccRCC) is the most common (75%) and aggressive type of RCC, which has a high tendency to metastasize and a poor prognosis. The 5-year and 10-year cancer-specific survival rates for ccRCC are 71% and 62% respectively, and distant metastases-free survival rates are 76% and 69% respectively[53-56]. Histologically, ccRCC is characterized by clear cytoplasm, and associated with loss or silencing of either one or both Von Hippel-Lindau (*VHL*) alleles in 60-90% of sporadic cases[25]. Inactivation of *VHL* results in upregulation of hypoxia inducible factors 1α (*HIF1α*) and 2α (*HIF2α*), which drive angiogenesis[26]. Due to upregulated angiogenesis promoting *VEGFA*, *KDM5C* and *KDM6A*, ccRCCs are highly vascularized[26]. As a result, ccRCC shows clusters of tumor cells, surrounded by networks of capillaries. Large genome-wide characterization studies, including the Cancer Genome Atlas (TCGA) Research Network and the TRACERx Renal study, have revealed the molecular landscape of ccRCC[57-62]. A critical genetic event in over 90% of ccRCCs is the loss of chromosome 3p, which holds four genes involved in chromatin remodeling, that are often inactivated in the remaining chromosomal copy. These include mutations in *VHL*; 60-70% of cases, *PBRM1*; 40% of cases, *BAP1*; 10% of cases, and *SETD2*; 10% of cases[57, 58, 60]. Additional chromosomal aberrations associated with ccRCCs are the gain of chromosome 5q (65-70% of cases) and the less frequent loss of chromosomes 8p, 9p and 14q[9, 58-60, 63, 64]. In addition to these sporadic ccRCC mechanisms, Von Hippel-Lindau disease is a hereditary disease associated with developing ccRCC through germline mutations in *VHL*[12, 65, 66].

Papillary RCC (pRCC) is less prevalent (15% of all RCCs) and aggressive compared to ccRCC. The 5-year and 10-year cancer-specific survival rates are 91% and 86% respectively, and distant metastases-free survival rates are 94% and 91% respectively[53-56]. Morphologically, pRCC can be subdivided into type 1 and type 2; apart from necrosis as a general histological feature, these pRCC subtypes present differently[26]. Type 1 pRCC is characterized by papillae lined with pale cytoplasm and low-grade nuclei tumor cells. In comparison, type 2 pRCC shows eosinophilic cytoplasm and large nuclei[64]. Although germline mutations in proto-oncogene *MET* are frequent in hereditary pRCC (75%), only approximately 6% of sporadic pRCC are associated with mutated *MET*[63, 64]. Its activated form drives cell growth, motility, migration, and differentiation[9]. Type 2 pRCC is associated with the Hereditary Leiomyomatosis and Renal Cell Carcinoma (HLRCC) syndrome, and is characterized by a germline mutation in *FH*[67]. Loss of this gene leads to accumula-

tion of fumarate in the cytoplasm of renal cells, resulting in inactivation of the *HIF1α* pathway as described above[64, 67].

Even though chromophobe RCC (chRCC; 5% of RCCs) is a malignant tumor, patients generally have a favorable prognosis compared to other RCCs (5-year and 10-year cancer-specific survival rates of 88% and 86% respectively, and distant metastases-free survival rates of 92% and 88% respectively[53-56]). Histologically, chRCC presents with large cells, clear cell borders and atypical nuclei with perinuclear halo[9]. PTEN alterations have been clearly linked to sporadic chRCC[25, 26]. More frequent genetic events in chRCC are the loss of chromosome 1, 2, 6, 10, 13, 17, 21 and X[9, 25, 26]. Birt-Hogg-Dubé patients often present with chRCCs as a result of germline mutations in *FLCN*. Although the function of *FLCN* is not yet fully understood, it seems to be a modulator of mTOR activity[64, 68].

Recently, several novel RCC subtypes have been proposed. Amongst others, succinate dehydrogenase-deficient RCC and thyroid-like follicular carcinoma of the kidney have already been acknowledged in the 2016 WHO classification of urological tumors, whereas additional subtypes are emerging and might be acknowledged in the near future[69].

In contrast to the limited amount of common genetic events in RCC summarized above, epigenetic alterations like DNA methylation are more frequent and early events in renal carcinogenesis. DNA methylation is the addition of a methyl group to the 5-carbon position of a cytosine, resulting in the inaccessibility of DNA for transcription and to gene silencing[63, 70]. Global methylation analyses in the TCGA database showed that high methylation correlated to higher stage and grade of all RCC subtypes, and the hypermethylated phenotype of all subtypes were correlated with poorer survival compared to their unmethylated counterparts ($P<0.0001$)[58, 71, 72]. In addition, a rare subset (5.6%) of pRCC tumors featuring a genome-wide CpG island methylator phenotype (CIMP) has been identified[58, 71, 72]. Despite being pRCC, this CIMP phenotype has been correlated to early onset and high stage disease, and was associated with the poorest survival among all RCC subtypes[58, 71, 73]. The *VHL* gene, but also other genes involved in the VHL-HIF signaling pathway involved in angiogenesis, like *PTEN*, *GREM1*, and *TIMP3*, are also commonly inactivated through hypermethylation[63]. In literature, a wide range of 3-42% of ccRCC cases are described to be affected by hypermethylated and thereby inactivated *VHL*[63]. In a TCGA network study, 7% of ccRCC were hypermethylated for *VHL*[72]. In the same study, an additional 289 additional genes were identified to be silenced through hypermethylation in at least 5% of RCCs and therefore considered functionally involved in RCC tumorigenesis. The most prominently methylated gene correlated to gene silencing in the TCGA study was *UQCRH* (methylated in 36% of all RCCs), which had already been recognized as a tumor-suppressor gene, but had never been linked to RCC[72]. A recent study by Luo *et al.* found that the loss of *UQCRH* expression by hypermethylation promotes RCC tumorigenesis by gaining a metabolic advantage through accelerating mitochondrial function decline[74]. In addition, hypermethylation of WNT

pathway genes, including *WIF1*, the *SFRP*s and *DKKs* (methylated in 8-73%, 9-80% and 7-58% respectively) dysregulates cell proliferation and differentiation, and can thereby induce tumorigenesis[58, 63, 73, 75-77]. DNA methylation of additional key genes involved in cell proliferation, differentiation and adhesion, amongst others *PBRM1*, *CDH1*, *FBN2* and *APC* (methylated in 41%, 6-83%, 21-52% and 5-54% respectively), are known to promote epithelial-to-mesenchymal transition and subsequent invasion and metastasis in RCC[63].

DNA METHYLATION BIOMARKERS FOR RENAL CELL CARCINOMA AND ITS CHALLENGES

As the molecular landscape of RCC has become more clear over the years, molecular markers involved in RCC such as (epi)genetic alterations have been investigated for the diagnosis, prognosis and disease prediction of localized RCC[14, 78, 79]. In addition, (epi) genetic biomarkers might contribute to better discrimination of benign and malignant SRMs prior to nephrectomy, thereby preventing surgical resection of benign SRMs. Current diagnostic procedures such as CT and MRI scans are costly and may be perceived as unpleasant because of scan duration, noise and space- and motion restriction[80, 81]. Next to that, they are considered time-consuming, not only because of the duration of the scan, but also because patients have to travel to and from the hospital[80]. To limit and potentially substitute part of such imaging procedures, researchers have been aiming to improve cancer diagnostics by focusing on molecular markers in liquid biopsies. These minimally-invasively collected bodily fluids like blood, stool or urine are assumed to represent the molecular composition of a malignant tumor, including its (epi)genetic make-up, and are therefore considered valuable sample types[82, 83]. Cell-free DNA (cfDNA), including circulating tumor DNA (ctDNA), is released into the blood stream as a result of apoptosis and necrosis of a solid tumor. Only small cfDNA and ctDNA fragments (~100 bp) can pass glomerular filtration and also end up in urine[84]. Next to ctDNA, other cancer-derived components such as proteins, circulating tumor cells (CTCs), RNA, and extracellular vesicles can be detected in liquid biopsies, providing information about the transcriptomic, proteomic, genetic and epigenetic features of a tumor[85]. As DNA methylation is a frequent and early event in carcinogenesis, it remains stable over time and can be analyzed by relatively simple, accurate and low-cost techniques, it is very suitable to act as a biomarker[63]. Even though for many types of cancer, several (epi)genetic biomarkers measured in liquid biopsies like blood and urine have been described, the translation rate of these biomarkers to a clinical setting is very low[86-88]. As advocated by several researchers, the field of (cancer) biomarkers produces a substantial amount of research waste, mainly caused by inappropriate research methodology, including a lack of validation, lack of standardization and lack of reproducibility of biomarkers[87-90].

Ioannidis *et al.* described the current biomarker development process as 'a tortuous series of linearly connected pipes' with several phases; biomarker discovery, validation, translation, evaluation and implementation[66]. All of these phases harbor their own issues, which hamper biomarker development and clinical translation[89]. For instance, in the research-oriented biomarker discovery and validation phase, non-empirically identifying candidate biomarkers, and how and where to measure these biomarkers, can hamper identification of suitable candidate biomarkers. A major technical consideration is designing an optimal assay regarding the technique, the primers and the genomic location of the assay. In addition, inappropriate study design such as low sample sizes and sampling bias may limit applicability of the biomarker in the general population. The fact that very few published biomarkers eventually reach clinical care emphasizes the importance of appropriate and standardized biomarker research methodology.

AIM AND OUTLINE OF THIS THESIS

The aim of this thesis is to identify and evaluate the utility of DNA methylation biomarkers for the non-invasive diagnosis of RCC. In addition, we aimed to evaluate reasons for the lack of clinical translation of diagnostic DNA methylation biomarkers and discuss how to overcome these.

In **Chapter 2**, we performed a systematic literature review in which we provided an overview of all published diagnostic DNA methylation biomarkers for RCC and summarized their current Level of Evidence (LoE). In addition, we identified issues that may hamper clinical translation of these biomarkers. In **Chapter 3**, we evaluated technical considerations in PCR-based assay design for diagnostic DNA methylation biomarkers. We specifically looked into the genomic location of the assay and assessed the primer and probe quality of included assays.

The availability of large study cohorts of appropriate samples, complemented by extensive and well-annotated clinical and pathological patient data is crucial for fast and adequate validation of biomarkers. Therefore, in **Chapter 4** we elaborated on considerations for establishing new biobanks, as well as for using existing biobanks, both in general and specific for certain specimen types, in order to develop optimal conditions for validation of biomarkers for early detection of cancer.

We concluded that the lack of clinically useful diagnostic DNA methylation biomarkers for RCC might be attributed to, amongst others, the lack of empirical biomarker identification. Therefore, in **Chapter 5,** we used a novel *in silico* approach to identify RCC specific DNA methylation biomarkers for the early detection of RCC.

The lack of reproducibility of biomarkers could be caused by amongst others the choice of inappropriate control samples. The fact that normal appearing tissues adjacent to the tumor might be molecularly predisposed to become malignant, emphasizes the importance of carefully selecting appropriate control tissues in biomarker studies. In **Chapter 6**, we therefore aimed to evaluate the existence of a DNA methylation field effect in RCC, and to illustrate the impact of this field effect and choice of control tissues in biomarker identification and development.

In the general discussion of **Chapter 7**, the findings of this thesis are discussed and reflected upon. In addition, we provide future perspectives and recommendations relevant to the development of clinically useful diagnostic DNA methylation biomarkers for cancer management.

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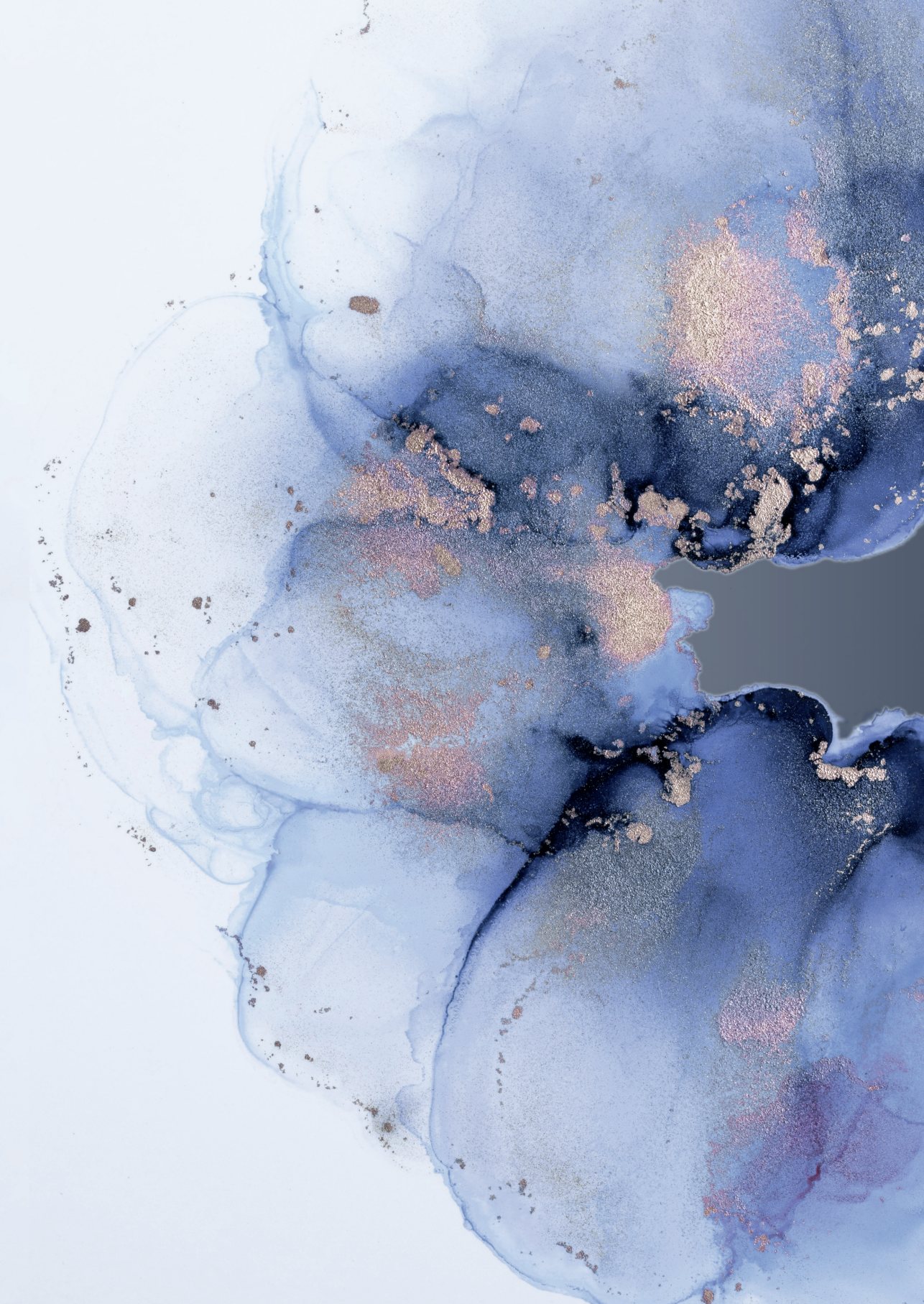

CHAPTER 2

DIAGNOSTIC DNA METHYLATION BIOMARKERS FOR RENAL CELL CARCINOMA: A SYSTEMATIC REVIEW

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European Urology Oncology. 2021;4(2):215-26

ABSTRACT

Context: The 5-year survival of early stage renal cell carcinoma (RCC) is approximately 93%, but once metastasized, the 5-year survival plummets to 12%, indicating that early RCC detection is crucial to improve 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 summarize 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 summarize 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 multi-marker panels were included; however, only 15 biomarkers were independently validated. STARD scores varied from 4-13 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.

Conclusions: None of the reported biomarkers exceeded LoE III, indicating limited clinical utility. Moreover, study reproducibility and further development of these RCC biomarkers is greatly hampered by inadequate reporting.

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%) that present advanced disease at time of diagnosis[2,3]. This is attributed to the typical lack of symptoms of the primary RCC, leading to a substantial number of metastasized 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-year survival rates of early stage RCC are around 93%, patients presenting with metastasized RCC have poor 5-year survival rates, around 12%[6]. These numbers indicate the great importance to accurately diagnose RCC at an early stage. Because 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 non-invasive 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 (≤4 cm) is discovered[9,10,11].

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 abberations[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 to 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 4 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 summarize current evidence for these biomarkers.

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].

Search strategy, eligibility criteria & study selection

Electronic literature searches (up to January 2019) of PubMed, EMBASE, Medline and Google Scholar were conducted (supplementary table 3). Articles eligible for this systematic review were all original articles on diagnostic DNA methylation biomarkers in RCC. Other inclusion criteria were: English language; specific genes being evaluated; biomarker potential was 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. Because 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 (figure 1).

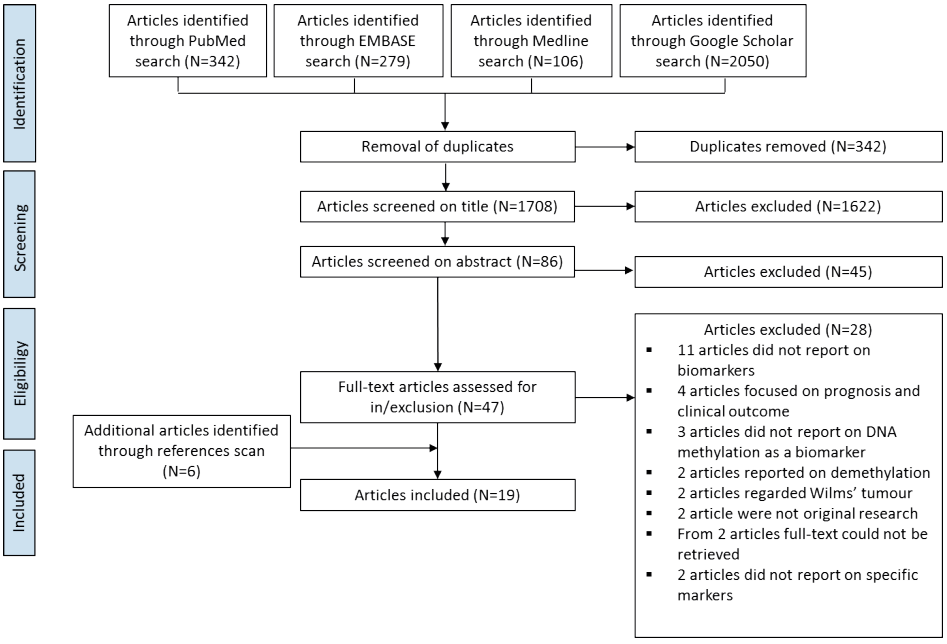


Figure 1. PRISMA flow diagram visualizing the study selection process
PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses.

Data extraction

All data were extracted by two independent authors (KL and KS) using a standardised data extraction sheet. In addition, articles were assessed for reporting quality using STARD 2015[20], which considers 34 items for good reporting of diagnostic accuracy studies. Each of these items were 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 4), the maximum reporting score was 23 points. Mutual consensus was reached whenever inter-observer variation occurred. The risk of potential bias across or within studies was analysed per study using the STARD scores (supplementary table 2). 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, 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 or not reported, potential measurement bias risk was increased (supplementary table 2).

To obtain a summary of current evidence on diagnostic DNA methylation biomarkers in RCC, the 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.

Forest plots

Forest plots were created to summarize 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.

EVIDENCE SYNTHESIS

Study characteristics

Nineteen articles (published between 2003 and 2017) were included in this systematic review using a standardised selection procedure (figure 1). Four (21%) studies described one single biomarker, whereas 15 (79%) reported on multiple markers. A total of 44

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

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	

Table 1. Characteristics of the 19 studies included in this systematic review. (continued)

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 %*	
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	
Costa, 2007[24]	85	PTT	Fresh frozen	ccRCC, pRCC, chRCC, oncocytomas	qMSP	*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	

Table 1. Characteristics of the 19 studies included in this systematic review. (continued)

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 %*	
Costa, 2011[30]	33	PTT	Fresh frozen	PTT: ccRCC, pRCC, chRCC, oncocytomas Urine: general RCC	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	
						Panel of TCF21, PCDH17	PTT 67[b], urine 32[b]	PTT 100[b], urine 100[b]	
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	

Table 1. Characteristics of the 19 studies included in this systematic review. (continued)

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 %*	
Dulaimi, 2004[25]	100	PTT	Imbedded in OCT	ccRCC, pRCC, chRCC, collecting duct, RCC unclassified, oncocytomas, TCC renal pelvis, Wilms' tumour	MSP	*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

Table 1. Characteristics of the 19 studies included in this systematic review. (continued)

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 %*	
Hauser, 2014[39]	35	Blood	NR	ccRCC, pRCC, chRCC	Restriction endonucle-ase 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. Characteristics of the 19 studies included in this systematic review. (continued)

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]	

Table 1. Characteristics of the 19 studies included in this systematic review. (continued)

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 %*	
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]	

Table 1. Characteristics of the 19 studies included in this systematic review. (continued)

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 %*	
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	
Urakami, 2006[35]	62	PTT	FFPE	ccRCC, granular cell RCC, ccRCC/granular cell RCC mix	MSP	*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

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

[b] Actual sensitivity and specificity

[c] Compared to oncocytomas

Abbreviations: 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/ionization 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

individual biomarkers were studied and sample sizes ranged from 21-196 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%) on pRCC patients and three (16%) studies did not specify RCC subtype. Study characteristics are summarized in table 1.

STARD reporting assessment and potential bias

The Standards for Reporting of Diagnostic Accuracy Studies (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 4-13 out of the maximum of 23 points, with a median of 10 points (supplementary table 1). Only items 3 and 4 were partially or completely described in every study, whereas items 13a, 18 and 19 were not reported in any study (figure 2). None of the included studies obtained the maximum quality score. The risk of potential selection- and measurement bias in the included studies is summarized in supplementary table 2, showing that most studies suffer from selection and measurement bias.

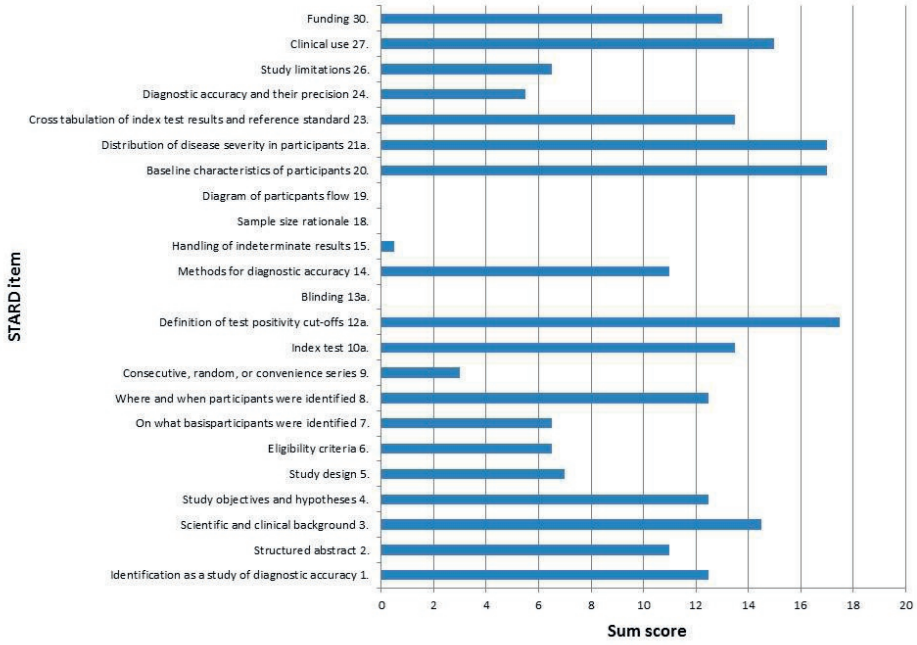
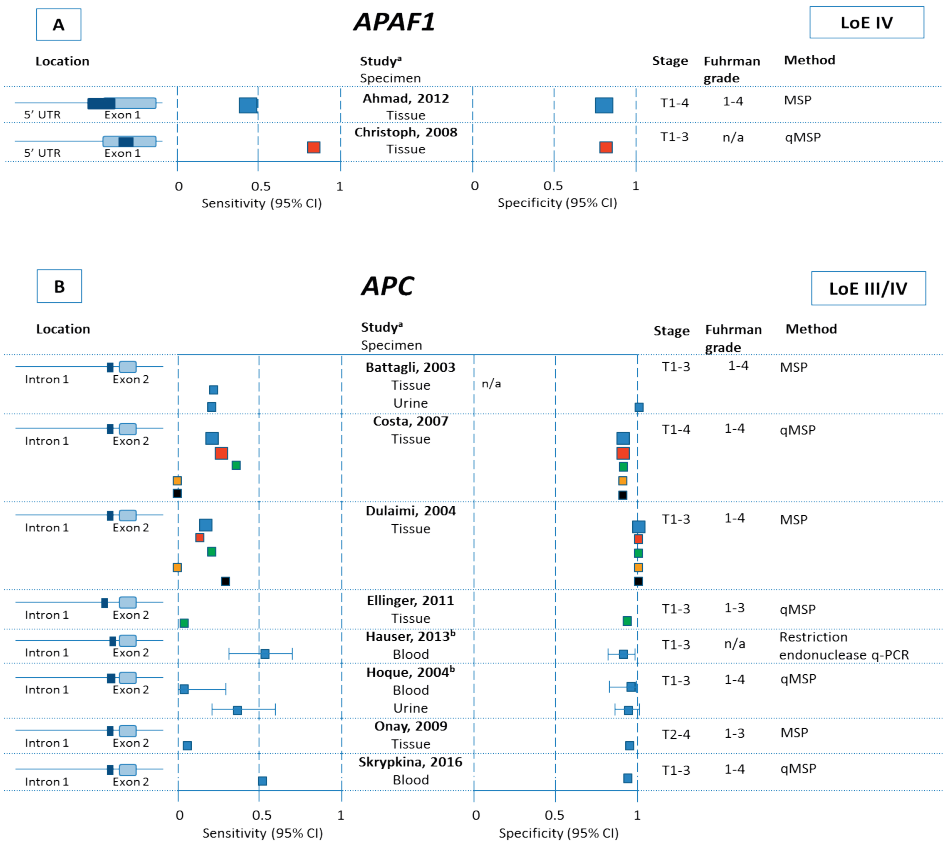


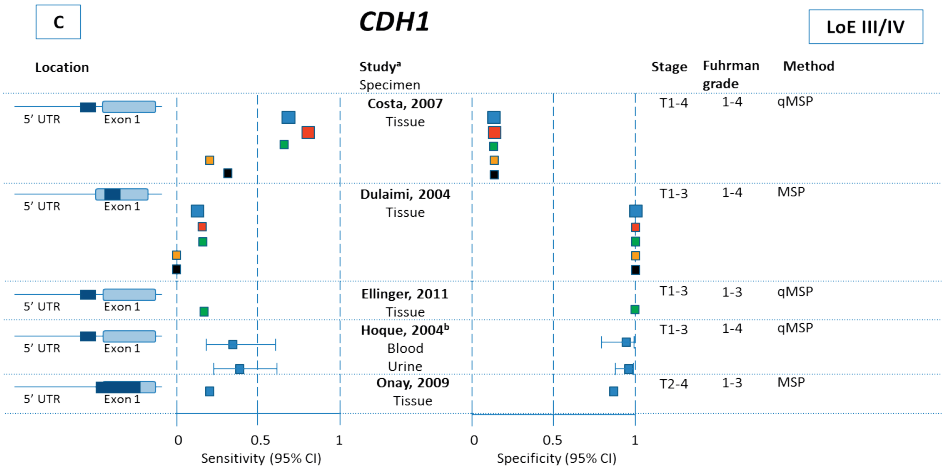
Figure 2. Sum score of all articles per STARD item
STARD: Standards for Reporting of Diagnostic Accuracy Studies.

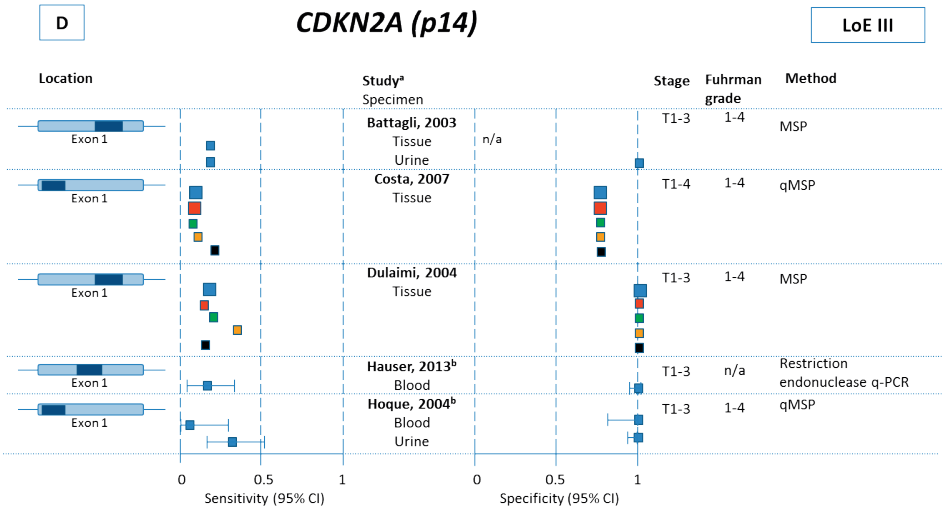
Study findings

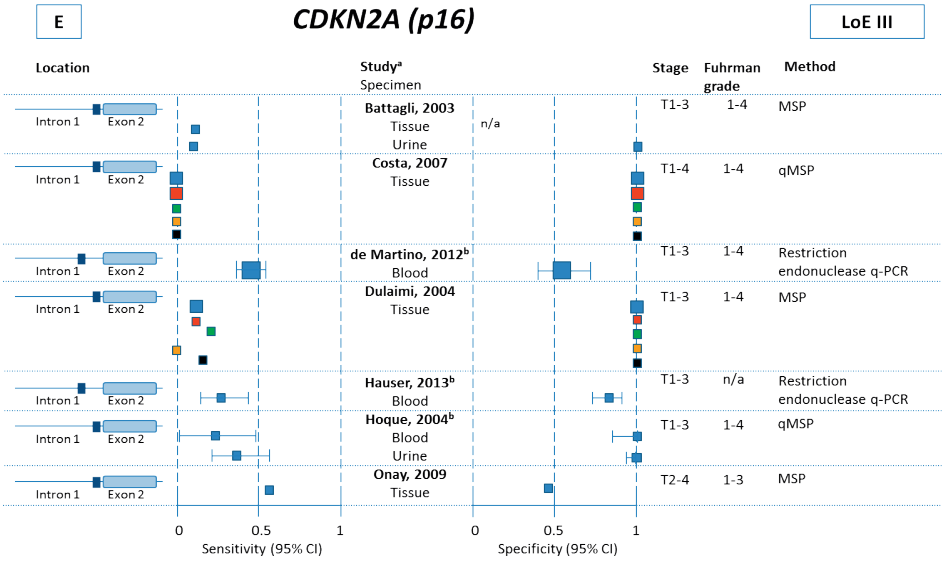
Fifteen DNA methylation biomarkers were studied in at least two independent study populations (figure 3). Results of biomarkers without independent validation are shown in supplementary figure 1.

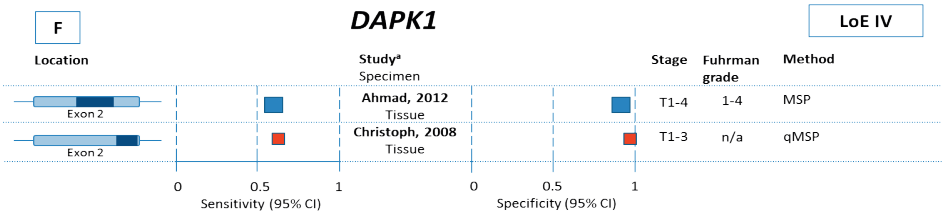
Overall, large methodological differences were observed between studies, including differences in DNA methylation detection techniques, study population and subgroup analyses. Most studies (n=15; 79%) measured DNA methylation using methylation specific polymerase chain reaction (MSP) or quantitative MSP (qMSP). 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 (figure 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].

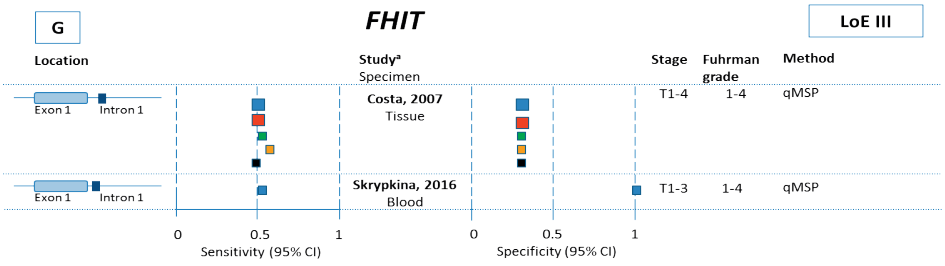


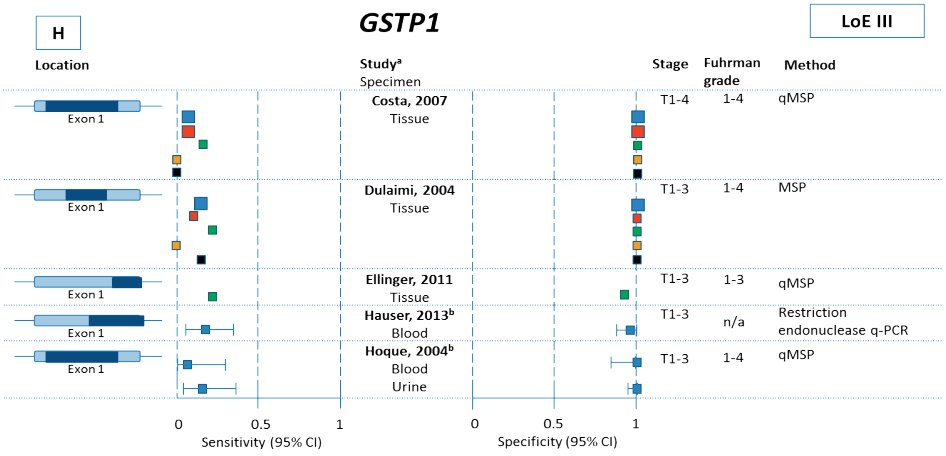

C
CDH1
LoE III/IV
Location
Study[a]
Specimen
Stage
Fuhrman grade
Method
5′ UTR
Exon 1
Costa, 2007
Tissue
T1-4
1-4
qMSP
Dulaimi, 2004
Tissue
T1-3
1-4
MSP
Ellinger, 2011
Tissue
T1-3
1-3
qMSP
Hoque, 2004[b]
Blood
Urine
T1-3
1-4
qMSP
Onay, 2009
Tissue
T2-4
1-3
MSP
0
0.5
1
Sensitivity (95% CI)
Specificity (95% CI)

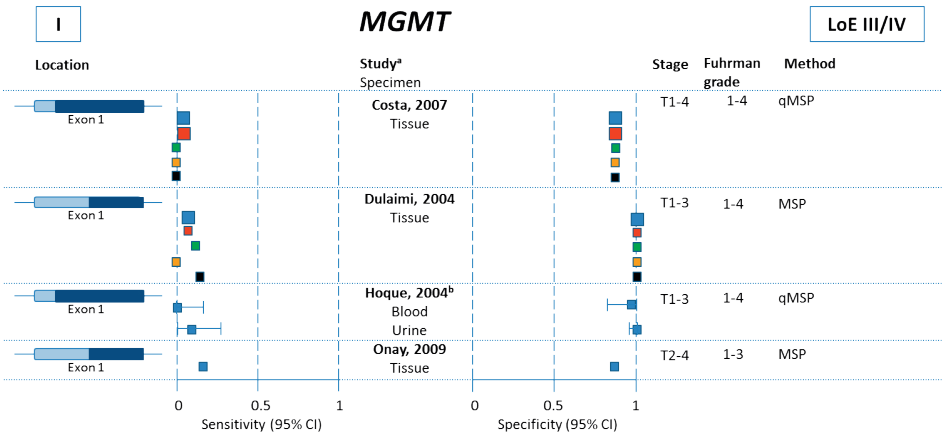

D
CDKN2A (p14)
LoE III
Location
Study[a]
Specimen
Stage
Fuhrman grade
Method
Exon 1
Battagli, 2003
Tissue
Urine
n/a
T1-3
1-4
MSP
Costa, 2007
Tissue
T1-4
1-4
qMSP
Dulaimi, 2004
Tissue
T1-3
1-4
MSP
Hauser, 2013[b]
Blood
T1-3
n/a
Restriction endonuclease q-PCR
Hoque, 2004[b]
Blood
Urine
T1-3
1-4
qMSP
0
0.5
1
Sensitivity (95% CI)
Specificity (95% CI)

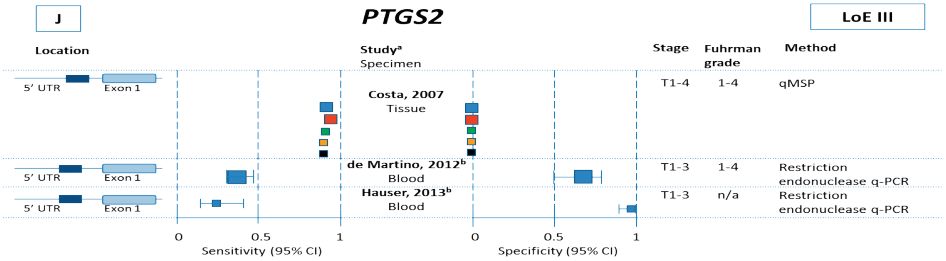

E
CDKN2A (p16)
LoE III
Location
Study[a]
Specimen
Stage
Fuhrman grade
Method
Intron 1
Exon 2
Battagli, 2003
Tissue
Urine
n/a
T1-3
1-4
MSP
Costa, 2007
Tissue
T1-4
1-4
qMSP
de Martino, 2012[b]
Blood
T1-3
1-4
Restriction endonuclease q-PCR
Dulaimi, 2004
Tissue
T1-3
1-4
MSP
Hauser, 2013[b]
Blood
T1-3
n/a
Restriction endonuclease q-PCR
Hoque, 2004[b]
Blood
Urine
T1-3
1-4
qMSP
Onay, 2009
Tissue
T2-4
1-3
MSP
0
0.5
1
Sensitivity (95% CI)
Specificity (95% CI)

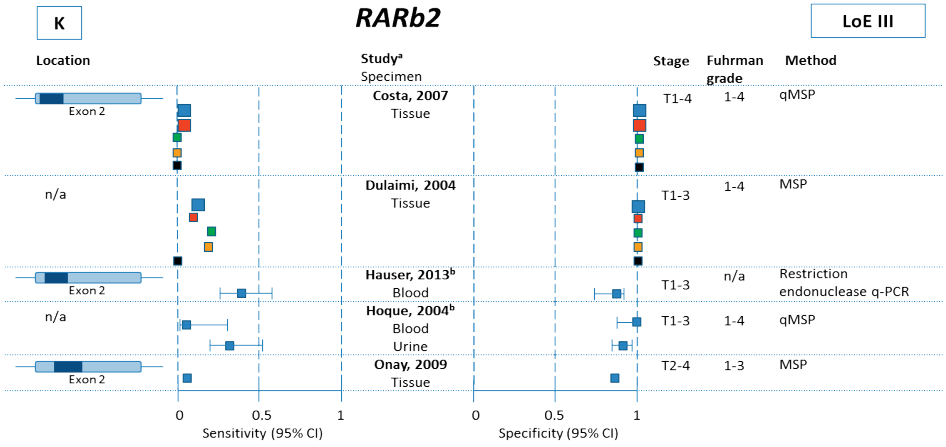

F
DAPK1
LoE IV
Location
Study[a]
Specimen
Stage
Fuhrman grade
Method
Exon 2
Ahmad, 2012
Tissue
T1-4
1-4
MSP
Christoph, 2008
Tissue
T1-3
n/a
qMSP
0
0.5
1
Sensitivity (95% CI)
Specificity (95% CI)

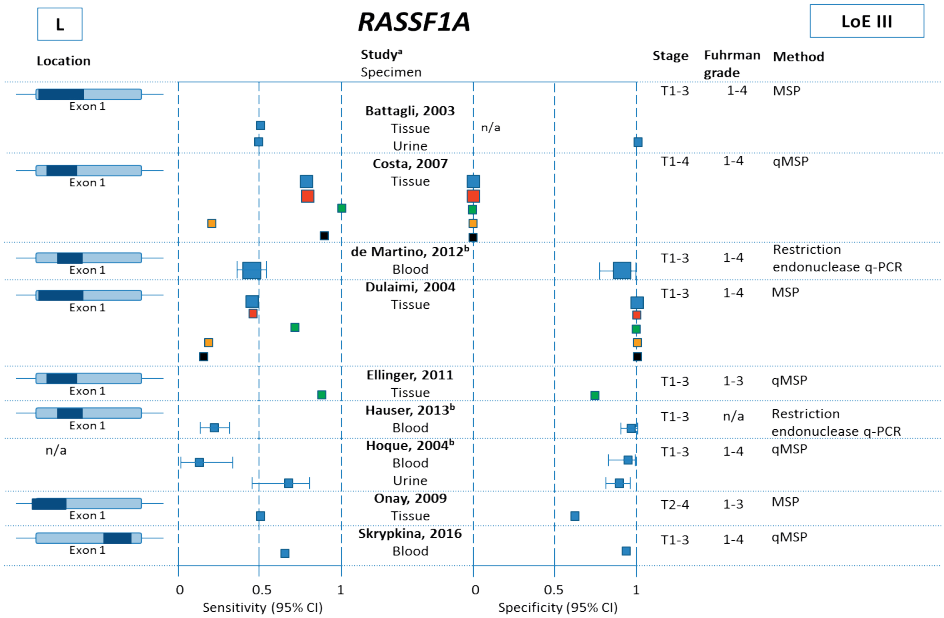

G
FHIT
LoE III
Location
Study[a]
Specimen
Stage
Fuhrman grade
Method
Exon 1
Intron 1
Costa, 2007
Tissue
T1-4
1-4
qMSP
Skrypkina, 2016
Blood
T1-3
1-4
qMSP
0
0.5
1
Sensitivity (95% CI)
Specificity (95% CI)

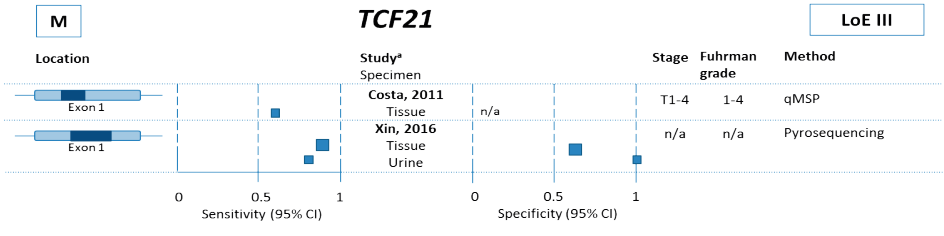

H
GSTP1
LoE III
Location
Study[a]
Specimen
Stage
Fuhrman grade
Method
Exon 1
Costa, 2007
Tissue
T1-4
1-4
qMSP
Dulaimi, 2004
Tissue
T1-3
1-4
MSP
Ellinger, 2011
Tissue
T1-3
1-3
qMSP
Hauser, 2013[b]
Blood
T1-3
n/a
Restriction endonuclease q-PCR
Hoque, 2004[b]
Blood
Urine
T1-3
1-4
qMSP
0
0.5
1
Sensitivity (95% CI)
Specificity (95% CI)


I
MGMT
LoE III/IV
Location
Study[a]
Specimen
Stage
Fuhrman grade
Method
Exon 1
Costa, 2007
Tissue
T1-4
1-4
qMSP
Dulaimi, 2004
Tissue
T1-3
1-4
MSP
Hoque, 2004[b]
Blood
Urine
T1-3
1-4
qMSP
Onay, 2009
Tissue
T2-4
1-3
MSP
0
0.5
1
Sensitivity (95% CI)
Specificity (95% CI)


J
PTGS2
LoE III
Location
Study[a]
Specimen
Stage
Fuhrman grade
Method
5' UTR
Exon 1
Costa, 2007
Tissue
T1-4
1-4
qMSP
de Martino, 2012[b]
Blood
T1-3
1-4
Restriction endonuclease q-PCR
Hauser, 2013[b]
Blood
T1-3
n/a
Restriction endonuclease q-PCR
0
0.5
1
Sensitivity (95% CI)
Specificity (95% CI)


K
RARb2
LoE III
Location
Study[a]
Specimen
Stage
Fuhrman grade
Method
Exon 2
Costa, 2007
Tissue
T1-4
1-4
qMSP
n/a
Dulaimi, 2004
Tissue
T1-3
1-4
MSP
Exon 2
Hauser, 2013[b]
Blood
T1-3
n/a
Restriction endonuclease q-PCR
n/a
Hoque, 2004[b]
Blood
Urine
T1-3
1-4
qMSP
Exon 2
Onay, 2009
Tissue
T2-4
1-3
MSP
0
0.5
1
Sensitivity (95% CI)
Specificity (95% CI)


L
RASSF1A
LoE III
Location
Study[a]
Specimen
Stage
Fuhrman grade
Method
Exon 1
Battagli, 2003
Tissue
Urine
n/a
T1-3
1-4
MSP
Exon 1
Costa, 2007
Tissue
T1-4
1-4
qMSP
Exon 1
de Martino, 2012[b]
Blood
T1-3
1-4
Restriction endonuclease q-PCR
Exon 1
Dulaimi, 2004
Tissue
T1-3
1-4
MSP
Exon 1
Ellinger, 2011
Tissue
T1-3
1-3
qMSP
Exon 1
Hauser, 2013[b]
Blood
T1-3
n/a
Restriction endonuclease q-PCR
n/a
Hoque, 2004[b]
Blood
Urine
T1-3
1-4
qMSP
Exon 1
Onay, 2009
Tissue
T2-4
1-3
MSP
Exon 1
Skrypkina, 2016
Blood
T1-3
1-4
qMSP
0
0.5
1
Sensitivity (95% CI)
Specificity (95% CI)


M
TCF21
LoE III
Location
Study[a]
Specimen
Stage
Fuhrman grade
Method
Exon 1
Costa, 2011
Tissue
n/a
T1-4
1-4
qMSP
Exon 1
Xin, 2016
Tissue
Urine
n/a
n/a
Pyrosequencing
0
0.5
1
Sensitivity (95% CI)
Specificity (95% CI)

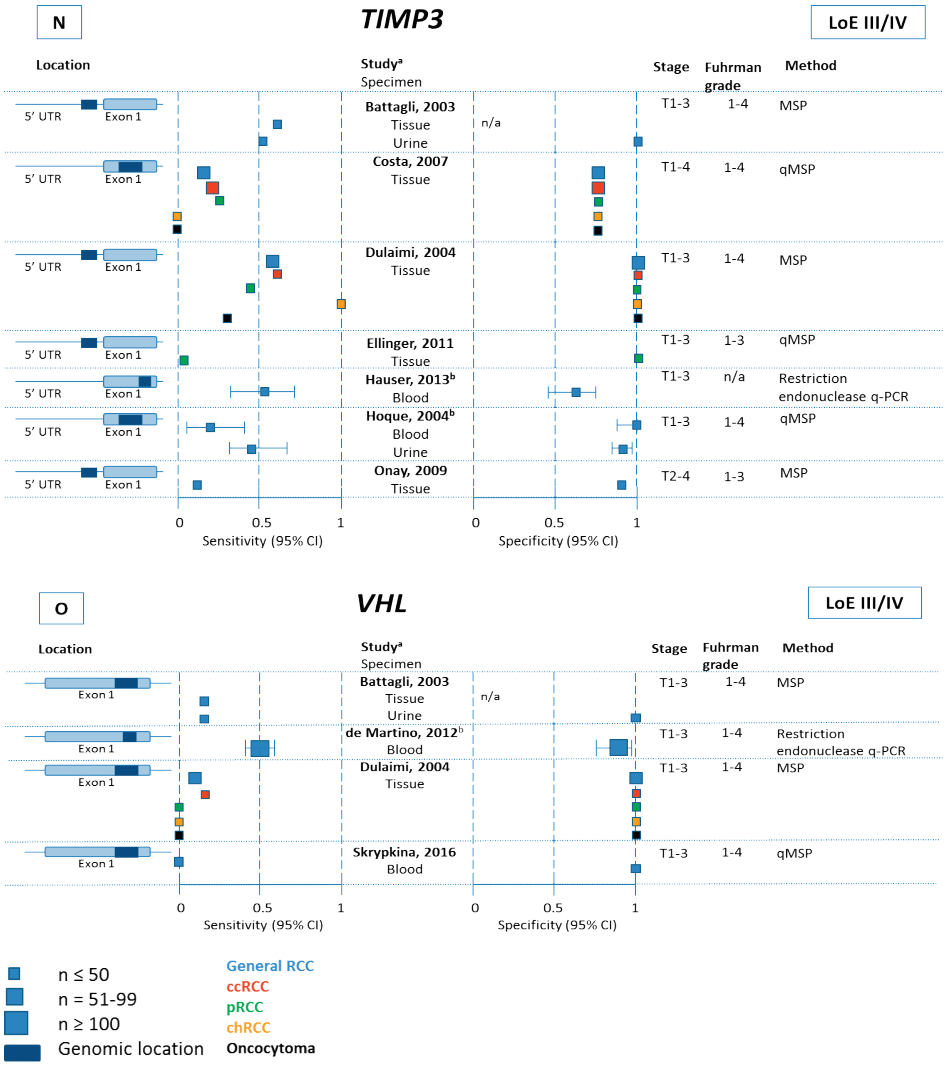


Figure 3. Forest plots of diagnostic RCC methylation markers that are independently validated in at least two studies.

Forest plots of genomic location, sensitivity and specificity associated with RCC diagnosis for *APAF1* (A), *APC* (B), *CDH1* (C), *CDKN2A (p14)* (D), *CDKN2A (p16)* (E), *DAPK1* (F), *FHIT* (G), *GSTP1* (H), *MGMT* (I), *PTGS2* (J), *RARb2* (K), *RASSF1A* (L), *TCF21* (M), *TIMP3* (N), and *VHL* (O) and corresponding LoE.

[a] Sensitivities and specificities calculated from percentage of methylated samples. [b] Actual sensitivities and specificities.

ccRCC, clear cell renal cell carcinoma; chRCC, chromophobe renal cell carcinoma; CI, confidence interval; LoE, Level of Evidence; MSP, methylation specific polymerase chain reaction; pRCC, papillary renal cell carcinoma; qMSP, quantitative methylation specific polymerase chain reaction; qPCR, quantitative polymerase chain reaction; RCC, renal cell carcinoma.

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 2 (11%) studies examined both tissue and urine (table 1) [5,36]. Fourteen single tissue markers were independently studied in at least two populations. The remaining 27 single tissue markers and four tissue multi-marker panels were only reported 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, wide ranges of sensitivities and specificities were observed by different research groups, e.g. for *RASSF1A* sensitivities of 45-80% and specificities of 0-100% were reported in RCC overall (figure 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 utilizing *OXR1* and *MST1R* appeared to be the best performing multi-marker panel for general RCC tissue samples with 98% sensitivity and 100% specificity, yet also this panel requires validation and assessment in liquid biopsies before any statement regarding diagnostic potential can be made[33].

Findings in liquid biopsies

Less invasive sample types such as blood or urine were investigated in 6 studies[5,36,38-41] with 3 (16%) studies reporting on blood[38,39,41], one (5%) describing both blood and urine [40], and two (11%) studies 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 multi-marker panels, 11 single blood biomarkers and seven blood multi-marker panels were only reported on once, without validation. In general, reported sensitivities for most markers were lower for liquid biopsies compared to tissue samples, with the highest reported single-marker sensitivity for *TCF21* (79%) in urine (specificity 100%; figure 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: e.g. sensitivities for *RASSF1A* ranged from 11% to 62.9% in blood (specificities of 93-98%)[38-41] and 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 46-52%

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.* was able to detect similar results regarding DNA methylation in *APC*, *CDKN2A (p14), CDKN2A (p16), RASSF1A, TIMP3* and *VHL* between tissue and urine[5]. Xin *et al.*, however, reported 89% sensitivity and 62% specificity in tissue, and 79% sensitivity and 100% specificity in urine[36]. Hoque *et al.* 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*[40]. 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%) (figure 3). *LRRC3B* (74% sensitivity, 66.7% specificity)[41] appeared to be the most promising individual marker, but independent validation is lacking (supplementary figure 1). Although several multi-marker panels studied by Hauser *et al.*, Battagli *et al.* and Skrypkina *et al.* showed promising results in liquid biopsies (sensitivities 74.3-92.3%; specificities 77.8-100%) (supplementary figure 1), independent validation studies are not available[5,39,41].

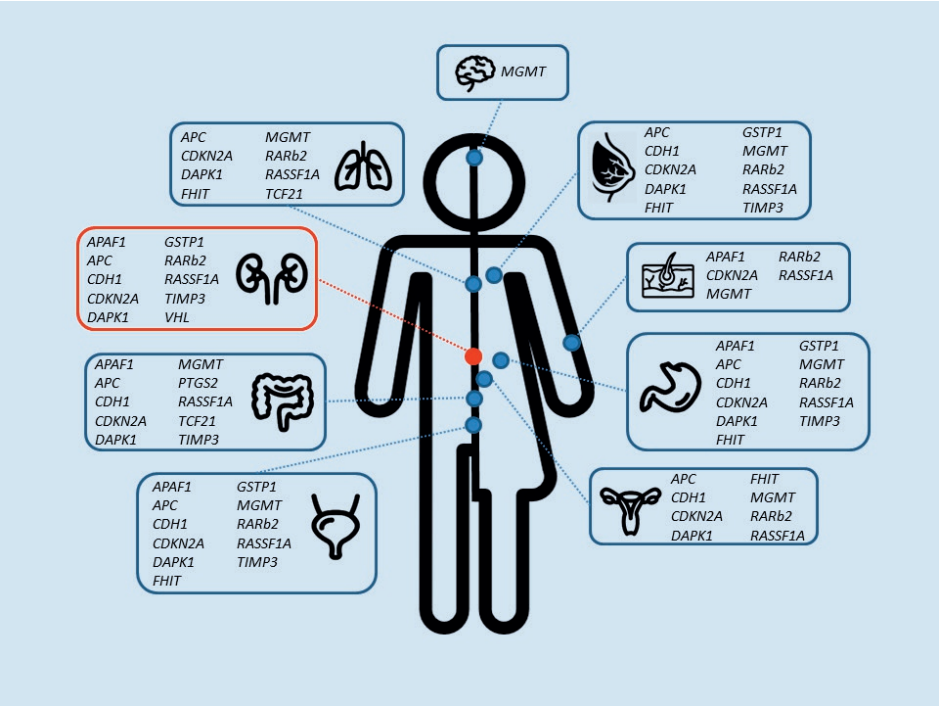


Figure 4. Independently validated markers known to be involved or methylated in several human cancers

Biomarker selection procedure

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

Level of Evidence

Finally, we estimated the current Level of Evidence (LoE) of the included biomarkers. For 18 biomarkers, the current LoE is III, 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 to consent to clinical implementation.

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 clinical value. Among these 44 biomarkers, only a few individual markers (*LRRC3B*[41] and *TCF21*[36]) or multi-marker panels (investigated by Battagli *et al.*, Hauser *et al.*, and Skrypkina *et al.*)[5,39,41] showed sensitivities higher than 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 independently validated 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 (figure 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, e.g. by using publically 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, a non-invasive 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 large numbers 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]. Analysing 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 multi-marker panel. In general, multi-marker panels outperform single markers as these panels better reflect the inter- and intratumour heterogeneity in cancer[48]. Consistently, multi-marker 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 are 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 inter-individual study comparison and further biomarker validation.

At this moment, none of the studied biomarkers exceed LoE III, indicating 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.

CONCLUSIONS

DNA methylation biomarkers may facilitate RCC diagnosis in patients presenting with unidentifiable renal masses and screening of people at high risk for 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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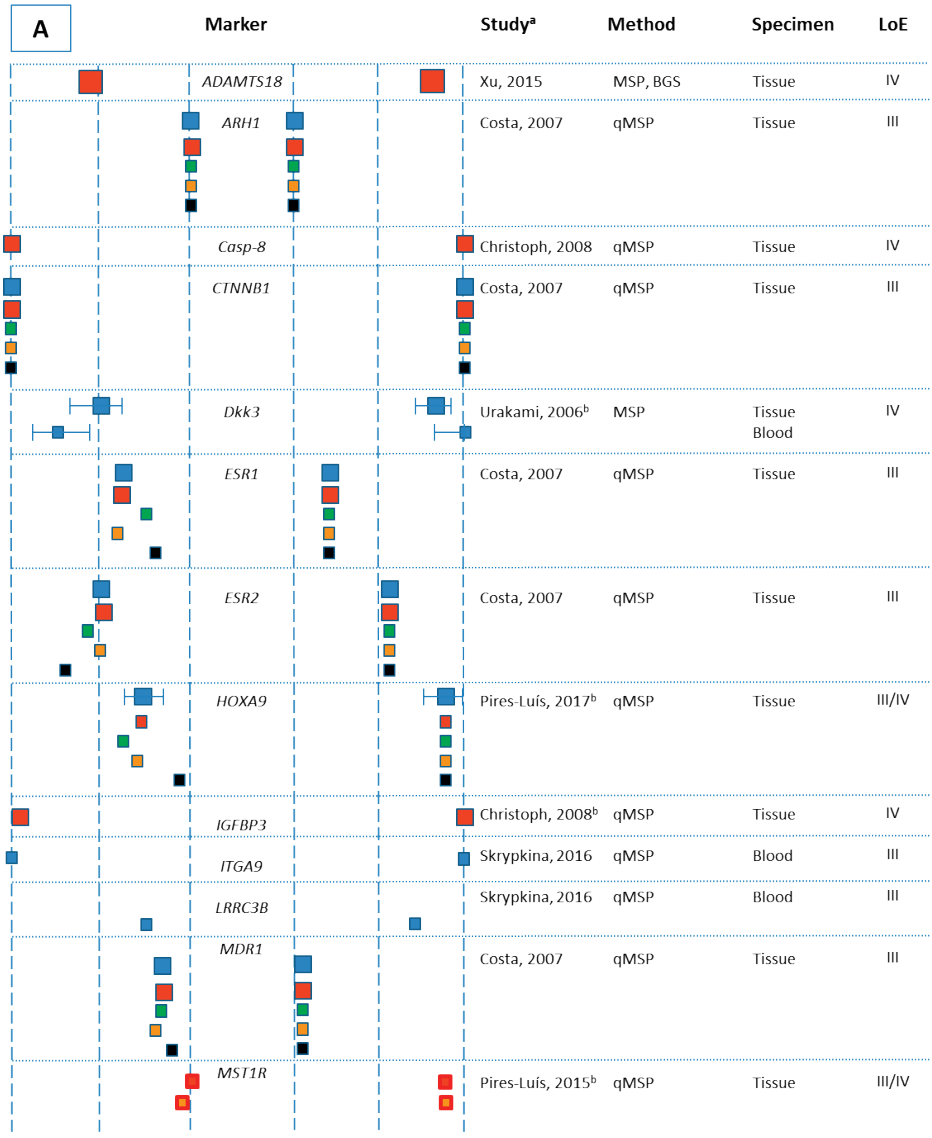
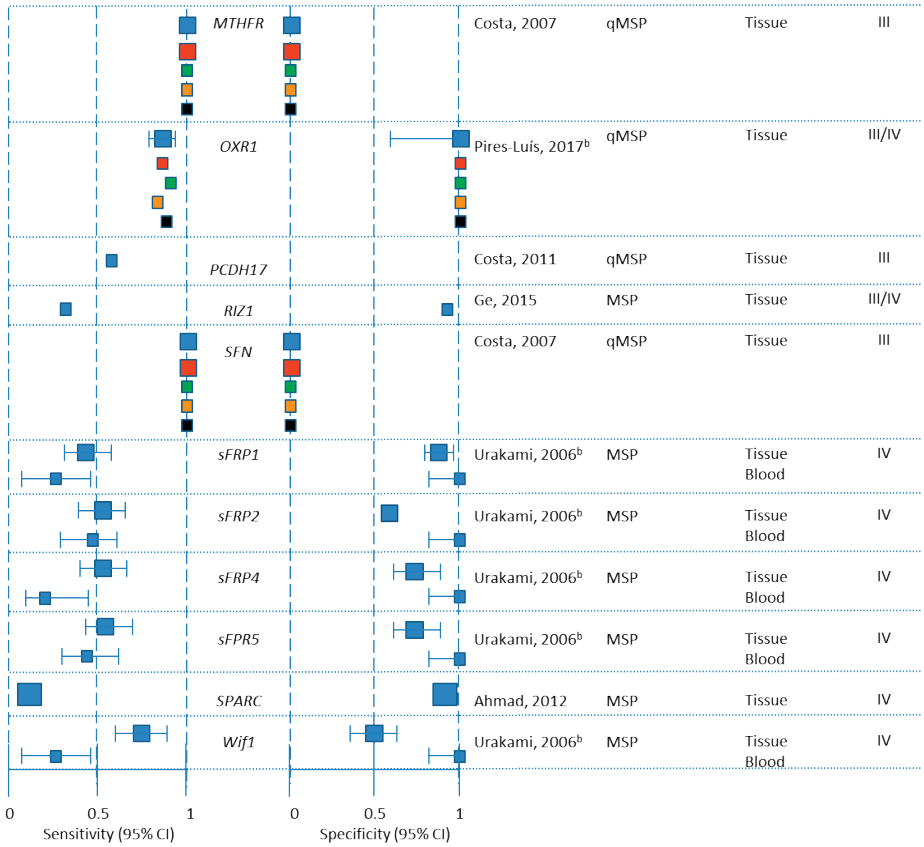
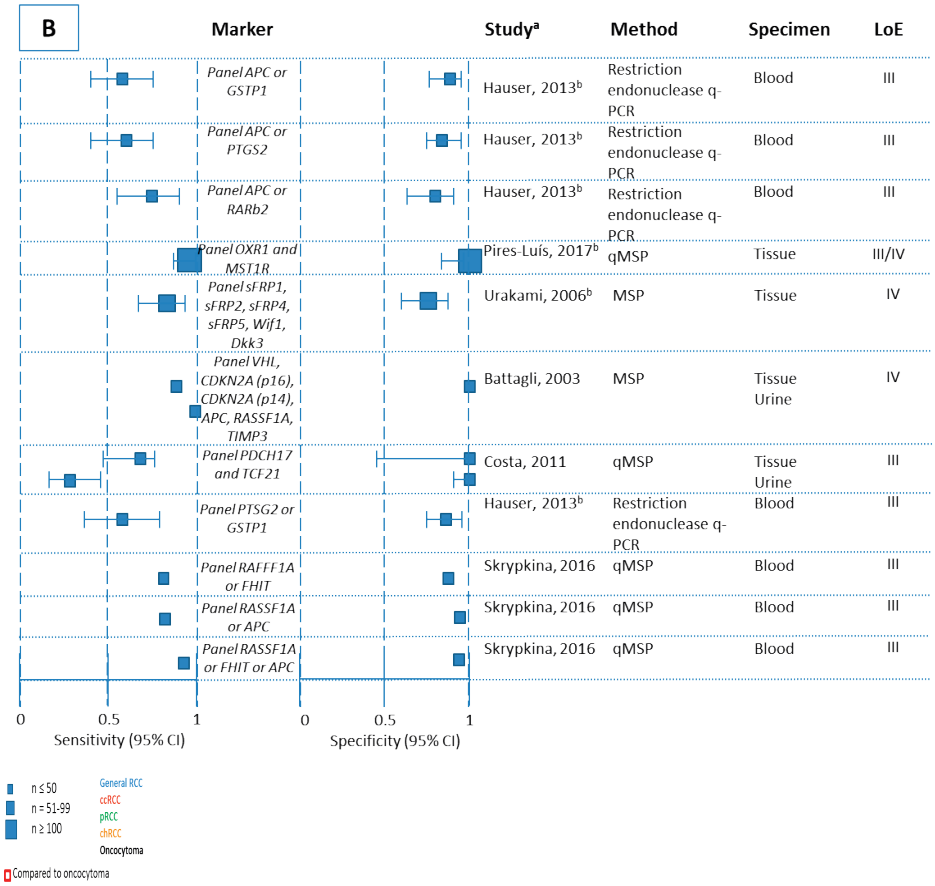


A	Marker	Study[a]	Method	Specimen	LoE
	ADAMTS18	Xu, 2015	MSP, BGS	Tissue	IV
	ARH1	Costa, 2007	qMSP	Tissue	III
	Casp-8	Christoph, 2008	qMSP	Tissue	IV
	CTNNB1	Costa, 2007	qMSP	Tissue	III
	Dkk3	Urakami, 2006[b]	MSP	Tissue Blood	IV
	ESR1	Costa, 2007	qMSP	Tissue	III
	ESR2	Costa, 2007	qMSP	Tissue	III
	HOXA9	Pires-Luís, 2017[b]	qMSP	Tissue	III/IV
	IGFBP3	Christoph, 2008[b]	qMSP	Tissue	IV
	ITGA9	Skrypkina, 2016	qMSP	Blood	III
	LRRC3B	Skrypkina, 2016	qMSP	Blood	III
	MDR1	Costa, 2007	qMSP	Tissue	III
	MST1R	Pires-Luís, 2015[b]	qMSP	Tissue	III/IV


MTHFR
Costa, 2007
qMSP
Tissue
III
OXR1
Pires-Luís, 2017b
qMSP
Tissue
III/IV
PCDH17
Costa, 2011
qMSP
Tissue
III
RIZ1
Ge, 2015
MSP
Tissue
III/IV
SFN
Costa, 2007
qMSP
Tissue
III
sFRP1
Urakami, 2006b
MSP
Tissue
Blood
IV
sFRP2
Urakami, 2006b
MSP
Tissue
Blood
IV
sFRP4
Urakami, 2006b
MSP
Tissue
Blood
IV
sFPR5
Urakami, 2006b
MSP
Tissue
Blood
IV
SPARC
Ahmad, 2012
MSP
Tissue
IV
Wif1
Urakami, 2006b
MSP
Tissue
Blood
IV
0
0.5
1
Sensitivity (95% CI)
0
0.5
1
Specificity (95% CI)



Supplementary figure 1. Forest plot of diagnostic RCC methylation markers and multi-marker panels that were investigated in a single study.

Forest plots of genomic location, sensitivity and specificity associated with RCC diagnosis for single markers (A) and multi-marker panels (B) and corresponding LoE.

[a] Sensitivities and specificities calculated from percentage of methylated samples. [b] Actual sensitivities and specificities.

ccRCC, clear cell renal cell carcinoma; chRCC, chromophobe renal cell carcinoma; CI, confidence interval; LoE, Level of Evidence; MSP, methylation specific polymerase chain reaction; pRCC, papillary renal cell carcinoma; qMSP, quantitative methylation specific polymerase chain reaction; qPCR, quantitative polymerase chain reaction; RCC, renal cell carcinoma.

Supplementary table 1. Individual STARD scores per study.

		Identification as a study of diagnostic accuracy	Structured abstract	Scientific and clinical background	Study objectives and hypotheses	Study design	Eligibility criteria	On what basis participants were identified	Where and when participants were identified	Consecutive, random, or convenience series	Index test	Definition of test positivity cut-offs	Blinding	Methods for diagnostic accuracy	Handling of indeterminate results	Sample size rationale	Diagram of participants flow	Baseline characteristics of participants	Distribution of disease severity in participants	Cross tabulation of index test results and reference standard	Diagnostic accuracy and their precision	Study limitations	Clinical use	Funding	**Total score**
STARD item		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10a**	**12a**	**13a**	**14**	**15**	**18**	**19**	**20**	**21a**	**23**	**24**	**26**	**27**	**30**	
Marker	**Study**																								
APAF1, DAPK1, SPARC	Ahmad, 2012[29]	0.5	0	0.5	0.5	0.5	1	1	1	0	1	1	0	0	0.5	0	0	1	1	1	0.5	0	1	1	**10**
VHL, CDKN2A (p16), CDKN2A (p14), APC, RASSF1A, TIMP3, panel of VHL and CDKN2A (p16) and CDKN2A (p14) and APC and RASSF1A and TIMP3	Battagli, 2003[5]	1	0	0.5	0.5	0.5	1	1	0	0	0.5	1	0	1	0	0	0	1	1	1	0.5	0	1	1	**10**
APAF1, CASP8, DAPK1, IGFBP3	Christoph, 2008[30]	0.5	1	0.5	1	0.5	1	1	1	0	0.5	1	0	0	0	0	0	1	1	0.5	0	0	0	0	**8**
APAF1, CASP8, DAPK1, IGFBP3	Christoph, 2008[30]	0.5	1	0.5	1	0.5	1	1	1	0	0.5	1	0	0	0	0	0	1	1	0.5	0	0	0	0	**8**
APC, ARH1, CDH1, CTNNB1, SFN, CDKN2A (p14), CDKN2A (p16), RASSF1A, GSTP1, MDR1, MTHFR, PTGS2, TIMP3, ESR1, ESR2, FHIT, MGMT, RARb2	Costa, 2007[25]	0.5	1	1	0.5	0.5	1	1	1	1	0.5	1	0	0	0	0	0	1	1	1	0.5	1	0.5	1	**12**

Supplementary table 1. Individual STARD scores per study. (continued)

STARD item		1	2	3	4	5	6	7	8	9	10a	12a	13a	14	15	18	19	20	21a	23	24	26	27	30	Total score
Identification as a study of diagnostic accuracy																									
Structured abstract																									
Scientific and clinical background																									
Study objectives and hypotheses																									
Study design																									
Eligibility criteria																									
On what basis participants were identified																									
Where and when participants were identified																									
Consecutive, random, or convenience series																									
Index test																									
Definition of test positivity cut-offs																									
Blinding																									
Methods for diagnostic accuracy																									
Handling of indeterminate results																									
Sample size rationale																									
Diagram of participants flow																									
Baseline characteristics of participants																									
Distribution of disease severity in participants																									
Cross tabulation of index test results and reference standard																									
Diagnostic accuracy and their precision																									
Study limitations																									
Clinical use																									
Funding																									
Marker	**Study**																								
TCF21, PCDH17, panel of TCF21 and PCDH17	Costa, 2011[31]	1	0	1	0.5	0.5	0	0	0.5	0	1	1	0	1	0	0	0	1	1	1	1	0	1	1	**11**
SFRP1, SCNN1B, SYT6, TFAP2A, DACH1, MT1G_001	Dalgin, 2008[32]	0	1	0.5	0.5	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	**4**
RASSF1A, VHL, PTGS2, CDKN2A (p16)	de Martino, 2012[39]	1	1	1	0.5	1	1	1	1	1	1	0	0	1	0	0	0	1	1	0	0	1	0.5		**13**
VHL, RASSF1A, CDKN2A (p16), CDKN2A (p14), APC, MGMT, GSTP1, RARb2, CDH1, TIMP3	Dulaimi, 2004[26]	0	1	0.5	0.5	0.5	0	0.5	0.5	0	0.5	1	0	0	0	0	0	1	1	1	0	0	1	1	**7**
APC, CDH1, GSTP1, RASSF1A, TIMP3	Ellinger, 201128	1	1	0.5	0.5	0.5	0	1	1	0	0.5	1	0	1	0	0	0	1	1	0	1	0.5	1	1	**11**
RIZ1	Ge, 2015[33]	0	1	0.5	1	0.5	0	0	0.5	0	0.5	1	0	0	0	0	0	1	1	1	0	0.5	1	0	**7**

Supplementary table 1. Individual STARD scores per study. (continued)

		Identification as a study of diagnostic accuracy	Structured abstract	Scientific and clinical background	Study objectives and hypotheses	Study design	Eligibility criteria	On what basis participants were identified	Where and when participants were identified	Consecutive, random, or convenience series	Index test	Definition of test positivity cut-offs	Blinding	Methods for diagnostic accuracy	Handling of indeterminate results	Sample size rationale	Diagram of participants flow	Baseline characteristics of participants	Distribution of disease severity in participants	Cross tabulation of index test results and reference standard	Diagnostic accuracy and their precision	Study limitations	Clinical use	Funding	**Total score**
STARD item		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10a**	**12a**	**13a**	**14**	**15**	**18**	**19**	**20**	**21a**	**23**	**24**	**26**	**27**	**30**	
Marker	**Study**																								
APC, GSTP1, CDKN2A (p14), CDKN2A (p16), RASSF1A, TIMP3, PTGS2, RARb2, panel APC or GSTP1, panel APC or PTGS2, panel APC or RARb2, panel PTGS2 or GSTP1	Hauser, 2014[40]	1	1	1	0.5	1	1	0	1	0	1	0.5	0	1	0	0	0	1	1	1	1	0.5	0	0	**12**
APC, CDKN2A (p14), CDH1, GSTP1, MGMT, CDKN2A (p16), RARb2, RASSF1A, TIMP3	Hoque, 2004[41]	1	0	1	0.5	1	0	0	0	0	0.5	1	0	1	0	0	0	1	1	1	0	0	1	1	**10**
RASSF1A, CDH1, TIMP3, APC, MGMT, CDKN2A (p16), RARb2	Onay, 2009[27]	0	0	0.5	1	0	0	0	0	0	0.5	1	0	0	0	0	0	1	1	1	0	1	1	0	**7**
MST1R	Pires-Luís, 2015[35]	1	1	1	1	0	0	0	1	0	1	1	0	1	0	0	0	1	1	0	0	0.5	1	1	**12**
HOXA9, OXR1, panel OXR1 and MSTR1	Pires-Luís, 2017[34]	1	1	1	1	0	0	0	1	0	1	1	0	1	0	0	0	1	1	0	0	1	1	1	**13**

Supplementary table 1. Individual STARD scores per study. (continued)

STARD item		1	2	3	4	5	6	7	8	9	10a	12a	13a	14	15	18	19	20	21a	23	24	26	27	30	Total score
		Identification as a study of diagnostic accuracy	Structured abstract	Scientific and clinical background	Study objectives and hypotheses	Study design	Eligibility criteria	On what basis participants were identified	Where and when participants were identified	Consecutive, random, or convenience series	Index test	Definition of test positivity cut-offs	Blinding	Methods for diagnostic accuracy	Handling of indeterminate results	Sample size rationale	Diagram of participants flow	Baseline characteristics of participants	Distribution of disease severity in participants	Cross tabulation of index test results and reference standard	Diagnostic accuracy and their precision	Study limitations	Clinical use	Funding	
Marker	**Study**																								
LRRC3B, APC, FHIT, RASSF1A, VHL, ITGA9, panel RASSF1A or FHIT or APC, panel RASSF1A or FHIT, panel RASSF1A or APC	Skrypkina, 2016[42]	1	0	1	0.5	0	0	0	1	0	1	1	0	1	0	0	0	1	1	1	0	0.5	0.5	1	**10**
sFRP1,sFRP2, sFRP4, sFRP5, Wif1, Dkk3	Urakami, 2006[36]	1	1	1	1	0	0	0	0	0	0.5	1	0	1	0	0	0	0	1	1	1	0	1	1	**12**
TCF21	Xin, 2016[37]	1	0	1	0.5	0	0	0	1	1	1	1	0	1	0	0	0	0	0	1	0	0	1	1	**10**
ADAMTS18	Xu, 2015[38]	0	0	0.5	0.5	0	0.5	0	1	0	1	1	0	0	0	0	0	1	1	1	0	0	0.5	0	**6**

Supplementary table 2. Risk of potential bias of the included studies. Studies indicated by • potentially have an increased risk of bias. Studies indicated by • potentially have a decreased risk of bias.

Study	Selection bias	Assay method (measurement bias)	Outcome assessment (measurement bias)	Other variable assessment (measurement bias)	STARD score
Ahmad, 2012	•	•	•	•	13
Battagli, 2003	•	•	•	•	12.5
Christoph, 2008	•	•	•	•	10.5
Costa, 2007	•	•	•	•	15
Costa, 2011	•	•	•	•	12.5
Dalgin, 2008	•	•	•	•	5
de Martino, 2012	•	•	•	•	14
Dulaimi, 2004	•	•	•	•	10
Ellinger, 2010	•	•	•	•	13.5
Ge, 2015	•	•	•	•	9.5
Hauser, 2013	•	•	•	•	13.5
Hoque, 2004	•	•	•	•	11
Onay, 2009	•	•	•	•	8
Pires-Luís, 2015	•	•	•	•	12.5
Pires-Luís, 2017	•	•	•	•	13
Skrypkina, 2016	•	•	•	•	11.5
Urakami, 2006	•	•	•	•	12.5
Xin, 2016	•	•	•	•	10.5
Xu, 2015	•	•	•	•	8

Supplementary table 3. Search strategy for this systematic review.

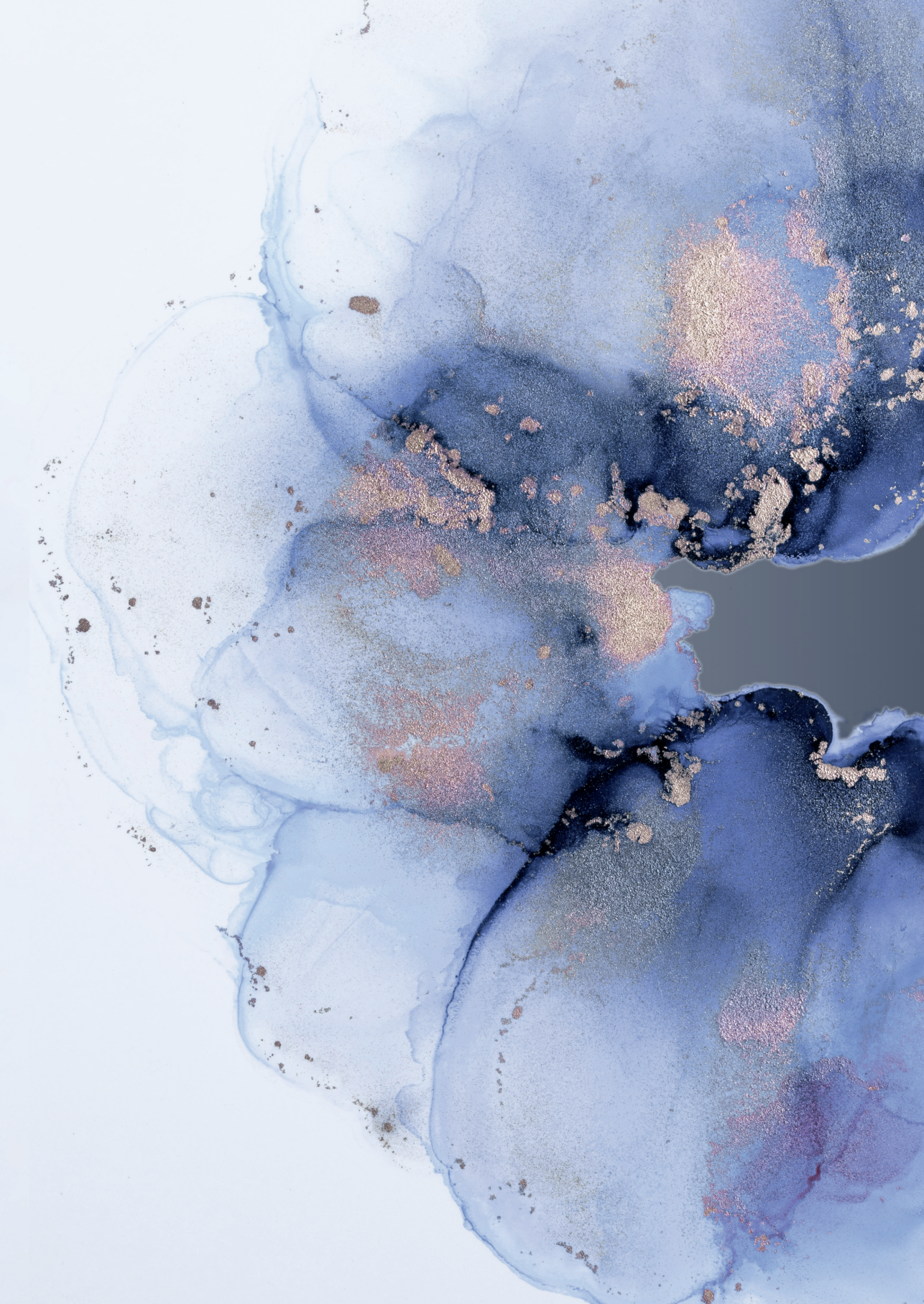
	PubMed	EMBASE	Medline	Google Scholar
1	"carcinoma, renal cell"[MeSH Terms] OR "carcinoma"[All Fields] AND "renal"[All Fields] AND "cell"[All Fields] OR "renal cell carcinoma"[All Fields] OR "renal"[All Fields] AND "cell"[All Fields] AND "carcinoma"[All Fields] OR "renal neoplasms"[All Fields] OR "renal cancer"[All Fields] OR "kidney carcinoma"[All Fields] OR "kidney neoplasms"[MeSH Terms] OR "kidney"[All Fields] AND "neoplasms"[All Fields] OR "kidney neoplasms"[All Fields] OR "kidney cancer"[All Fields]	'renal cell carcinoma' OR 'renal neoplasms' OR 'renal cancer' OR 'kidney carcinoma' OR 'kidney neoplasms' OR 'kidney cancer'	'renal cell carcinoma' OR 'renal neoplasms' OR 'renal cancer' OR 'kidney carcinoma' OR 'kidney neoplasms' OR 'kidney cancer'	kidney cancer, renal cell carcinoma, hypermethylation, methylation, epigenetics, epigenetic, diagnostic, diagnostic testing, cancer diagnosis, marker, biomarker, screening, clinical test
2	"dna methylation"[MeSH Terms] OR "dna"[All Fields] AND "methylation"[All Fields] OR "dna methylation"[All Fields] OR "methylation"[MeSH Terms] OR "methylation"[All Fields]	'DNA methylation' OR 'methylation'	'DNA methylation' OR 'methylation'	
3	"diagnosis"[Subheading] OR "diagnosis"[All Fields] OR "diagnosis"[MeSH Terms] OR "cancer diagnosis"[All Fields] OR "cancer screening"[All Fields] OR "screening"[All Fields]	'diagnosis' OR 'cancer diagnosis' OR 'screening' OR 'cancer screening'	'diagnosis' OR 'cancer diagnosis' OR 'screening' OR 'cancer screening'	
Search	1 AND 2 AND 3	1 AND 2 AND 3	1 AND 2 AND 3	1

Supplementary table 4. STARD criteria list with interpretation and scoring remarks.

Section	No	Item	Interpretation and scoring
Title or abstract			
	1	Identification as a study of diagnostic accuracy using at least one measure of accuracy (such as sensitivity, specificity, predictive values, or AUC)	
Abstract			
	2	Structured summary of study design, methods, results, and conclusions (for specific guidance, see STARD for Abstracts)	
Introduction			
	3	Scientific and clinical background, including the intended use and clinical role of the index test	
	4	Study objectives and hypotheses	
Methods			
Study design	5	Whether data collection was planned before the index test and reference standard were performed (prospective study) or after (retrospective study)	
Participants	6	Eligibility criteria	
	7	On what basis potentially eligible participants were identified (such as symptoms, results from previous tests, inclusion in registry)	
	8	Where and when potentially eligible participants were identified (setting, location, and dates)	
	9	Whether participants formed a consecutive, random, or convenience series	
Test methods	10a	Index test, in sufficient detail to allow replication	In case the article referred to another article for primer and probe sequences, 0.5 point was awarded.
	10b	Reference standard, in sufficient detail to allow replication	Excluded. Not applicable to biomarker studies.
	11	Rationale for choosing the reference standard (if alternatives exist)	Excluded. Not applicable to biomarker studies.
	12a	Definition of and rationale for test positivity cut-offs or result categories of the index test, distinguishing pre-specified from exploratory	
Test methods	12b	Definition of and rationale for test positivity cut-offs or result categories of the reference standard, distinguishing pre-specified from exploratory	Excluded. Not applicable to biomarker studies.
	13a	Whether clinical information and reference standard results were available to the performers or readers of the index test	
	13b	Whether clinical information and index test results were available to the assessors of the reference standard	Excluded. Not applicable to biomarker studies.

Supplementary table 4. STARD criteria list with interpretation and scoring remarks. (continued)

Section	No	Item	Interpretation and scoring
Analysis	14	Methods for estimating or comparing measures of diagnostic accuracy	
	15	How indeterminate index test or reference standard results were handled	
	16	How missing data on the index test and reference standard were handled	Excluded. Not applicable to biomarker studies.
	17	Any analyses of variability in diagnostic accuracy, distinguishing pre-specified from exploratory	Excluded. Not applicable to biomarker studies.
	18	Intended sample size and how it was determined	
Results			
Participants	19	Flow of participants, using a diagram	
	20	Baseline demographic and clinical characteristics of participants	
	21a	Distribution of severity of disease in those with the target condition	
	21b	Distribution of alternative diagnoses in those without the target condition	Excluded. Not applicable to biomarker studies.
	22	Time interval and any clinical interventions between index test and reference standard	Excluded. Not applicable to biomarker studies.
Test results	23	Cross tabulation of the index test results (or their distribution) by the results of the reference standard	
	24	Estimates of diagnostic accuracy and their precision (such as 95% confidence intervals)	
	25	Any adverse events from performing the index test or the reference standard	Excluded. Not applicable to biomarker studies.
Discussion			
	26	Study limitations, including sources of potential bias, statistical uncertainty, and generalizability	In case only one limitation was mentioned or limitations were mentioned without explanation, 0.5 point was awarded.
	27	Implications for practice, including the intended use and clinical role of the index test	
Other information			
	28	Registration number and name of registry	Excluded. Not applicable to biomarker studies.
	29	Where the full study protocol can be accessed	Excluded. Not applicable to biomarker studies.
	30	Sources of funding and other support; role of funders	



CHAPTER 3

TECHNICAL CONSIDERATIONS IN PCR-BASED ASSAY DESIGN FOR DIAGNOSTIC DNA METHYLATION CANCER BIOMARKERS

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Adapted from:
Clinical Epigenetics. 2022 Apr 27;14(1):56.

ABSTRACT

Introduction: DNA methylation biomarkers for early detection, risk stratification and treatment response in cancer have been of great interest over the past decades. Nevertheless, clinical implementation of these biomarkers is limited, as only <1% of the identified biomarkers is translated into a clinical or commercial setting. Technical factors such as a suboptimal genomic location of the assay and inefficient primer or probe design have been emphasized as important pitfalls in biomarker research. Here, we use eleven diagnostic DNA methylation biomarkers for colorectal cancer (*ALX4, APC, CDKN2A, MGMT, MLH1, NDRG4, SDC2, SFRP1, SFRP2, TFPI1* and *VIM*), previously described in a systematic literature search, to evaluate these pitfalls.

Results: To assess the genomic assay location, the optimal genomic locations according to TCGA data were extracted and compared to the genomic locations used in the published assays for all eleven biomarkers. In addition, all primers and probes were technically evaluated according to several criteria, based on literature and expert opinion. Both assay location and assay design quality varied widely amongst studies.

Conclusions: Large variation in both assay location and design hinders the development of future DNA methylation biomarkers as well as inter-study comparability.

INTRODUCTION

DNA methylation biomarkers for early detection, risk stratification and treatment response have been of great interest in the clinical management of cancer. Over the past decades, the focus in DNA methylation biomarkers research has expanded from tissue to liquid biopsies. Since then, some of these biomarkers have been incorporated in commercially available diagnostic tests[1]. In a recent systematic literature review, 100 potentially published DNA methylation biomarkers for colorectal cancer (CRC) were identified in bodily fluids (Feng *et al.* unpublished data). Only three of these (*NDRG4*, *BMP3* and *SEPT9*) have been translated into commercial tests currently available for the early detection of CRC[1]. Various reasons for this suboptimal clinical translation have been postulated[1-3]; many of these focus on issues such as a suboptimal study design, lack of validation and lack of clinical relevance. However, technical factors such as a suboptimal genomic location of the assay and inefficient primer or probe design have been emphasized as important pitfalls in biomarker research as well [1, 3-5]. The choice of which genomic location to study in the evaluation of DNA methylation biomarkers can influence the conclusion on the clinical value of this biomarker. Koch *et al.* previously described the importance of selecting the optimal genomic location, for example by using publicly available data such as The Cancer Genome Atlas (TCGA) or whole-genome sequencing data[1, 6]. These data can be used to identify the genomic location with the largest methylation differences between sample groups, associated with the clinical outcome of interest. For example, we assume that the genomic locations with the largest difference in methylation between normal and tumor samples can be used to discriminate tumor tissue/patients from normal tissue/healthy individuals, as suggested in several of our previous publications[1, 7, 8].

In addition to the identification of these extracted locations with the largest difference between normal and tumor tissue, several technical assay design issues are crucial for optimal DNA methylation biomarker development and subsequent chances for successful clinical translation, including assay type and primer- and probe design. For DNA methylation analysis, the most widely used technique is (quantitative) methylation-specific PCR (MSP/qMSP), which requires primer and probe design on the bisulfite-converted sequence of the biomarker of interest[9]. Although MSP primer design tools (including Bisearch, Methprimer and PrimerSuite) are available[10], these tools do not incorporate publicly available genomic data, and therefore do not preselect the most optimal genomic region for assay design.

Here we analyzed the diagnostic CRC methylation biomarkers identified in a previously conducted systematic literature search in order to provide an overview of the genomic locations. Moreover, we evaluated the quality of the described primers and probes and define recommendations that can guide assay design within the DNA methylation biomarker field.

METHODS

Search strategy and study selection

A systematic search until December 2020 was performed in Pubmed, Embase, Cochrane library and Google Scholar, to identify all diagnostic DNA-methylation biomarker studies for CRC. Only original articles in the English language were considered; reviews, editorials and conference abstracts were excluded. Only articles studying DNA methylation through MSP (nested/direct) and qMSP (probe/SYBR), which provided the assay sequences in the article, and studied liquid biopsies (blood, serum, plasma, stool or urine) were included. Articles discussing hereditary cancer syndromes were excluded. From all diagnostic DNA methylation markers for CRC reported in the included studies, eleven were selected for further evaluation (*ALX4, APC, CDKN2A, MGMT, MLH1, NDRG4, SDC2, SFRP1, SFRP2, TFPI1* and *VIM*) as they were described in at least five studies. Diagnostic performance (sensitivity and specificity) was extracted for all genes when available. Although it is one of the most commonly studied biomarkers for early CRC detection, *SEPT9* was excluded due to the fact that most studies (62%) used one of the two commercial assays to measure *SEPT9* methylation.

Identification optimal genomic location within TCGA data

In order to identify the genomic location where the methylation difference between normal and tumor tissue is the largest, the online available TCGA data visualization tool MEXPRESS[11, 12] was used. TCGA methylation data of the genes of interest in the CRC patient dataset (COAD) was assessed. MEXPRESS visualizes data for specific genes, and all Illumina 450K methylation array CpGs that have been linked to that gene. All CpGs, irrespective of their location relative to the gene, were assessed. The three locations with the largest methylation difference between normal and tumor tissue (tumor hypermethylated compared to normal in all genes, except for *MGMT*) were extracted and will be referred to as 'the extracted locations' throughout this manuscript.

Primer and probe quality assessment

In order to assess primer and probe quality, two independent observers (M.M. & K.L.) scored all primers and probes according to criteria were constructed based on both literature and expert experience (Table 1). Although we are aware that designing the perfect primers and probes is challenging, and many different criteria have been postulated, we attempted to evaluate the optimal design criteria.

All criteria apply to (q)MSP primers and probes on the bisulfite-converted sequence of the gene of interest, and to the methylation-specific primer set in case of (q)MSP without probe. As bisulfite-conversion changes unmethylated cytosines to uracil, while methylated cytosines remain unchanged[9], primers and probes should be designed to

distinguish methylated from unmethylated DNA and to anneal efficiently. Therefore, at least 2-3 CpG dinucleotides and 4-5 non-CpG cytosines should be included in the primer or probe[9, 13-16]. For optimal annealing, a CpG dinucleotide should be at the most 3' end of each primer, and preferably the other CpGs are also at the 3' end of the primer[9, 13-16]. Also, the ideal primer length is 20-30 bases[15,] and preferably the forward and reverse primer should have a similar Tm (calculated using Gene Runner software). When using a probe, ideally it is 20-30 bases long, and the Tm is 5-10˚C above the primers' Tm (calculated using Gene runner software); when using an MGB probe, its length is preferentially 12-20 bases long, and the Tm of MGB probes should preferably be 5-15˚C above the primers' Tm (calculated using Primer Express software)[17-21]. Additionally, the most 5' end of the probe cannot be a G, as this might quench the fluorophore[18]. Last, liquid biopsies mostly carry highly fragmented cell-free DNA of maximum 160 bp (depending on sample type), and DNA is additionally fragmented by bisulfite conversion. Therefore, amplicon size was

Table 1. Primer and probe assessment definitions.

Criterium		Score
≥ 2-3 CpG dinucleotides per primer/probe	<2 CpG dinucleotides per primer/probe	🔴
	2 CpG dinucleotides per primer/probe	🟡
	>2 CpG dinucleotides per primer/probe	🟢
≥ 4-5 non-CpG cytosines per primer/probe	<4 non-CpG cytosines per primer/probe	🔴
	4 non-CpG cytosines per primer/probe	🟡
	>4 non-CpG cytosines per primer/probe	🟢
CpG dinucleotide at most 3' end of primer, other CpGs also at 3' end of the primer	0 CpG dinucleotide at most 3' end	🔴
	1 CpG dinucleotide at most 3' end	🟡
	1 CpG dinucleotide at most 3' end of primer, other CpGs also at 3' end of the primer	🟢
Length primer/probe 20-30 bases; length MGB probe 12-20 bases	< 17 or > 36 bases, MGB probe <12 or >20 bases	🔴
	17-19 or 31-36 bases	🟡
	20 - 30 bases, MGB probe 12-20 bases	🟢
Forward and reverse primers similar Tm (gene runner software)	> 2°C difference	🔴
	< 2°C difference	🟢
Tm probe 5-10˚C above Tm primers; Tm MGB probe 5-15˚C above Tm primers	< 5°C difference or > 10°C difference, MGB probe < 5 °C difference or > 15°C difference	🔴
	5 - 10°C difference, MGB probe 5-15 °C difference	🟢
	Molecular beacon probe, Tm not assessed	⚫
Most 5' end of probe is not a G	Most 5' end of probe is a G	🔴
	Most 5' end of probe is not a G	🟢
Amplicon size <100 bp	Amplicon size ≥ 160 bp	🔴
	Amplicon size 121-159 bp	🟡
	Amplicon size ≤ 120	🟢

evaluated, with the preferred amplicon size being a maximum of 120 bp[22-26]. All primers and probes were scored according to these criteria (defined in Table 1). In case a nested approach was used, the inner assay was evaluated. For specific types of probes, such as molecular beacon probes, one of our criteria might not be completely suitable, as (to our knowledge) no design tools exist to calculate the Tm of these probes. Therefore, we were unable to assess the Tm of these probes, and they were specifically marked within Figure 2. Green dots represent optimal design, orange dots represent suboptimal, but acceptable design. Red dots do not necessarily mean a primer or probe does not work, but rather that there is an increased risk of technical problems with the primer or probe for that specific criterium (Table 1). Black dots mean that the criterium was not assessed for that probe, as it was a molecular beacon probe.

RESULTS & DISCUSSION

Dataset characteristics

Here, we provided an overview of the studied genomic locations, the extracted locations according to TCGA data, and the quality of used primers and probes for the 11 most studied diagnostic DNA methylation biomarkers in CRC (*ALX4, APC, CDKN2A, MGMT, MLH1, NDRG4, SDC2, SFRP1, SFRP2, TFPI2, VIM)*. All genes were evaluated in a minimum of five (*TFPI2*) and a maximum of 12 (*SFRP2*) independent studies (Table 2). Markers had been studied in a variety of bodily fluids including stool, serum, plasma, and urine. Diagnostic performance (sensitivity and specificity) showed considerable variation between individual studies evaluating the same marker, which might be attributed to sample type differences. *MGMT* showed the largest sensitivity range of 5.7-90.0% across sample types, with specificities varying from 93.8-100%. *MLH1* showed the smallest range in sensitivity (30.0-45.1%), however the specificity range was substantial (56.9-97.6%). Despite using identical assays, diagnostic performance of these studies varied widely; e.g. 20-80% sensitivity and 96.8-100% specificity for *CDKN2A*, 60-94.2% sensitivity and 54-100% specificity for *SFRP2* and 32.6-81% sensitivity and 82-100% specificity for *VIM* (Figure 1C, I, K). This might be attributed to sample type differences, as illustrated by the relatively low sensitivities of *CDKN2A* methylation in stool (20-40%), compared to serum (59-80%), plasma (61.1%) and peripheral blood (55.4%) using the same assay (Table 2; Figure 1C). Similarly, the diagnostic performance of *sFRP2* in stool varied more widely (sensitivity 60-94.2%, specificity 54-100%) compared to serum (sensitivity 66.9-86.8%, specificity 93.7%) using the same assay (Table 2; Figure 1I). As stool contains PCR inhibitors like complex polysaccharides and bile salts, undigested debris, and an abundance of e.g. bacterial DNA over human DNA, this can explain the lack of performance in these samples, compared to blood-based samples[27]. In addition, plasma seems to perform

worse compared to serum using the same assay for most markers (Table 2; Figure 1), which is in line with literature suggesting that DNA is more abundant and stable in serum compared to plasma[28, 29].

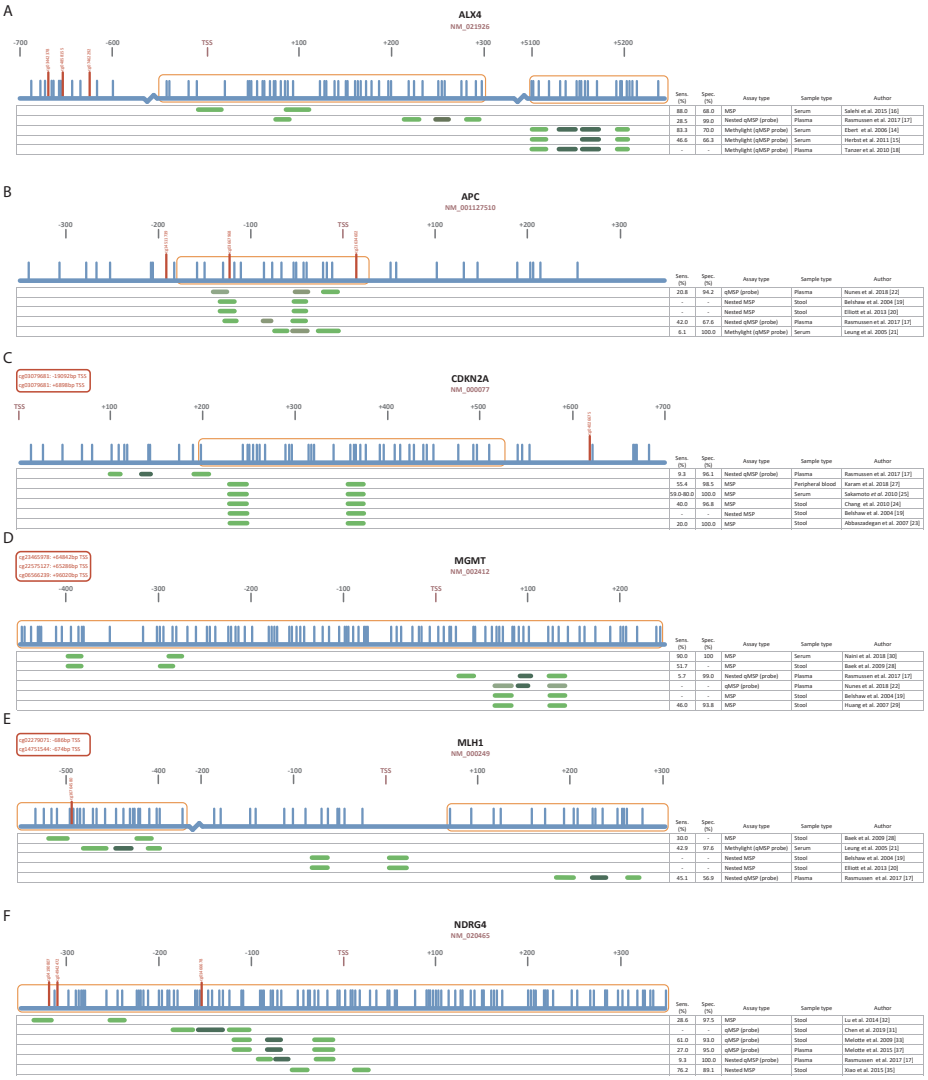
Table 2. Summary of the 11 most studied DNA methylation biomarkers for CRC in liquid biopsies.

Gene	Number of articles	Liquid biopsy type	Sensitivity range	Specificity range
ALX4	3 [30-32]	Serum	46.6-88.0%	66.3-70.0%
	2 [33, 34]	Plasma	28.5-80.0%	41.0-99.0%
APC	2 [35, 36]	Stool	-	-
	1 [37]	Serum	6.1%	100%
	2 [33, 38]	Plasma	20.8-42-0%	67.6-94.2%
CDKN2A	3 [35, 39, 40]	Stool	20.0-40.0%	96.8-100%
	1 [41]	Serum	59.0-80.0	100%
	2 [33, 42]	Plasma	9.3-61.1%	96.1%
	1 [43]	Peripheral blood	55.4%	98.5%
MGMT	3 [35, 44, 45]	Stool	46.0-51.7%	93.8%
	1 [46]	Serum	90.0%	100%
	2 [33, 38]	Plasma	5.7%	99.0%
MLH1	3 [35, 36, 44]	Stool	30.0%	-
	1 [37]	Serum	42.9%	97.6%
	1 [33]	Plasma	45.1%	46.9%
NDRG4	6 [47-52]	Stool	28.6-76.2%	80.0-97.5%
	2 [33, 53]	Plasma	9.3-27.0%	95.0-100%
	1 [51]	Total blood	54.8%	78.1%
	1 [51]	Urine	72.6%	85.0%
SDC2	2 [52, 54]	Stool	81.1%	93.3%
	2 [47, 55]	Serum	71.2-87.0%	95.2-95.6%
	1 [33]	Plasma	24.4%	94.1%
	1 [56]	White blood cells	-	-
sFRP1	2 [32, 57]	Stool	52.0-89.0%	86.0-92.0%
	1 [58]	Serum	77.7%	70.0%
	2 [33, 59]	Plasma	21.8-62.9%	91.7-93.1%
sFRP2	9 [40, 45, 48, 50, 60-64]	Stool	57.1-94.2%	54.0-100%
	2 [61, 64]	Serum	66.9-86.8%	93.7%
	2 [33, 65]	Plasma	20.2-54.4%	72.3-82.4%
TFPI2	4 [50, 52, 66, 67]	Stool	31.4-89.0%	79.0-100%
	1 [33]	Plasma	7.3%	98.0%
VIM	4 [44, 68-70]	Stool	38.3-81.0%	82.0-100%
	2 [31, 71]	Serum	31.1-32.6%	60%
	1 [33]	Plasma	17.6%	88.2%

Overview of genomic and extracted locations of selected assays

In the 11 most studied diagnostic DNA methylation biomarkers for CRC, multiple genomic locations were studied. An overview of all genomic locations in the individual studies is presented in Figure 1. In addition, the extracted locations that we identified from TCGA data were compared to the locations used in all published assays.

For *ALX4, APC, MGMT, sFRP1*, *sFRP2 TFPI2* and *VIM*, the least variation in genomic locations was observed amongst studies (three different genomic locations; Figure 1A, B, D, H, I, J, K). For *APC* and *VIM*, most assays (4/5 and 5/7 respectively) included at least one of the extracted locations as identified in TCGA data (Figure 1B, K). In contrast, none of



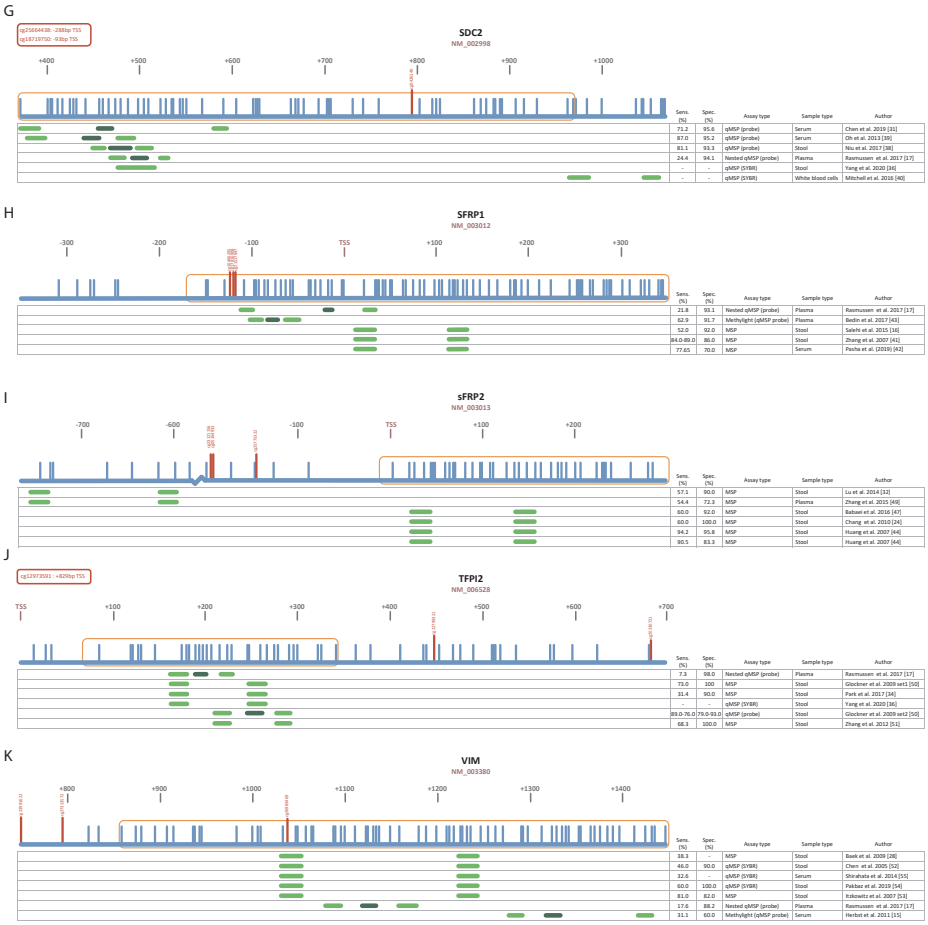


Figure 1 (A-K): Genomic locations, extracted CG's (from TCGA) and diagnostic performances of the investigated assays per marker.

: CpG islands, : CGs, : extracted CGs (obtained from TCGA), : primers, : probes,

the assays investigating *ALX4, MGMT, sFRP1, sFRP2* and *TFPI2* included an extracted CG (Figure 1A, D, H, I, J). Although *MLH1* and *NDRG4* were studied in four and five genomic locations respectively, most assays did not contain an extracted CG (3/4 for *MLH1*, 3/5 for *NDRG4*; Figure 1E, F). Largest variation in genomic location was observed for *SDC2*; however, none of these included an extracted CG (Figure 1G).

These results show that there is a large variation in the investigated genomic locations among the different assays, whereas most studies do not specify a specific rationale for their used genomic location. Next to these variations in genomic location, we also observed a large variation in diagnostic performance even within the same genes. As previously postulated, the exact studied genomic location could influence the

diagnostic performance of a biomarker, emphasizing the importance of considering genomic location of the assay upfront[1, 7, 8]. Currently, to our knowledge, no guidelines for identifying the optimal genomic location for diagnostic DNA methylation biomarkers are described. However, we previously recommended using TCGA data to identify the genomic location where the difference in methylation between normal and tumor tissue is largest. In theory, these locations might represent the most clinically relevant methylation sites for diagnostic purposes. Even though TCGA is a very accessible data source, it is limited in the amount of covered CGs. TCGA data is based on Infinium 450K microarrays, of which the probes do not necessarily cover the most relevant CGs amongst the genome[1]. However, all genomic regions illustrated in this overview were covered by Illumina 450K methylation array probes according to MEXPRESS. To assure full genomic coverage, sequencing prior to deciding on the genomic location covered in the methylation assay would be required. This has not always been feasible in the past, especially for small research groups with limited funding. The decreased sequencing costs and the availability of sequencing facilities (in both academic and commercial setting) combined with publicly available DNA methylation and gene expression data now provide opportunities to identify the most optimal genomic location for a DNA methylation marker[1]. Unfortunately, these sequencing data are rarely publicly available, which did not allow us to consider these in this manuscript.

Primer and probe assessment

Of the 47 assays used to measure the 11 included markers, 16 (34%) were MSP assays, 25 (53%) were qMSP assays with probe, and 6 (13%) were qMSP assays with SYBR (Figure 2). As bisulfite-conversion changes unmethylated cytosines to uracil, while methylated cytosines remain unchanged, the CpG dinucleotides and non-CpG cytosines in the primers define the discriminative power of the primers to distinguish methylated from unmethylated DNA[9]. As an alternative to this damaging and fragmenting bisulfite-conversion, an enzymatic modification kit to enable distinguishing methylated from unmethylated DNA has become available that is less damaging to the DNA in terms of fragmentation[72]. This novel enzymatic conversion could therefore impact assay design. However, as specific issues of e.g. bisulfite conversion have been described in detail before[73], they are not evaluated in this manuscript.

In the MSP assays, 1 forward and 1 reverse primer (6.25%) failed to include at least 2 CpG dinucleotides (Figure 2A), whereas 2 forward primers and 1 reverse primer (4.8%) failed to include at least 2 CpG dinucleotides in the qMSP assays (Figure 2B). All probes met this criterium (Figure 2B). Additionally, in the MSP assays 2 forward primers and 1 reverse primer (9.4%), and 5 forward primers and 9 reverse primers (22.6%) in the qMSP assays failed to include at least 4 non-CpG cytosines (Figure 2). Almost half of all probes (48%) in the qMSP assays with probe did not meet this criterium (Figure 2B). Not meeting

these criteria could lead to inefficient annealing and unspecific binding of the primers and probes, resulting in inconclusive findings. Inefficient annealing could result in false negatives due to the lack of amplification, even when the target sequence is available. Unspecific binding could result in false positives due to binding even when the target sequence is not fully complementary to the primer or probe[9, 13-16.]

Next, 6 reverse primers (18.8%) in the MSP assays, and 6 forward and 14 reverse primers (32.2%) in the qMSP assays did not carry a CpG dinucleotide at the most 3' end of the primer (Figure 2). Not including a CpG dinucleotide at the most 3' end of the primer might also result in inefficient or a lack of annealing, and unspecific binding, which could induce both false negative and false positive results[9, 13-16]. Optimal primer/probe length of 20-30 bases and 12-20 bases for minor groove binder (MGB) probes was met in 84.4% of the MSP primers, 69.4% of qMSP primers and 67% of the probes (Figure 2). An additional 15.6% of the MSP primers, 29.2% of qMSP primers and 33% of probes were suboptimal in length (17-19 or 31-36 bases, < 12 or > 20 bases for MGB probes; Figure 2). Among the probes suboptimal in length, 87.5% were molecular beacon probes. MGB probes generally allow a shorter probe sequence because of the increase in Tm by the MGB addition, which was accounted for in the results[74]. Molecular beacon probes carry an additional 5-7 bases complementary to each other at the start and end of the sequence, which means these probes are generally longer compared to Taqman probes[75]. In order to take these specific characteristics into account, the primer/probe length criterium was extended to 17-19 bases and to 31-36 bases. Nevertheless, 12.5% of these molecular beacon probes did not comply to the extended primer length criterium. This could potentially lead to inefficient or lack of annealing as well.

Eighty-one percent of MSP primer sets, and 55% of the qMSP primer sets had a similar melting temperature (Tm), meeting the criterium (i.e., Tm forward and reverse primers ≤2°C difference). For the qMSP assays with Taqman probe, 75% of the probes met the criterium (i.e., Tm 5-10°C higher than the corresponding primer set), and all of the MGB probes met the criterium (i.e., Tm 5-15°C higher than the corresponding primer set; Figure 2B). Not adhering to these Tm criteria could again lead to inefficient annealing of (one of) the primers or probe. For probes, an additional criterium was assessed (i.e., no G base at the most 5' end of the probe), which was met in 95.8% of the included probes. A G base at the most 5' end of the probe might prematurely quench the fluorophore, resulting in false negative results[18].

Last, optimal amplicon sizes of maximum 120 bp were used in 62.5% of the MSP assays, and 76.7% of the qMSP assays with probe or SYBR. An additional 25% of MSP assays and 13.3% of qMSP assays with probe or SYBR used suboptimal amplicon sizes (121-159 bp), and 12.5% of MSP assays, and 10% of qMSP assays exceeded the acceptable 160 bp amplicon size (Figure 2). As DNA in liquid biopsies mostly originates from apoptotic and necrotic cells and in this case has to undergo bisulfite conversion, it is highly fragmented

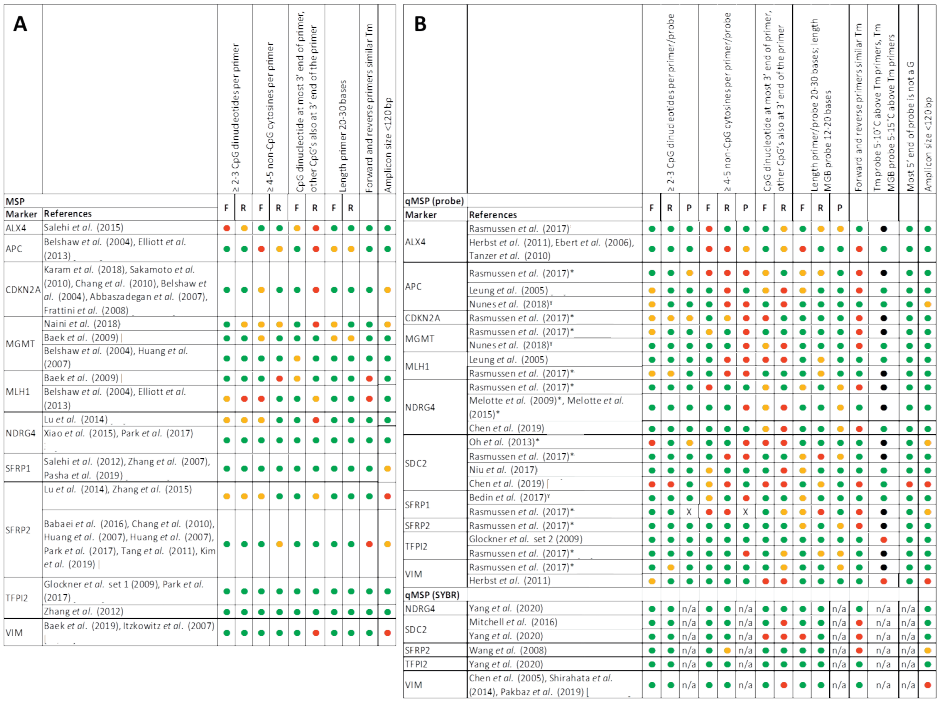


Figure 2. Primer and probe quality assessment of all markers and studies in MSP (A), qMSP assays (probe/SYBR) (B). F: forward primer, R: reverse primer, P: probe, n/a: not applicable, *: Molecular beacon probe, additional bases included in evaluation, ¥: Minor groove binder probe, X: probe sequence could not be mapped back to gene

• : optimal design, • : suboptimal, but acceptable design, • : increased risk of technical problems with the primer or

with an estimated maximum of ~160 bp. However, depending on sample type, DNA in liquid biopsies can be as small as <100 bp which should be taken into account when designing an assay[22-26].

Although assay design varied widely, the major criteria to distinguish methylated from unmethylated DNA were covered in most assays. However, several factors should receive additional consideration, such as primer length and Tm (Figure 2). Probe design factors tend to score poorer compared to primer design factors, and generally, qMSP assays scored worse compared to MSP assays across all criteria (Figure 2). In addition to the scored items, it is important to adhere to general primer/probe design criteria like a CG content of 30-80% and to ensure that no dimers or hairpin loops form[17, 18, 76, 77]. Further, it is important to consider genetic background and to make sure no prevalent single nucleotide polymorphisms (SNPs) appear at the 3' end of the primer, to allow efficient annealing[78]. Moreover, assays including appropriate controls and a reference gene are most likely to generate reliable results[79].

To measure DNA methylation, several different techniques are currently available for research purposes, of which MSP and qMSP are most widely used. In general, qMSP assays with probe revealed more design flaws compared to both MSP and qMSP with SYBR assays. In the assays with a probe, especially the items regarding probe design showed low scores (Figure 2). This emphasizes the difficulty of designing qMSP assays where the addition of a probe introduces another layer of complexity to the designing process. However, it can be questioned whether it is a necessity to fully optimize all separate subcomponents of primer and probe design, as assays with suboptimal scores for some criteria may also work. For example, if one of the primers in a set fails to meet the criterium of including ≥ 4-5 non-CpG cytosines per primer, the other primer could compensate, and the assay might work without any problems. This emphasizes that primers and probes should be designed as an assay, rather than single components.

Often, (nested) MSP assays are used in biomarker studies because they require substantially less DNA input compared to qMSP assays. Because of its quantitative nature and the specific binding properties of the utilized probe, qMSP with probe might be preferred over MSP assays for specific research questions. However, qMSP assays with SYBR are prone to false-positive results, as SYBR is an intercalating dye that binds to all double-stranded DNA[80].

After designing an assay, it is advised to perform an *in silico* analysis of this assay to check for dimers, hairpins and 3'-end primer stability, as extensively described by Davidović *et al*[15]. In addition, assays should be optimized in terms of PCR conditions, such as PCR component concentrations and annealing temperature, using gradient PCRs. Bisulfite-converted fully methylated, fully unmethylated and no template controls, as well as non-converted DNA and a non-converted no template control should be used in the assay optimization process[81, 82]. Next, pilot studies using small sample sets of interest can evaluate the feasibility of an assay for cancer diagnosis, and minimize false positive and false negative results. Additionally, when analyzing quantitative data, it is important to select an appropriate cutoff value to determine whether a sample is methylated or unmethylated, and several methods to determine the optimal cutoff have previously been postulated[83, 84]. Different cutoffs amongst studies examining the same assays could, amongst others, explain the large variation in diagnostic performance, and could therefore hamper comparability of studies[83, 84].

CONCLUSIONS

In this study, using CRC markers as an example, we emphasized the importance of assay design for diagnostic DNA methylation biomarkers indicating that a rational choice of genomic location and proper primer/probe design upfront are crucial when striving towards a clinically relevant and useful biomarker. This not only applies for diagnostic

biomarkers, but for all DNA methylation markers intended to discriminate between two patient categories, such as prognostic and predictive biomarkers. However, only using the recommendations summarized in Box 1 does not guarantee a successful clinically relevant assay. Next to the factors discussed in this article, additional experimental factors can influence the diagnostic performance of DNA methylation biomarkers, such as sample type, quality and composition, assay amplicon size, and bisulfite conversion efficiency[27, 73, 85-90], as well as methodological factors such as sample size, using appropriate controls and statistical analyses. Nevertheless, considering both assay location and assay design upfront could greatly improve future DNA methylation biomarker development and inter-study comparability. To achieve this, future research should focus on linking the technical considerations discussed here to diagnostic parameters and clinical outcome. By optimizing these technical considerations in DNA methylation biomarker development, clinically relevant DNA methylation biomarkers are more likely to be developed.

Box 1. Recommendations for DNA methylation assay design.

DNA methylation assay design recommendations
Genomic location
Before designing a DNA methylation biomarker assay, make a rational choice for the genomic location of the assay
Identify the optimal genomic location
e.g. sequencing or publicly available data such as TCGA
Primer- and probe design
Ensure the primers and probes are able to discriminate unmethylated from methylated DNA
Appropriate amount of CpG dinucleotides and non-CpG cytosines in primers and probe
Ensure the primers and probes have the ability to anneal efficiently
CpG dinucleotides at most 3' end of primer, primer length, avoiding premature quenching of probe fluorophore
Ensure primers and probes are designed as an assay, rather than single primers and probes
Similar Tm between primers and appropriate Tm of probe relative to the Tm of the primers
Consider sample type in assay development
For liquid biopsies, the total assay amplicon size should be maximum 120 bp
Assay optimization
In silico analysis of assay
Optimize PCR conditions
Use appropriate controls
Perform pilot studies
Determine cutoff

In this article, we evaluated diagnostic DNA methylation biomarkers for CRC, previously described in a systematic literature search, to evaluate technical pitfalls in DNA methylation biomarker research. Even though we evaluated CRC markers to prove our point, we believe that suboptimal genomic location of the assay and inefficient primer and probe design are also factors that contribute to the lack of clinical translation of DNA methylation biomarkers in other cancers, including renal cell cancer.

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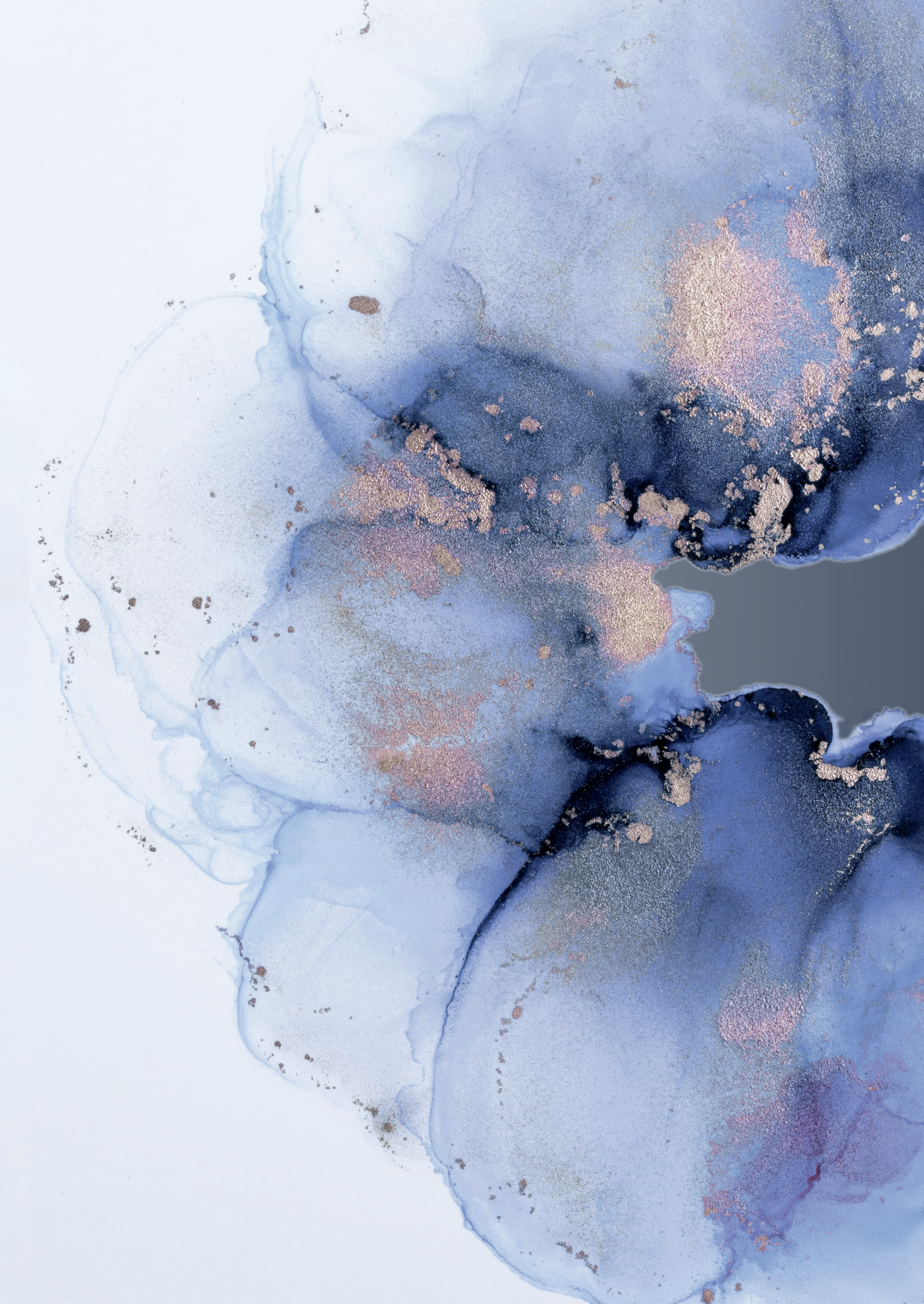

CHAPTER 4

BIOBANKING IN MOLECULAR BIOMARKER RESEARCH FOR THE EARLY DETECTION OF CANCER

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Cancers. 2020, *12*, 776.

ABSTRACT

Although population-wide screening programs for several cancer types have been implemented in multiple countries, screening procedures are invasive, time-consuming and often perceived as a burden for patients. Molecular markers measurable in non-invasively collected samples (liquid biopsies) could facilitate screening, as they could have incremental value on early diagnosis of cancer, but could also predict prognosis or monitor treatment response. Although the shift towards biomarkers from liquid biopsies for early cancer detection was initiated some time ago, there are many challenges that hamper the development of such biomarkers. One of these challenges is large-scale validation that requires large prospectively collected biobanks with liquid biopsies. Establishing those biobanks involves several considerations, such as standardization of sample collection, processing and storage within and between biobanks. In this perspective, we will elaborate on several issues that need to be contemplated in biobanking, both in general and for certain specimen types specifically, to be able to facilitate biomarker validation for early detection of cancer.

INTRODUCTION

Over the past decades, early diagnosis of cancer has become a main focus in research, and population-wide screening programs have been implemented for several cancer types (such as breast, colorectal and cervical cancer) in multiple countries[1]. However, current screening procedures are often invasive or perceived as unpleasant. In addition, they can lead to false positives and might pressure health care, because of time-consuming and costly screening methods. Because of this, many researchers have been aiming to improve cancer screening by focusing on measuring molecular markers in liquid biopsies; non-invasively collected samples, such as stool, blood, sputum, urine or other bodily fluids that are thought to represent the molecular composition of the tumor[2, 3]. However, for the clinical translation of biomarkers, large prospectively collected biobanks with corresponding patient data are necessary for biomarker validation after initial publication. Large-scale, independent validation will make sure the biomarker performance can be generalized across populations, which is essential to be able to be translated into clinical practice[4].

Although non-invasive biomarkers for early detection of cancer have become a popular research subject over the past decades, there have been many underlying challenges that hamper their translation into clinical practice. Research indicates that less than 1% of all published biomarkers are eventually implemented in clinical care[5, 6]. In agreement with prior publications[4, 7], we previously described various problems hindering clinical translation of biomarkers, including a lack of available and appropriate samples, lack of standardized research methodology and a lack of validation[8].

Historically, biobanks were merely gatherings of biological samples supporting specific research projects. Nowadays, biobanks are being established to be ongoing infrastructures with large sophisticated collections of biological samples, complemented with extensive and well-annotated clinical and pathological patient data, sometimes even including medical imaging and pathological histology[9, 10]. Depending on the research question, either general or specialized biobanking may be appropriate. General biobanks are often collected population-wide, and therefore suitable for broad research questions. For specific or rare diseases, a more specialized biobanking approach is demanded to ensure that the samples suit the research question and that sufficient samples are available to ensure statistical power.

As modern biobanks use increasingly advanced technology and automated sample processing, and are often not exclusively established to answer specific research questions, large-scale analysis of these samples can be performed for several purposes, making these biobanks more universally applicable. However, samples from different biobanks cannot always be used and interpreted interchangeably, amongst others due to different national governmental guidelines regarding patient and data protection[11],

but also due to technical differences between biobanks[12]. Various methods for collecting, processing and storing samples, as well as corresponding data, result in heterogeneity between biobanks[12] which can make it more difficult to compare research results from samples originating from existing biobanks.

In this perspective, we will elaborate on several issues that need to be considered when establishing a new biobank, as well as when utilizing an existing biobank, both in general and specific for certain specimen types, to be able to develop and validate biomarkers for early detection of cancer.

Establishing a novel biobank for molecular marker research questions

The commitment of prolonged storage of formalin-fixed paraffin embedded (FFPE) tissue from cancer patients for diagnostic and clinical purposes facilitates researchers to relatively easily obtain tissue samples, and ample material is available even though collecting the corresponding clinical data may be challenging. Although many liquid biopsies are collected during routine clinical care as well, they are not commonly stored for clinical and future research purposes[13]. Biobanking (specific parts of) these samples could facilitate large-scale validation and clinical translation of liquid biopsy biomarkers for early detection of cancer or other research questions. However, developing biobanks with routinely collected liquid biopsies would require huge efforts, both financially and in work-load, and is therefore probably only feasible for specific patient groups and specific research questions. Although implementing biobanking activities in clinical workflows is logistically challenging, several biobanks have described their clinical workflow in illustrative diagrams which could serve as guidelines to others[9, 14, 15].

Collection, processing and storage of liquid biopsies for biobanking

Due to the composition of liquid biopsies, validating candidate biomarkers in these samples has proven to be challenging; some of these challenges need to be considered upfront, when designing the biobank protocol. Depending on sample and biomarker type, both sample properties and protocol components could hinder optimal biobanking and future analyses[16-18]. Partially, this could be overcome by carefully selecting stabilization, pretreatment and processing protocols tailored to the future purpose of the samples, and translating this into an optimal logistical process for each biobank (Figure 1). Moreover, it is important to optimize fast processing, short-term storage and stabilization in a way that is logistically convenient for the personnel involved, without harming the sample integrity before long-term storage. In order to warrant both the quantity (e.g. DNA yield) and the quality (e.g. intact DNA) of the sample, pretreatments of the original sample like centrifugation, or the addition of stabilizing agents to inhibit degradation of the sample, have to be considered. For molecular analysis of DNA and RNA biomarkers for example, it is important that a sufficient amount of DNA or RNA can be yielded from

the samples. Therefore, DNases and RNases should preferably be eliminated from the samples to avoid degradation (Figure 1). Addition of e.g. EDTA for preserving DNA, and e.g. RNAlater for preserving RNA in the sample, should be considered as these preservatives inhibit DNase and RNase respectively[19, 20], and could thereby facilitate higher DNA or RNA yields from the sample. For molecular markers, PCR-based techniques are commonly used to assess biomarker status. Here, PCR inhibitors (organic or inorganic, soluble or dissolved substances) can disrupt the PCR process at any step, affecting the amplification efficiency and thus resulting in a suboptimal technical assay. Removing PCR inhibitors from the liquid biopsy samples will yield more reliable and reproducible results, but also add another processing step[21, 22]. In addition, the DNase/RNase inhibitor of choice, in the chosen concentration, should not act as a PCR inhibitor[22].

Apart from processing the samples, both short-term and long-term storage conditions are important to warrant both quantity and quality of the samples. The optimal time frame between sample deposition and long-term storage, and the optimal conditions within this time frame, should be defined and standardized within a biobank (Figure 1). For this short-term storage, it is important to determine how long the samples can be at room temperature or 4°C before long-term storage[23]. For long-term storage, most sample types are known to remain stable at -80°C[23]. To avoid sample degradation from freezing and thawing, the original sample could be aliquotted in smaller volumes before long-term storage.

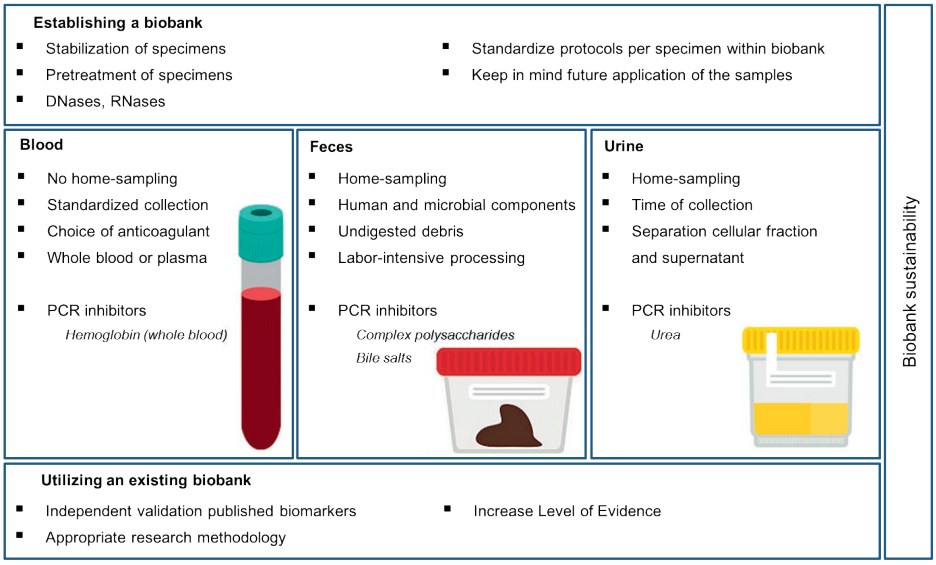

Figure 1. Summary of crucial considerations for establishing a biobank, handling of specific sample types and utilizing existing biobanks.

Quality of the methods used to collect and store samples, and thereby the sample quality, should be ascertained by implementing standard operating procedures and quality control systems into the biobank workflow. Although possibly financially unfeasible for small biobank initiatives, large biobanks should consider international ISO9001 and ISO 20387 accreditation in order to assure sample quality[24].

Besides the general considerations that are applicable to all sample types, most non-invasive sample types also have specific properties that require extra awareness. In the following paragraph, we will give some suggestions for general sample collection and storage protocols for liquid biopsies, and elaborate on crucial properties of specific non-invasive sample types which should be taken into account when collecting and processing these samples for storage in a biobank (Figure 1). Although several protocols for liquid biopsy collection and processing have been described before, future purpose of the samples should always be taken into account and general protocols should, where applicable, be adapted to that purpose.

Blood

As blood is often collected for routine care purposes, it has proven to be the most popular sample type for non-invasive biomarker studies. For the same reason, protocols for different fractions of blood collection (e.g. whole blood, serum, plasma) and processing have been highly standardized over time (Figure 1)[25-27]. For anticoagulated blood, a maximum storage of 24-72 hours at room temperature is recommended before long-term storage at low temperatures[28-30]. Timing of blood collection does not seem to have an impact on e.g. DNA or RNA yield; however, in case of metabolomics analyses, the timing of blood collection should certainly be considered as metabolites are more abundant after high metabolic activity[31].

Whole blood collection requires anticoagulation tubes, of which the most conventional ones are EDTA and heparin tubes. Despite the fact that heparin tubes are widely used to determine hormone or cholesterol levels in routine clinical care, they are not preferred for molecular analyses. EDTA tubes are preferred over heparin tubes, because of the property to preserve cells and inhibit DNase activity; using these tubes will yield higher DNA concentrations of equal quality[32]. Although high concentrations EDTA can inhibit PCR efficiency by depleting magnesium ions, heparin is known to act as a PCR inhibitor at much lower concentrations suggesting that EDTA tubes should be preferred for blood collections aimed at molecular or DNA research (Figure 1)[22]. Next to whole blood, fractions like serum or plasma can be used for molecular analyses. Serum or plasma is preferred over whole blood for cell-free DNA (cfDNA), protein or hormone analyses because the removed cellular (solid) fraction cannot interfere with the results. Although it shares most characteristics regarding PCR inhibitors and DNase/RNase activity with whole blood, serum and plasma are mostly depleted of the known PCR inhibitor hemo-

globin[33]. Therefore, serum or plasma, collected in EDTA tubes seem to be the preferred sample types for storage in a biobank aimed at molecular markers (Figure 1).

Blood collection requires venipuncture, certified skills and special collection tubes, and is therefore considered the most invasive of all non-invasive samples (Figure 1)[31]. Nonetheless, as blood collection is often part of routine clinical care, collecting an additional blood sample for storage in biobanks does not substantially burden the patient. Therefore, biobanking blood samples is relatively uncomplicated. Examples of large biobanks with blood sample collections from cancer patients are the UK Biobank[34], Biobank Japan[35], the Victorian Cancer Biobank[36] and the Canadian Tumor Repository Network[37].

Feces

Although for blood collection and processing, standardization was established over time, that does not hold true for the other non-invasive sample types, like feces. Even though several articles describing their collection and processing protocol for feces, no standardized protocol is currently available[38-40]. Generally, fecal samples are recommended to be stored at room temperature for a maximum of 24-72 hours before long-term storage at low temperatures[41-43]. Several molecular components can be analyzed from feces, such as human and microbial DNA, proteins and metabolites; however, collection of whole stool samples requires relatively large containers and thereby poses a logistical challenge. The collected feces samples need to be homogenized, preferably using a buffer stabilizing the samples' components before freezing. PCR inhibitors to take into consideration when later processing stool samples are mostly bile salts and complex polysaccharides, which could be inactivated by addition of an absorbing buffer (mostly provided in commercial DNA isolation kits), or using a Taq polymerase that is insensitive to these substances in subsequent PCR experiments (Figure 1)[22]. The addition of a PCR facilitator, like spermidine, could partially overcome PCR inefficiency due to the above mentioned PCR inhibitors[44]. Other challenges when processing fecal samples are the abundance of e.g. bacterial DNA over host DNA and undigested debris (Figure 1). Depending on dietary intake and its biological variability, the inorganic fraction of feces contains 25-54% bacterial biomass, which could interfere with multiple downstream analyses, including human fecal DNA and protein extraction[17, 45]. Additional cleanup steps are recommended when processing fecal samples in order to overcome these issues. For DNA isolation, adding an additional precipitation step to purify the sample before starting the extraction using a commercial kit could increase yield. In addition, the technology behind the chosen DNA/RNA/protein isolation kit may influence the yield. Cleanup of these isolations are often based on spin columns; however, an additional DNA/RNA/protein capturing step involving e.g. magnetic beads could further purify the sample[46]. Noteworthy, bacterial enzymes may contribute to degradation of human DNA and RNA in fecal samples[47].

From a biobank sample-storage perspective, fecal samples tend to take up large amounts of space because of their volume, which is a financial burden as well. An advantage of feces collection is that it can be executed at home, as it does not require specialized skills or (apart from relatively large collection containers) equipment (Figure 1)[31]. Although fecal samples have not been collected from cancer patients in any large publicly-funded biobank setting yet, it is becoming a more popular sample type in research settings for several cancer-related studies, as well as for fecal microbiota transplantation studies, as summarized by Terveer *et al.*[48].

Urine

Although urine has been used to diagnose several diseases and infections for a long time, measuring molecular markers for cancer in urine has only emerged over the last decades. Therefore, similar to feces, no standardized way of collecting and processing urine for storage in biobanks has been established yet. Generally, storing urine at room temperature for a maximum of 4 hours is recommended before long-term storing at low temperatures[49, 50]. A general protocol for urine collection and storage was published by the UK biobank[26, 27]. Molecular components like DNA, RNA, proteins and metabolites can all be measured in urine, but the quantity of these components in urine fluctuates throughout the day. In the morning, a concentrated urine sample can be obtained in terms of e.g. DNA; in contrast, metabolites are more abundant after high metabolic activity (Figure 1). Although urea can act as a PCR inhibitor, its concentration is usually too low to affect future analyses (Figure 1)[22]. Depending on the purpose of the samples, urine can be centrifuged to separate the cellular fraction and the supernatant, containing tumor-derived cfDNA. After separation, both fractions can be stored separately (Figure 1). Su *et al.* described that different types of cfDNA can be found in urine; only low molecular weight DNA is derived from the tumor while high molecular weight DNA is not[51]. It is suggested that only the small size DNA fraction in urine rather than the total DNA should be used to increase assay sensitivity for cancer-related DNA biomarkers in urine[51].

Storing urine in biobanks has some advantages over other sample types, as urine collection and processing does not require much time, effort or specialized equipment, and home-sampling is enabled. Moreover, urine is a non-invasive liquid biopsy to obtain, which will increase willingness of participants to donate samples, allowing the establishment of relatively large collections within a limited amount of time (Figure 1). Examples of large biobanks that have collected urine samples from cancer patients are the UK biobank[34] and the Canadian Tumor Repository Network[37].

Other

Less conventional non-invasive sample types include sputum, saliva, mucinous swabs, hairs, nails and exhaled breath. Sputum, saliva and mucinous swabs are liquid samples thought to carry cells that shed from epithelial lining, which is a non-invasive way to examine a potentially cancerous site. Solid sources like nails or hairs have proven to be especially useful for retrospective metabolite or protein evaluation (widely used in forensic sciences)[52, 53], but also DNA or RNA can be isolated from these samples[54]. A potential threat of these solid sources is that the hair or nail has grown over time with possibly changing exposures, and depending on the measurement, the results should be interpreted with caution. Moreover, these sources can exclusively be used to examine germline DNA/RNA, rather than cancer-induced changes[55]. Although exhaled breath has been used to study volatile organic compounds as an indicator of cancer, clinical trials with standardized sampling methodology are required before this technique can be implemented for non-invasive cancer biomarkers[56-58].

Utilizing existing biobanks for validation of potential molecular markers

Apart from relatively small research-driven biobanks established by researchers, several very extensive and renowned, often publicly funded, biobanks that are accessible for research purposes exist. Examples of such biobanks have been summarized by Vaught *et al.* and Patil *et al.*[59, 60] respectively. Although these biobanks carry many valuable samples and data, only few biobanks include liquid biopsies and are oriented towards cancer research. The UK Biobank[34], BioBank Japan[35], the Victorian Cancer Biobank[36] and the Canadian Tumor Repository Network[37] are examples of large biobanks which include non-invasively collected samples from cancer patients. Existing biobanks could for example support translational research aimed at identifying those patients that would benefit from immune therapy for cancer as they could facilitate relatively fast validation of promising research data obtained through pilot studies.

Level of Evidence

The Level of Evidence (LoE) represents the current evidence for clinical utility of a biomarker, with LoE I representing the highest evidence and LoE V representing the poorest evidence for clinical utility of that particular biomarker[61]. We previously demonstrated that after initial publication of a potential biomarker, subsequent studies do not substantially add to the LoE and clinical translation of potential biomarkers[8]. The quest for novelty has become a paradigm for many researchers; however, this will not benefit the biomarker field in terms of clinical translation. It is therefore encouraged to further validate published biomarkers that showed promising results upon initial publication, in order to bridge biomarkers from initial publication to clinical use, and subsequently reduce research waste. To improve the LoE, prospective cohorts including sufficient,

appropriate samples are required. Selecting appropriate samples from biobanks that fit the research design could facilitate relatively fast validation (Figure 1). Especially in case researchers have easy access to such large cohorts of non-invasive samples, an effort should be made to ensure validation of previously published potential biomarkers independent of the initial research group as this is an essential step in obtaining a sufficient LoE for a potential biomarker[62].

Sample selection

As biobanks will generally include heterogeneous patient populations (including all TNM stages and grades in case of cancer patients' samples), it is important to select only those samples from the biobank that fit a specific research question (Figure 1). For early detection of cancer for example, it is crucial that the biomarker is also assessable in early stage and grade cancers; analyzing subgroups could therefore reveal the true potential of a biomarker. In order to both select an appropriate sample population from a biobank, and perform subgroup analyses, the availability of correct and adequately annotated clinical data to complement the biological samples is of great importance.

Standardization

Generally, it is considered important to have a certain level of standardization regarding processing and storage of liquid biopsies within and between biobanks to be able to perform validation experiments[4, 5]. Although standardized processing and storage within one biobank is a requirement, strict standardization between different biobanks is practically and logistically challenging. However, preferably, biomarkers should be robust and perform equally, independent of the sample pretreatment or assessment procedure, as illustrated by routinely used biomarkers evaluated in various sample types with various techniques, such as *KRAS* and *BRAF* mutations in colorectal cancer[63]. Standardization regarding methods and sample quality can be achieved by implementing standard operating procedures, and by adhering to ISO 9001 and ISO 20387 accreditation, as described before[24].

Biobank sustainability

Although funding providers might presume that after initial investments, biobanks should be self-sustainable, this is challenging for multiple reasons. Warranting solid sources of funding, standardizing procedures to assure sample quality, and complying with legal and privacy related regulations are critical factors to ensure biobank sustainability (Figure 1). Establishing biobanks in a way that they adhere to the FAIR (Findable-Accessible-Interoperable-Reusable) principles could encourage biobank sustainability[64].

Underestimating the costs to establish and maintain a biobank poses a problem, as these do not exclusively include storage costs but also e.g. employees, soft- and

hardware, maintaining and replacing ultra-cold freezers, and storage room rental[65]. For sustainability, it is crucial to make sure that the biobank is cost-effective and visible, promoting the biobank is necessary to generate additional financial funds[65-67].

Standardization of protocols and procedures to assure quality is advocated in all facets of biobanking. Accreditation and standard operating procedures in a biobank could increase utilization by researchers, but also increase willingness of participants to donate samples[65, 66]. Standard operating procedures can also provide adherence to local, national and international law and regulations[65]. Standardization and accreditation could result in interoperability of samples, which means that samples from different biobanks could be pooled to increase statistical power of a study. Although accreditation poses several advantages for biobank sustainability, it might not be feasible to implement for small biobanks because of accreditation costs.

To stimulate funding acquisition, well thought-through cost-benefit analyses, preliminary data obtained from the biobank and close partnership with biobank users, who could include funding requests for biobanking in their grant proposals, are desirable[65].

CONCLUSIONS

This perspective describes some of the challenges in biobanking, both in general and dependent on the collected sample type, also summarized in figure 1. Biobanks could facilitate relatively fast validation of research findings like diagnostic biomarkers for cancer, provided that the utilized samples match the research question. Considering the future purpose of samples is crucial before implementing standardized procedures and logistics to process and store them. Interoperability of samples from different biobanks could facilitate larger sample sizes and thereby increase statistical power of a study. However, as biomarkers should be robust, the degree of standardization between biobanks necessary for biomarker research remains uncertain. Next to establishing biobanks, researchers should make an effort to utilize existing biobanks and ensure independent validation of previously published potential biomarkers, as this is an essential step for clinical implementation of biomarkers. Apart from technical and methodological considerations, biobank sustainability should be considered throughout all phases of biobanking. We therefore highly recommend biobanks to adhere to the FAIR principles and register in directories like https://directory.bbmri-eric.eu/ for European biobanks and https://specimencentral.com/biobank-directory/ for large biobanks worldwide to create visibility and stimulate utilization.

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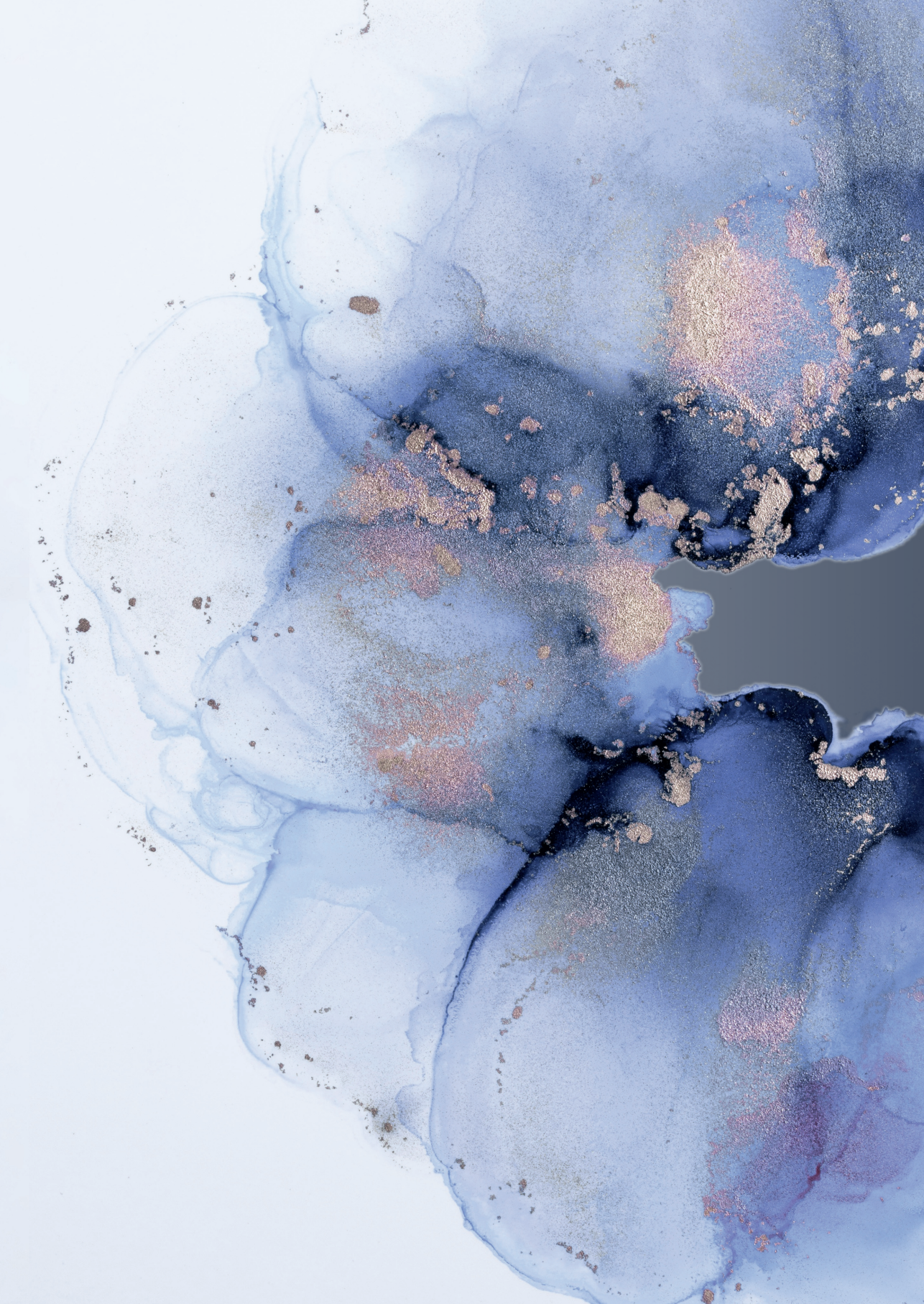

CHAPTER 5

NOVEL DIAGNOSTIC DNA METHYLATION BIOMARKERS FOR RENAL CELL CARCINOMA

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In preparation

ABSTRACT

Background: As the 5-year survival of metastasized renal cell carcinoma (RCC) is low (14%) in advanced disease stages, early RCC diagnosis is crucial to increase survival rates. Urine is regarded as a non-invasive source of early detection biomarkers, such as DNA methylation biomarkers, that might have a clinical impact on early RCC diagnosis. However, published potential urinary DNA methylation biomarkers for RCC do not exceed 65% sensitivity and 89% specificity with Level of Evidence III, making them inapplicable for clinical use. In order to investigate whether we could identify DNA methylation markers with a better diagnostic performance, we used a recently developed *in silico* approach to identify DNA methylation biomarkers for the early detection of RCC.

Methods: Publicly available data from The Cancer Genome Atlas was used to identify potential RCC DNA methylation markers. These markers were validated in 85 RCC tissue samples, and 63 normal, healthy kidney tissue samples with quantitative methylation specific PCR to select the most promising biomarkers for validation. These were further validated in cell-free DNA from an independent population consisting of 92 RCC patients' urine samples, and 115 urine samples from individuals without cancer. Receiver operating characteristic (ROC) curves were created and analyzed to determine methylation cutoffs. Subsequently, a diagnostic model was created using stepwise backward logistic regression analysis. To internally validate the model and correct for optimism, bootstrapping was used.

Results: Twelve potential diagnostic DNA methylation biomarkers were identified through TCGA analysis; nine biomarkers were suitable for subsequent validation in clinical samples. Individual sensitivities ranged from 13-84%, and individual specificities ranged from 44-98% in tissue samples. The six most promising biomarkers were selected for further evaluation in urine samples. In urine samples, individual sensitivities ranged from 3-86% and individual specificities ranged from 7-99%. The final diagnostic model consisted of 4 biomarkers, sex and age, with an optimism-corrected area under the curve of 0.84.

Conclusion: This DNA methylation biomarker panel for diagnosing RCC in urine showed to be a robust model in the sample set studied here. Therefore, it serves as a promising starting point for further validation and extension by addition of other types of biomarkers to further improve this model.

INTRODUCTION

Worldwide, 430 000 people were diagnosed with renal cell carcinoma (RCC) and approximately 180 000 people died of this disease in 2020[1]. Five-year survival rates of early stage RCC are around 93%, and due to new systemic treatments the survival rates of advanced RCC have improved, but patients presenting with metastasized RCC still have poor 5-year survival rates, around 14%[2]. As indicated by these numbers, accurately diagnosing RCC at an early stage is of great importance and could lead to a substantial reduction of RCC health burden. As the disease often presents without symptoms, a considerable amount of metastasized RCC cases (up to 17% at time of diagnosis) may be prevented if diagnosed early[3,4]. Due to the increased utilization of imaging procedures, nowadays many RCC patients are diagnosed after the coincidental finding of a renal mass, i.e. an incidentaloma[5]. Especially for small renal masses (SRM; ≤4 cm), distinguishing benign from malignant lesions based on imaging procedures has proven to be challenging, as these can be detected with only 50% specificity[6,7]. The possibility to diagnose RCC using a non-invasive liquid-biopsy based molecular test, to replace imaging in the future, could not only enhance early diagnosis, but also facilitate differentiation of benign and malignant masses in case a small renal mass is discovered.

Recently, The Cancer Genome atlas (TCGA) and the TRACERx Renal study studied the genetic and epigenetic landscape of RCC. Next to the well-known genetic mutations in *VHL*, *PBRM1*, *BAP1*, and *SETD2* for clear cell RCC (ccRCC), and mutations of *MET* or *FH* in papillary RCC (pRCC) and mutations of *PTEN* or *FLCN* in chromophobe RCC (chRCC), DNA hypermethylation was more pronounced and frequently observed in all RCC subtypes[8-10]. 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[11,12]. This is illustrated by the successful implementation of 7 DNA methylation biomarkers in 4 clinical diagnostic tests for prostate, colorectal and lung cancer[13].

Previously, we systematically reviewed available literature regarding diagnostic DNA methylation biomarkers for RCC, and summarized the current evidence for utilizing these biomarkers[14]. At that moment, no clinically useful diagnostic DNA methylation biomarker for RCC was available. A possible explanation is the repeated investigation of well-known tumor-suppressor genes involved in other cancers, instead of systematically identifying candidate biomarkers for the disease of interest[14]. Therefore, to identify potentially clinically useful RCC markers, we used an *in silico* approach to identify DNA methylation biomarkers for the early detection of RCC. In addition, we validated the identified DNA methylation biomarkers in one study population containing RCC and healthy persons' tumor tissue and another study population containing urine samples from RCC patients and healthy persons.

METHODS

Standards for reporting diagnostic accuracy studies (STARD) guidelines were applied in the process of writing this manuscript[15]. Items 10b, 11, 12b, 16, 17, 21b, 22, 25, 28 and 29 were not scored as they do not have additional value in diagnostic biomarker studies.

In silico DNA methylation marker discovery

TCGA DNA methylation data (Infinium Human Methylation 450K data) for the two most common RCC subtypes (covering >90% of RCCs) ccRCC (KIRC) and pRCC (KIRP) were analyzed to identify novel DNA methylation markers for early diagnosis of RCC. All TCGA data were filtered, so that only probe targets located in CpG islands in RCCs that were unmethylated ($\beta<0.2$) in normal kidney samples remained. For both KIRC and KIRP samples, two differential methylation analyses were performed; one in early stage RCC (stages I and II) and one in late stage RCC (stages III and IV). This ensured selection of probes that were methylated throughout tumorigenesis, as this discovery analysis was targeted towards diagnosing all stages. Next, probes with a statistically significant difference in DNA methylation between normal and RCCs were selected, and to further improve the confidence in the resulting probe set, only probes with an additional differentially methylated probe within 1500 base pairs were retained in order to evade false-positive results. Finally, the early and late stage probe lists were overlapped and the probes that appeared in both were selected. All analyses were performed using R programming language.

Sample collection and preparation

Tissue samples: A hospital-based series of 86 primary RCC tissue samples from adult patients treated with radical or partial nephrectomy without neo-adjuvant therapy was collected retrospectively. In addition, 63 histologically normal kidney tissue samples (FFPE) from patients without cancer were obtained. All samples were collected between 1995 and 2008 and retrospectively obtained from the archives of the Department of Pathology of the Maastricht University Medical Center+ (MUMC+) and the Department of Histopathology, University Hospital of Leuven. This study was approved by the Medical Ethical Committee of the MUMC+ (08-4-030, 2020-2371) and the University Hospital of Leuven (S62466).

Urine samples: A series of urine samples from 92 patients with RCC and 115 urine samples of patients without cancer were obtained from the Radboud Biobank (Radboudumc). All samples were collected between 2012 and 2015, and written informed consent was obtained from all patients. This study was approved by the Medical Ethical Committee of Radboudumc (METC: 2010/370).

DNA isolation and bisulfite conversion

Tissue samples: DNA was isolated from 5x20µm thick FFPE sections from all patients using the QIAamp DNA FFPE Tissue kit (Qiagen) according to the manufacturers' protocol.

Urine samples: Urine samples were centrifuged at 4°C for 10 minutes at 1800g. Cell-free DNA (cfDNA) was isolated from 4 ml of the supernatant using the QIAamp MinElute ccfDNA kit (Qiagen) according to the manufacturers' protocol.

Bisulfite conversion: Sodium bisulfite conversion was carried out on a maximum of 500ng genomic DNA isolated from FFPE tissue samples (FFPE Tissue Samples protocol) and a maximum of 500ng cfDNA isolated from urine samples (Low-Concentration Solutions protocol) using the Epitect bisulfite kit and QIAcube according to the manufacturers' protocol.

Nested methylation-specific PCR

To select the most promising candidate markers, nested methylation-specific PCR (MSP) was performed. This method requires two separate reactions; a pre-amplification reaction, followed by a reaction discriminating methylated from unmethylated DNA with specifically designed (un)methylated primer sets. For the pre-amplification, a reaction mix consisting of 8.15 µl sterile water, 2.5 µl Magic buffer (consisting of 0.13 µl 3.2M (NH4)2SO4, 0.84 µl 2.0M Tris pH 8.8, 0.17 µl 1.0M MgCl2, 1.35µl MiliQ), 1.25 µl deoxynucleotide triphosphates (dNTPs) (GE Healthcare), 2.5 µl forward flank mix primer, 2.5 µl reverse flank mix primer and 0.1 µl Immolase Taq (5U/µl; Bioline) was prepared per sample. Eight microliter of bisulfite converted DNA was transferred to a 96-wells plate on ice, and 17 µl of reaction mix was added. The product was amplified using a thermal cycler (3 minutes 95°C; 30 seconds 95°C; 30 seconds 56°C; 30 seconds 72°C; previous three steps were repeated 34 times, 4 minutes 72°C) (Bio-Rad). The amplified product was diluted 1:1000. The second reaction required two primer sets per gene; a primer set for the methylated and a primer set for the unmethylated sequence. Reaction mixes were prepared by combining 5.2 µl sterile water, 0.4 µl forward primer, 0.4 µl reverse primer and 10 µl SensiMix (Bioline) per sample. Four microliter of the diluted pre-amplification product was added to 16 µl of reaction mix. The product was amplified using a real-time thermal cycler (10 minutes 95°C; 15 seconds 95°C; 45 seconds primer specific temperature; previous two steps were repeated 39 times; 1 minute 90°C; 1 minute at 60°C) (Bio-Rad CFX96). Commercially available positive (methylated DNA) and negative (unmethylated DNA) controls were used (Qiagen). Delta Cq (Cq unmethylated reaction - Cq methylated reaction) was calculated. Samples were excluded from the analysis in case no U reaction took place, or whenever the melting temperature of that sample was >1.5 Cq values away from the corresponding control value. Primers were designed in close proximity of the TCGA-derived candidate marker probes using both Primer3 and Gene Runner, and were manufactured by Eurogentec.

Quantitative methylation-specific PCR

Quantitative methylation-specific PCR (qMSP) with hydrolysis probe was performed to analyze the diagnostic performance of candidate biomarkers. For tissue samples, reactions were performed in a final volume of 25µl, consisting of 4.0µl bisulfite converted DNA, 15.3 µl sterile H2O, 2.5µl Magic Buffer (consisting of 0.13 µl 3.2M (NH4)2SO4, 0.84 µl 2.0M Tris pH 8.8, 0.17 µl 1.0M MgCl2, 1.35µl MiliQ), 1.0 µl dNTPs (6.25mM; BIO-39026; Bioline), 0.75 µl forward primer (10µM), 0.75 µl reverse primer (10µM), 0.5 µl probe (5µM) and 0.2 µl HS Taq (5U/µl; BIO-21112; Bioline). Considering the scarce availability of cfDNA from urine samples, urine qMSPs were performed in 2 separate multiplex reactions targeting 3 genes each. For urine samples, reactions were performed in a final volume of 24µl, consisting of 8.0µl bisulfite converted DNA, 10.3 µl sterile H2O, 2.5µl Magic Buffer (consisting of 0.13 µl 3.2M (NH4)2SO4, 0.84 µl 2.0M Tris pH 8.8, 0.17 µl 1.0M MgCl2, 1.35µl MiliQ), 1.0 µl dNTPs (6.25mM; BIO-39026; Bioline), 0.75 µl forward primer mix (10µM), 0.75 µl reverse primer mix (10µM), 0.5 µl probe per target gene (5µM; 1.5 µl in total) and 0.2 µl HS Taq (5U/µl; BIO-21112; Bioline). The product was amplified using a real-time thermal cycler (Bio-Rad CFX96), and the results were normalized using ALU repeat elements as reference. Samples were excluded when ALU was not amplified. The PCR program was initiated with 3 minutes at 95°C, followed by 50 cycles of 15 seconds at 95°C, 1 minute at 60°C and 1 minute at 72°C. A serially diluted plasmid (Life Technologies) containing the target and reference amplicons was used as a standard curve (4.00E+07 copies/µl, 4.00E+06 copies/µl, 4.00E+05 copies/µl, 4.00E+04 copies/µl, 4.00E+03 copies/µl, 4.00E+02 copies/µl, 4.00E+01 copies/µl). Quantification of DNA methylation was analyzed by interpolating the Ct value of the unknown sample to the corresponding plasmid copy using Bio-Rad CFX manager 2.0 software (Bio-Rad). All assays were performed using commercially available fully unmethylated and fully methylated DNA (Qiagen), and a no-template control as control samples. Primers and probes were designed in close proximity of the TCGA-derived candidate marker probes using both Primer3 and Gene Runner, and were manufactured by Integrated DNA Technologies.

Data analysis

For nested MSP, receiver operating characteristic (ROC) curves with area under curve (AUC) and 95% confidence intervals (95%-CI) were created to assess the discriminative ability of the candidate markers. Cutoff values were based on the highest positive likelihood ratio (LR) through which sensitivities and specificities were derived. Samples were considered methylated when their delta Ct was higher than the predetermined cutoff.

For qMSP, ROC curves with corresponding AUC and 95%-CI were created to assess the diagnostic utility of the candidate markers in tissue samples first. A qMSP cutoff value for each markers was established based on the highest positive LR, through which sensitivity and specificity values were derived. A sample was considered methylated when its methylation value was higher than the predetermined cutoff. Next, the best performing markers

in tissue were identified. These markers were subsequently analyzed in urine samples. In order to assess the association between the candidate genes methylation status in tissue samples and clinicopathological features, Pearson's chi-square tests were performed for categorical variables (like stage, grade, and sex), and independent samples t-tests were performed for continuous variables (like age). Pearson's chi-square tests were also performed to compare methylation status of the candidate genes between RCC and non-cancer patients' samples in both tissue and urine. To build a multivariable prediction model in urine containing multiple methylation markers, we performed a backward stepwise elimination procedure using the likelihood-ratio test and a liberal α of 0.1 to prevent the exclusion of potentially important predictors from the model[16]. Age and sex were included in this model regardless of statistical significance. The resulting model was internally validated using 1000 bootstrap samples to estimate the amount of overfitting (by computing the average prediction slope) and to estimate optimism in performance. The initial model was then penalized using uniform shrinkage, and the optimism-corrected AUC was computed.

All statistical analyses were performed using Graphpad Prism, SPSS, R and Stata.

Table 1. Clinicopathological features of the RCC and NK tissue samples cohort.

Patient demographics		RCC tissue (n=86) n (%)	Normal kidney tissue (n=63) n (%)
Sex			
	Male	53 (62.6)	41 (65.1)
	Female	33 (38.4)	22 (34.9)
Age (years)			
	Mean age (SD)	58.05 (±12.8)	65.78 (±15.1)
Subtype			
	ccRCC	63 (73.3)	-
	pRCC	15 (17.4)	-
	chRCC	8 (9.3)	-
Cancer stage			
	Stage 1	49 (57.0)	-
	Stage 2	15 (17.4)	-
	Stage 3	21 (24.4)	-
	Stage 4	1 (1.2)	-
Fuhrman grade			
	Grade 1	3 (3.5)	-
	Grade 2	37 (43.0)	-
	Grade 3	39 (45.3)	-
	Grade 4	7 (8.1)	-

ccRCC; clear cell renal cell carcinoma, chRCC; chromophobe renal cell carcinoma, NK; normal kidney, pRCC; papillary renal cell carcinoma, RCC; renal cell carcinoma, SD; standard deviation.

RESULTS

Study characteristics

Characteristics of the tissue study population are shown in Table 1. Patients in the RCC and NK tissue samples population were similar in terms of gender, and differed slightly in age (Table 1). The ccRCC and pRCC samples comprised >90% of the RCCs included, and more than half of the samples were stage 1 (Table 1). Only one stage 4 RCC, and predominantly Fuhrman grade 2 and 3 were included (Table 1).

Characteristics of the urine study population are shown in Table 2. Similar to the tissue study population, the RCC patients and healthy persons in the urine study population were similar in terms of gender, and differed slightly in age (Table 2). The ccRCC and pRCC samples comprised >90% of included samples. Stage 1 and 3, and grade 2 and 3 were predominantly included (Table 2). Cancer stage was unknown for three patients, and Fuhrman grade was unknown for 9 other patients.

Identification of diagnostic DNA methylation markers for RCC

After performing a multistep in silico DNA methylation marker discovery for diagnosing RCC using publicly available TCGA data, 63 probes corresponding to 22 genes remained. These probes were hypermethylated throughout all stages in RCC samples and unmethylated in normal samples. For two of these genes, no appropriate primers could be designed; these genes were therefore excluded from further analyses

Methylation of the most promising DNA methylation markers in tissue

After performing nested MSPs, we identified nine DNA methylation markers (*Gene 1, Gene 2, Gene 3, Gene 4, Gene 5, Gene 6, Gene 7, Gene 8* and *Gene 9*) as the best performing markers in FFPE tissue samples in terms of sensitivity and specificity (data not shown). These markers were selected for further analysis using qMSP.

The qMSP results of the 9 DNA methylation markers showed statistically significantly different methylation frequencies between RCC and normal tissue in all genes (*Gene 1, Gene 2, Gene 3, Gene 4, Gene 5, Gene 6, Gene 7*: p=0.000, *Gene 8*: p=0.047, *Gene 9*: p=0.029; Figure 1A). As only one sample per gene was methylated, specificity was 98% in 7 out of 9 genes; specificity for *Gene 7* and *Gene 8* were 44% and 70% respectively (Figure 1A). Sensitivity of these genes varied widely; 36% for *Gene 1* , 56% for *Gene 2,* 38% for *Gene 3,* 36% for *Gene 4,* 52% for *Gene 5,* 34% for *Gene 6*, 84% for *Gene 7*, 48% for *Gene 8* and 13% for *Gene 9* (Figure 1A). Because of the high methylation of *Gene 7* and *Gene 8* in normal tissue samples, and the very limited methylation of *Gene 9* in RCC samples, these three genes were excluded for further validation analyses. The AUCs for *Gene 1, Gene 2, Gene 3, Gene 4, Gene 5,* and *Gene 6* were 0.66 (95%-CI: 0.57–0.74), 0.83 (95%-CI: 0.77-0.90), 0.70 (95%-CI: 0.61-0.78), 0.69 (95%-CI: 0.60-0.77), 0.66 (95%-CI: 0.57-0.76) and

0.56 (95%-CI: 0.47-0.66) respectively (Figure 1B). ROC curves for *Gene 7, Gene 8* and *Gene 9* are shown in supplementary figure 1. The association between methylation of all individual biomarkers and clinicopathological features in FFPE tissue samples is presented in supplementary table 1. *Gene 1, Gene 2, Gene 3, Gene 4, Gene 5,* and *Gene 6* were further analyzed in urine samples.

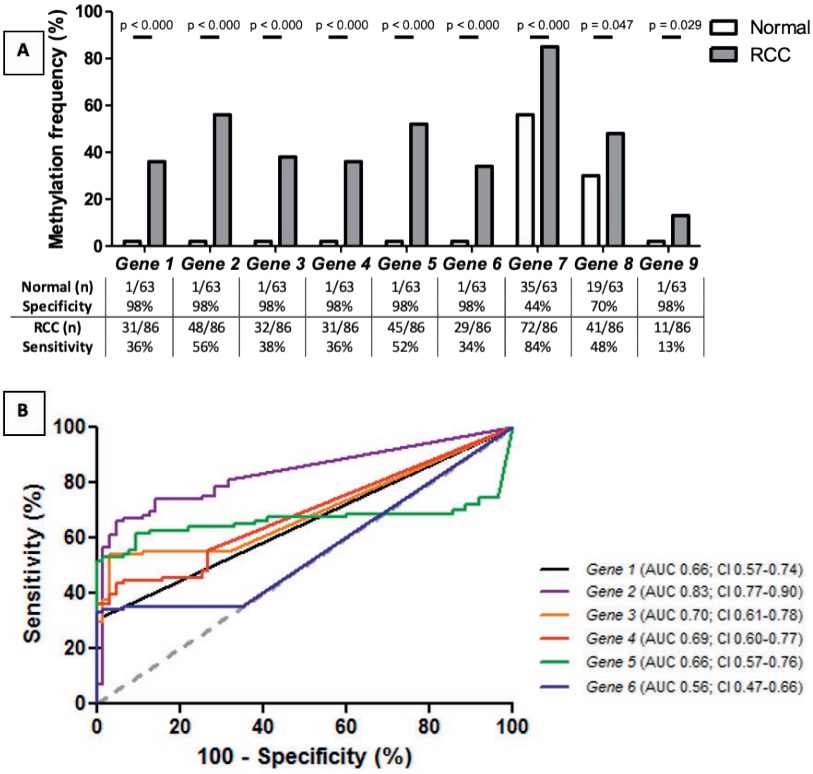

Figure 1. DNA methylation marker performance in healthy persons' and RCC patients' FFPE tissue DNA. A) DNA methylation frequency, sensitivity and specificity for *Gene 1, Gene 2, Gene 3, Gene 4, Gene 5, Gene 6, Gene 7, Gene 8* and *Gene 9* in healthy persons' (n=63) and RCC patients' (n=86) FFPE tissue DNA. B) ROC curves and corresponding AUC for diagnostic performance of *Gene 1, Gene 2, Gene 3, Gene 4, Gene 5,* and *Gene 6* as indicated by the corresponding colors. ROC curves for *Gene 7, Gene 8* and *Gene 9* can be found in supplementary figure 1.

Methylation of the most promising DNA methylation markers in urine

Statistically significantly different methylation frequencies between RCC patients' and healthy persons' urine cfDNA were observed for *Gene 1, Gene 2, Gene 3, Gene 5* and *Gene 6* (Figure 2). *Gene 3* and *4* did not show a methylation difference; methylation frequencies were very low (3% and 1%) in RCC patients' and healthy persons' urine cfDNA respectively for *Gene 3*, and very high (95% and 93%) in RCC patients' and healthy persons'

urine cfDNA respectively for *Gene 4* (Figure 2). Individual sensitivities ranged from 3% to 95%, and specificities ranged from 7% to 99%. Individual AUCs for *Gene 1* (0.51; 95%-CI: 0.42-0.60), *Gene 2* (0.63; 95%-CI: 0.51-0.71), *Gene 3* (0.60; 95%-CI: 0.53-0.68), *Gene 4* (0.50; 95%-CI: 0.42-0.58), *Gene 5* (0.53; 95%-CI: 0.45-0.61), and *Gene 6* (0.61; 95%-CI: 0.54-0.69) indicated limited discriminative power of the DNA methylation biomarkers alone. The association between methylation of all individual biomarkers and clinicopathological features in urine samples is presented in Table 3. Methylation of *Gene 6* in RCC patients' urine was more often found in men compared to women (p=0.003; Table 3). Methylation of *Gene 2* was borderline significantly associated with Fuhrman grade, but no clear direction in this trend could be observed (p=0.520; Table 3). No other differences between methylation of the studied biomarkers and clinicopathological features was found (Table 3).

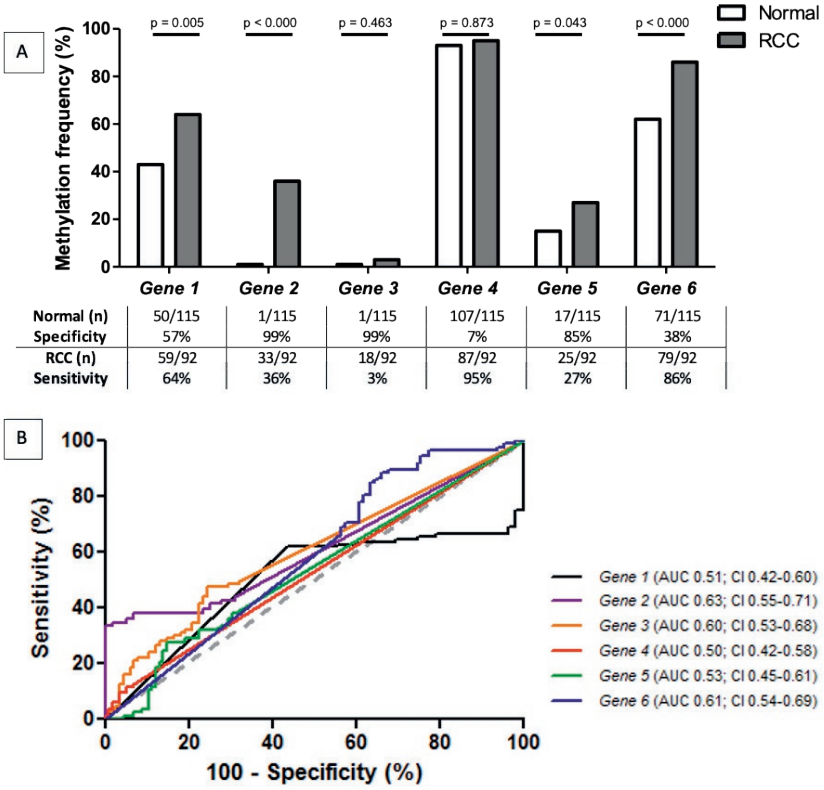

Figure 2. DNA methylation marker performance in healthy persons' and RCC patients' urine cfDNA. A) DNA methylation frequency, sensitivity and specificity for *Gene 1*, *Gene 2*, *Gene 3*, *Gene 4*, *Gene 5*, and *Gene 6* in healthy persons' (n=115) and RCC patients' (n=92) urine cfDNA. B) ROC curve and AUC for diagnostic performance of *Gene 1*, *Gene 2*, *Gene 3*, *Gene 4*, *Gene 5*, and *Gene 6* as indicated by the corresponding colors.

Table 3. DNA methylation biomarkers in RCC patients' urine cfDNA compared to clinicopathological features.

	Gene 1		*Gene 2*		*Gene 3*		*Gene 4*		*Gene 5*		*Gene 6*	
	M	U	M	U	M	U	M	U	M	U	M	U
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Gender												
Male	42 (71.2)	25 (75.8)	22 (66.7)	45 (76.3)	3 (100.0)	64 (71.9)	64 (73.6)	3 (60.0)	19 (76.0)	48 (71.6)	62 (78.5)	5 (38.5)
Female	17 (28.8)	8 (24.2)	11 (33.3)	14 (23.7)	0 (0.0)	25 (28.1)	23 (26.4)	2 (40.0)	6 (24.0)	19 (28.4)	17 (21.5)	8 (61.5)
p-value	0.636		0.321		0.282		0.507		0.676		**0.003**	
Age												
Mean age (±SD)	62.5 (±10.1)	62.1 (±7.8)	63.1 (±9.1)	62.0 (±9.5)	60.0 (±15.1)	62.5 (±9.2)	62.4 (±9.5)	61.6 (±6.4)	61.9 (±8.9)	62.7 (±9.5)	62.3 (±9.0)	62.6 (±11.1)
p-value	0.825		0.571		0.655		0.848		0.626		0.922	
RCC subtype												
ccRCC	47 (79.7)	22 (66.7)	26 (78.8)	43 (72.9)	3 (100.0)	66 (74.2)	64 (73.6)	5 (100.0)	19 (76.0)	50 (74.6)	56 (70.9)	13 (100.0)
ccRCC/pRCC	1 (1.7)	2 (6.1)	1 (3.0)	2 (3.4)	0 (0.0)	3 (3.4)	3 (3.4)	0 (0.0)	1 (4.0)	2 (3.0)	3 (3.8)	0 (0.0)
pRCC	8 (13.6)	8 (24.2)	6 (18.2)	10 (16.9)	0 (0.0)	16 (18.0)	16 (18.4)	0 (0.0)	5 (20.0)	11 (16.4)	16 (20.3)	0 (0.0)
chRCC	2 (3.4)	0 (0.0)	0 (0.0)	2 (3.4)	0 (0.0)	2 (2.2)	2 (2.3)	0 (0.0)	0 (0.0)	2 (3.0)	2 (2.5)	0 (0.0)
MiT family translocation RCC	1 (1.7)	1 (3.0)	0 (0.0)	2 (3.4)	0 (0.0)	2 (2.2)	2 (2.3)	0 (0.0)	0 (0.0)	2 (3.0)	2 (2.5)	0 (0.0)
p-value	0.355		0.669		0.905		0.779		0.793		0.283	
Stage												
Stage 1	27 (45.8)	14 (42.4)	16 (48.5)	25 (42.4)	1 (33.3)	40 (44.9)	39 (44.8)	2 (40.0)	9 (36.0)	32 (47.8)	36 (45.6)	5 (38.5)
Stage 2	3 (5.1)	3 (9.1)	2 (6.1)	4 (6.8)	0 (0.0)	6 (6.7)	6 (6.9)	0 (0.0)	1 (4.0)	5 (7.5)	6 (7.6)	0 (0.0)
Stage 3	26 (44.1)	13 (39.4)	14 (42.4)	25 (42.4)	2 (66.7)	37 (41.6)	36 (41.4)	3 (60.0)	13 (52.0)	26 (38.8)	32 (40.5)	7 (53.8)
Stage 4	3 (5.1)	0 (0.0)	1 (3.0)	2 (3.4)	0 (0.0)	3 (3.4)	3 (3.4)	0 (0.0)	1 (4.0)	2 (3.0)	3 (3.8)	0 (0.0)
Unknown	0 (0.0)	3 (9.1)	0 (0.0)	3 (5.1)	0 (0.0)	3 (3.4)	3 (3.4)	0 (0.0)	1 (4.0)	2 (3.0)	2 (2.5)	1 (7.7)
p-value	0.102		0.758		0.921		0.894		0.779		0.560	
Fuhrman grade												
Grade 1	4 (6.8)	1 (3.0)	2 (6.1)	3 (5.1)	0 (0.0)	5 (5.6)	5 (5.7)	0 (0.0)	2 (8.0)	3 (4.5)	4 (5.1)	1 (7.7)
Grade 2	15 (25.4)	12 (36.4)	14 (42.4)	13 (22.0)	0 (0.0)	27 (30.3)	25 (28.7)	2 (40.0)	7 (28.0)	20 (29.9)	23 (29.1)	4 (30.8)
Grade 3	24 (40.7)	11 (33.3)	8 (24.2)	27 (45.8)	1 (33.3)	34 (38.2)	33 (37.9)	2 (40.0)	11 (44.0)	24 (35.8)	30 (38.0)	5 (38.5)
Grade 4	8 (13.6)	8 (24.2)	8 (24.2)	8 (13.6)	2 (66.7)	14 (15.7)	15 (17.2)	1 (20.0)	4 (16.0)	12 (17.9)	13 (16.5)	3 (23.1)
Unknown	8 (13.6)	1 (3.0)	1 (3.0)	8 (13.6)	0 (0.0)	9 (10.1)	9 (10.3)	0 (0.0)	1 (4.0)	8 (11.9)	9 (11.4)	0 (0.0)
p-value	0.240		0.052		0.220		0.904		0.742		0.751	

Pearson's chi-square tests (gender, RCC subtype, stage and Fuhrman grade) and independent samples t-tests (age) were performed to calculate p-values. ccRCC; clear cell renal cell carcinoma, chRCC; chromophobe renal cell carcinoma, M; methylated, NK; normal kidney, pRCC; papillary renal cell carcinoma, RCC; renal cell carcinoma, SD; standard deviation, U; unmethylated.

Diagnostic performance of a multimarker panel

To determine the best performing diagnostic panel using *Gene 1*, *Gene 2*, *Gene 3*, *Gene 4*, *Gene 5* and *Gene 6*, backward stepwise logistic regression analysis was performed. The final diagnostic model consisted of 4 biomarkers (*Gene 1*, *Gene 2*, *Gene 5* and *Gene 6)*, sex and age. All coefficients and odds ratios (ORs) for this final model are presented in Table 4. The AUC for this 4-marker panel was 0.85 (95%-CI 0.80-0.90). As this model aims to identify all RCC subtypes, but the studied markers were not specifically identified for chRCC, the analyses were reran without including these chRCC cases, which did not change the model (data not shown).

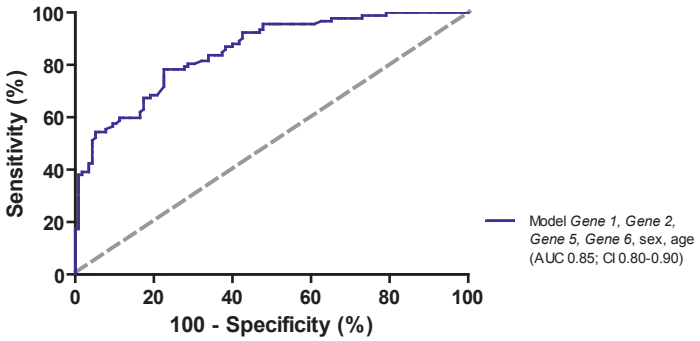

Figure 3. ROC curve for diagnostic performance of a marker panel including *Gene 1*, *Gene 2*, *Gene 5*, and *Gene 6*, age and sex.
Internal validation of this model shows a shrinkage factor of 0.82 that was used to penalize the initial model coefficients (sex: -0.19, age: 0.05 *Gene 1*: 1.07, *Gene 2*: 1.21, *Gene 5*: 3.39 *Gene 6*: 0.85). The optimism-corrected AUC was 0.84.

Table 4. Diagnostic performance of the selected marker panel in RCC patients' (n=92) and healthy persons' (n=115) urine samples.

	Coefficient	SE	OR (95%-CI)	p-value
Sex	-0.24	0.02	0.79 (0.33-1.90)	0.598
Age	0.06	0.02	1.06 (1.02-1.09)	0.001
Gene 1	1.30	0.40	3.68 (1.69-8.02)	0.001
Gene 2	1.47	0.49	4.36 (1.67-11.38)	0.003
Gene 5	4.13	1.06	62.14 (7.81-494.70)	<0.001
Gene 6	1.04	0.47	2.83 (1.12-7.14)	0.028
Intercept	-5.64	1.17	0.004 (0.00-0.04)	<0.001

SE; standard error, OR; odds ratio, 95%-CI; 95% confidence interval

DISCUSSION

This study, we utilized publicly available data from the TCGA database to identify potential DNA methylation biomarkers for the early detection of RCC, and validated these biomarkers in both FFPE tissue and urine. From this *in silico* TCGA analysis, a limited number of candidate biomarkers were identified. Six of 22 identified markers (*Gene 1, Gene 2, Gene 3, Gene 4, Gene 5* and *Gene 6*) showed good diagnostic performance in FFPE tissue samples and were therefore further investigated in urine samples. However, the diagnostic performance of these markers decreased in urine samples. The final diagnostic model contained 4 biomarkers (*Gene 1, Gene 2, Gene 5* and *Gene 6)*, sex and age.

The *in silico* analysis of TCGA data in this manuscript yielded 22 candidate diagnostic DNA methylation biomarkers for RCC, which is much lower as compared to similar analyses performed for e.g. colorectal cancer[17]. Previously, it has been suggested that epigenetic alterations are more abundant compared to genetic alterations in RCC[18]. However, both types of alterations are less often observed in RCC compared to other cancer types[8]. The results from our *in silico* TCGA analysis are in line with these previous observations. Nevertheless, as TCGA methylation data is based on Infinium 450K arrays, it does therefore not fully cover the genome, and bisulfite sequencing might yield additional or complementary candidate markers[19]. Nonetheless, our analyses identified 4 potential DNA methylation markers for the early detection of RCC.

The DNA methylation markers studied here have not been linked to (early detection of) RCC before, except for *Gene 5*. However, they have been implicated in other cancer types, such as bladder, pancreatic, oral squamous cell, head- and neck squamous cell, colorectal, urothelial, and hepatocellular carcinoma. Some of the markers have been suggested for diagnostic (*Gene 2*) or prognostic (*Gene 5, Gene 6*) purposes in these cancer types as well. In our recent systematic review, *Gene 5* was marked as a promising diagnostic DNA methylation biomarker for RCC, however it was not independently validated at that time[14]. Here, *Gene 5* performed inferior (52% sensitivity, 98% specificity) compared to the original study in tissue samples, however a different genomic location was studied. None of the examined markers examined in this manuscript (methylation of *Gene 1, Gene 2, Gene 3, Gene 4, Gene 5* and *Gene 6*) were studied for diagnostic potential in urine before. Although not directly linked to RCC, previously the function of these genes have been linked to cancer-related pathways such as cell adhesion, invasion, metastasis, apoptosis, oxidative stress and progression[20,21].

Diagnosing cancer using DNA methylation has been successful in the past, illustrated by the implementation of 7 DNA methylation biomarkers in 4 clinical diagnostic tests for colorectal, prostate and lung cancer[13]. However, we observed that the reported diagnostic performance in urine was lower compared to tissue samples. The drop in diagnostic performance in the translation of tissue to liquid biopsies has been observed in RCC

and other cancer types before[14,22]. This could be attributed to several factors; e.g. the small amounts of extracted cfDNA from liquid biopsies, the presence of PCR inhibitors, but it could also be caused by the liquid biopsy sample handling and storage. In urine, a preserving agent that inhibits DNAse activity (e.g. EDTA), can limit DNA degradation and facilitate higher cfDNA yield from these samples[23,24]. In addition, an abundance of normal cfDNA can disrupt the tumor-derived cfDNA signal. Therefore, only urine supernatant was used after centrifugation to ensure that most genomic DNA and cells were discarded[25]. It has previously been suggested that only low molecular weight (LMW) cfDNA was derived from the tumor, while high molecular weight (HMW) cfDNA was not[26]. Additional centrifugation steps might therefore further increase the fraction of tumor-derived cfDNA, and allow more accurate detection of the biomarkers in urine.

In addition to the limited amount available, cfDNA is known to be highly fragmented in all sample types. Especially cfDNA in urine is highly fragmented as glomerular filtration of plasma is very selective; only molecules <6.4 nm in diameter and a molecular weight under 70 kDa can pass the glomeruli, equating to approximately 100 bp stretches of DNA[25]. All biomarker assays in this manuscript are <97 bp, which could have increased the risk of false negative results. To overcome this, instead of qMSP, even more sensitive DNA methylation detection techniques like droplet digital PCR, bisulfite sequencing or Discrimination of Rare EpiAlleles by Melt (DREAMing) might allow for more accurate detection of these biomarkers in urine[27-32].

In conclusion, we developed a DNA methylation biomarker panel for the early detection of RCC in urine that shows to be a robust model. Therefore, it serves as a promising starting point for further validation in independent urine series and extension by addition of other types of biomarkers. Further validation should focus specifically on addition of a larger number of SRMs, as this model is expected to have the highest clinical implication for this patient group by distinguishing benign from malignant SRMs.

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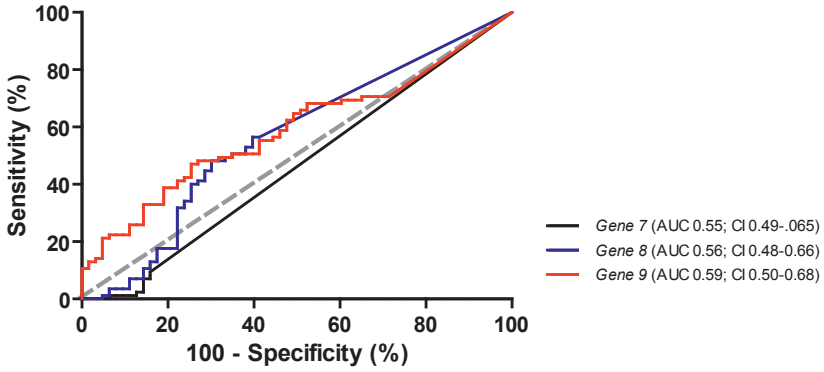
SUPPLEMENTARY INFORMATION



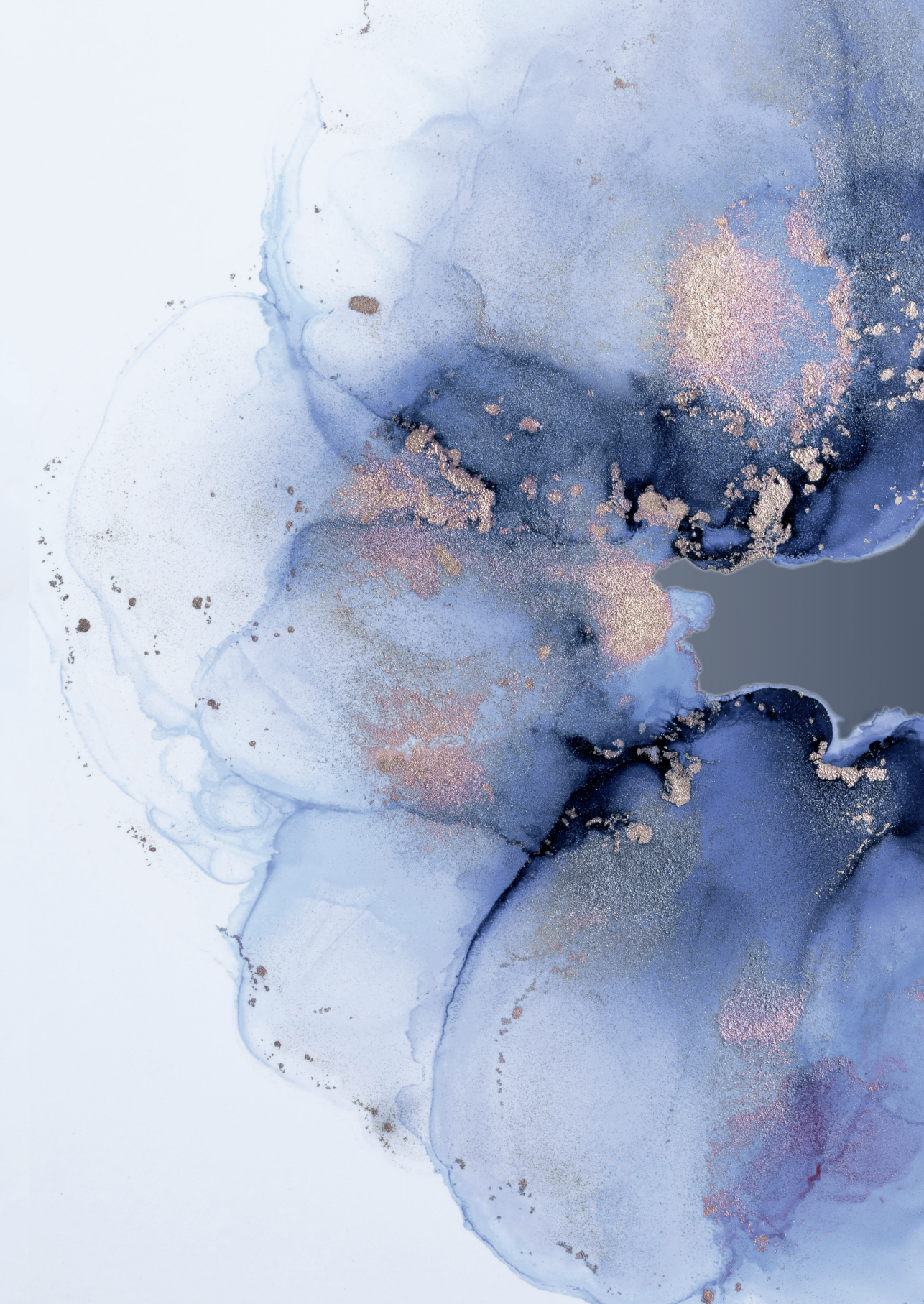
Supplementary figure 1. Receiver operating characteristic (ROC) curve for diagnostic performance of *Gene 7, Gene 8,* and *Gene 9* in healthy persons' (n=63) and RCC patients' (n=86) FFPE tissue DNA as indicated by the corresponding colors.

Supplementary table 1. DNA methylation biomarkers in RCC FFPE tissue DNA compared to clinicopathological features.

	Gene 1		***Gene 2***		***Gene 3***		***Gene 4***	
	M	U	M	U	M	U	M	U
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Gender								
Male	23 (74.2)	29 (46.3)	31 (64.6)	21 (56.8)	20 (62.5)	32 (60.4)	20 (66.7)	31 (58.5)
Female	8 (25.8)	25 (53.7)	17 (35.4)	16 (43.2)	12 (37.5)	21 (39.6)	10 (33.3)	22 (41.5)
p-value	0.127		0.557		0.716		0.750	
Age								
Mean age (±SD)	60.0 (±10.3)	54 (±56.6)	58.0 (±11.2)	57.7 (±14.7)	56.3 (±12.5)	58.8 (±13.0)	57.2 (±11.4)	58.8 (±13.4)
p-value	0.242		0.914		0.393		0.597	
RCC subtype								
ccRCC	22 (71.0)	40 (74.0)	39 (81.2)	23 (62.2)	20 (62.5)	42 (79.2)	28 (93.3)	33 (62.3)
pRCC	8 (25.8)	7 (13.0)	7 (14.6)	8 (21.6)	8 (25.0)	7 (13.2)	1 (3.3)	13 (24.5)
chRCC	1 (3.2)	7 (13.0)	2 (4.2)	6 (16.2)	4 (12.5)	4 (7.6)	1 (3.3)	7 (13.2)
p-value	0.371		0.261		0.517		**0.037**	
Stage								
Stage 1	16 (51.6)	33 (61.1)	25 (52.1)	24 (64.9)	22 (68.8)	27 (50.9)	15 (50.0)	34 (64.2)
Stage 2	4 (12.9)	10 (18.5)	6 (12.5)	8 (21.6)	4 (12.5)	10 (18.9)	5 (16.7)	10 (18.9)
Stage 3	10 (32.3)	11 (20.4)	16 (33.3)	5 (13.5)	6 (18.7)	15 (28.3)	9 (30.0)	9 (16.9)
Stage 4	1 (3.2)	0 (0.0)	1 (2.1)	0 (0.0)	0 (0.0)	1 (1.9)	1 (3.3)	0 (0.0)
p-value	0.213		0.104		0.259		**0.036**	
Fuhrman grade								
Grade 1	0 (0.0)	3 (5.6)	0 (0.0)	3 (8.2)	2 (6.3)	1 (1.9)	0 (0.0)	3 (5.7)
Grade 2	11 (35.5)	26 (48.1)	20 (41.7)	17 (45.9)	12 (37.5)	25 (47.2)	13 (43.3)	22 (41.5)
Grade 3	14 (45.2)	24 (44.4)	21 (43.8)	17 (45.9)	16 (50.0)	22 (41.5)	12 (40.0)	26 (49.0)
Grade 4	6 (19.3)	1 (1.9)	7 (14.5)	0 (0.0)	2 (6.3)	5 (9.4)	5 (16.7)	2 (3.8)
p-value	0.085		0.097		0.769		0.330	

Pearson's chi-square tests (gender, RCC subtype, stage and Fuhrman grade) and independent samples t-tests (age) were performed to calculate p-values. ccRCC; clear cell renal cell carcinoma, chRCC; chromophobe renal cell carcinoma, M; methylated, NK; normal kidney, pRCC; papillary renal cell carcinoma, RCC; renal cell carcinoma, SD; standard deviation, U; unmethylated

Gene 5		***Gene 6***		***Gene 7***		***Gene 8***		***Gene 9***	
M	U	M	U	M	U	M	U	M	U
n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
31 (72.1)	20 (50.0)	7 (24.1)	45 (80.4)	45 (62.5)	7 (53.8)	18 (43.9)	34 (77.3)	2 (18.2)	50 (67.6)
12 (27.9)	20 (50.0)	22 (75.9)	11 (19.6)	27 (37.5)	6 (46.2)	23 (56.1)	10 (22.7)	9 (81.8)	24 (32.4)
0.116		**0.000**		0.613		**0.005**		**0.005**	
59.3 (±12.0)	57.1 (±13.3)	57.0 (±12.2)	58.3 (±13.2)	58.5 (±12.7)	54.0 (±13.3)	57.5 (±13.5)	58.1 (±12.3)	53.0 (±15.6)	58.6 (±12.3)
0.430		0.676		0.241		0.830		0.179	
32 (62.8)	29 (72.5)	22 (75.9)	40 (71.4)	54 (75.0)	8 (61.5)	31 (75.6)	31 (70.5)	7 (63.6)	55 (74.3)
10 (23.3)	4 (10.0)	3 (10.3)	12 (21.4)	13 (18.1)	2 (15.4)	5 (12.2)	10 (22.7)	2 (18.2)	13 (17.6)
1 (2.3)	7 (17.5)	4 (13.8)	4 (7.1)	5 (6.9)	3 (23.1)	5 (12.2)	3 (6.8)	2 (18.2)	6 (8.1)
0.092		0.614		0.438		0.653		0.815	
27 (62.8)	22 (55.0)	16 (55.2)	33 (58.9)	43 (59.7)	6 (46.1)	19 (46.3)	30 (68.2)	3 (27.3)	46 (62.2)
9 (20.9)	6 (15.0)	5 (17.2)	9 (16.1)	12 (16.7)	2 (15.4)	10 (24.3)	4 (9.1)	2 (18.2)	12 (16.2)
7 (16.3)	11 (27.5)	7 (24.1)	14 (25.0)	16 (22.2)	5 (38.5)	12 (29.3)	9 (20.4)	6 (54.5)	15 (20.3)
0 (0.0)	1 (2.5)	1 (3.4)	0 (0.0)	1 (1.4)	0 (0.0)	0 (0.0)	1 (2.3)	0 (0.0)	1 (1.3)
0.053		0.339		0.367		0.087		0.071	
1 (2.3)	2 (5.0)	2 (6.9)	1 (1.8)	2 (2.8)	1 (7.7)	1 (2.4)	2 (4.6)	0 (0.0)	3 (4.1)
18 (41.9)	17 (42.5)	10 (34.5)	27 (48.2)	34 (47.2)	3 (23.1)	15 (36.6)	22 (50.0)	1 (9.1)	36 (48.6)
21 (48.8)	17 (42.5)	13 (44.8)	25 (44.6)	29 (40.3)	9 (69.2)	21 (51.2)	17 (38.6)	6 (54.5)	32 (43.2)
3 (7.0)	4 (10.0)	4 (13.8)	3 (5.4)	7 (9.7)	0 (0.0)	4 (9.8)	3 (6.8)	4 (36.4)	3 (4.1)
0.945		0.524		0.341		0.765		**0.007**	



CHAPTER 6

EXPLORING A DNA METHYLATION FIELD EFFECT IN RENAL CELL CARCINOMA AND ITS IMPLICATIONS FOR BIOMARKER RESEARCH

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In preparation

ABSTRACT

Background: A field effect, the phenomenon of molecular alterations (e.g. DNA methylation) in normally appearing tissue surrounding a malignant tumor, has been suggested to be associated with malignant transformation. Nevertheless, normally appearing tissue adjacent to the tumor is frequently used as control tissue in biomarker studies. Here, we aimed to evaluate DNA methylation alterations in tissue surrounding malignant renal cell carcinoma (RCC), and demonstrate the impact of these alterations on the evaluation of biomarker potential.

Methods: We evaluated six potential DNA methylation markers across several sampled areas of eleven kidneys using qMSP. To evaluate the impact of the field effect on biomarker performance, the percentage of methylated reference (%PMR) was calculated and samples were analyzed with ROC curve analysis using RCCs as cases with either adjacent normal (AN) or normal kidney (NK) tissues as controls.

Results: High methylation was present in RCC tissues (ranging from 34-56%), and matched AN tissues (ranging from 13-59%) as compared to NK (2% in all genes). All AN samples (100%) methylated for *Gene 4* corresponded to methylation in the RCC, whereas this holds true for only 0-55.6% of the other studied genes. The methylation rates varied widely between markers and patients. A gradual decline in $\%PMR_{\text{all genes}}$ was found when moving from the tumor (41.4%) towards the area furthest away from the tumor (0.2%). Analyzing biomarker performance using AN as control tissues yielded lower sensitivities for all biomarkers (7.1-37.7%) compared to using NK as controls (34.1-56.5%).

Conclusion: Here, we evaluated DNA methylation alterations in normally appearing tissue surrounding RCC, and generated the hypothesis that a DNA methylation field effect in RCC might exist. In addition, this data emphasizes the importance of using appropriate control tissues to avoid underestimation of biomarker potential. Therefore we would advocate to use normal tissue of non-cancerous patients as controls rather than AN in diagnostic biomarker studies, to avoid underestimation of biomarker performance.

INTRODUCTION

In 1944, Slaughter first described the concept of a field effect in cancer as being 'the remains of histologically normal, but molecularly predisposed tissue after surgical resection of oral carcinomas could explain the development of new primary or recurrent tumors'[1-3]. A field effect has since been studied in various cancer types, although the focus has shifted from histological tissue evaluation to molecular approaches[4]. Amongst others, a field effect has been demonstrated in gastrointestinal, lung, head- and neck, breast, skin and urological cancers[4]. The biological processes underlying the field effect have been linked to the tissue type from which the cancer arises[5]. For example, contiguous epithelial tumors, such as head-and-neck[6,7], skin[8], gastrointestinal[9-11] and bladder[12,13] cancers have been correlated to a monoclonal expansion model, whereas tumors in glandular tissues, such as prostate[14,15] and breast[16-18] cancer are often, although not always, linked to a polyclonal expansion model[19].

A clinical application of the field effect has been established for prostate cancer; the presence of *GSTP1*, *APC* and *RASSF1* promoter methylation in beningn-appearing prostate biopsies using the ConfirmMDx® test has been suggested as an approach to avoid unnecessary repeat biopsies[20]. Although the field effect has not been extensively studied in renal cell cancer (RCC), a previous comparison of the methylation status of RCC, matched normal renal tissues, and normal renal tissues from healthy volunteers, showed that the average number of methylated CpG islands was highest in the RCCs. However, a high methylation rate was also found in the matched normal tissues as compared to the normal tissues obtained from healthy individuals[21]. In addition, the amount of methylation in the matched normal tissue samples was statistically significantly correlated with a higher histological grade of the corresponding RCCs[21]. These findings indicate a possible field effect; the matched normal tissues seem to have accumulated DNA methylation (also present in the corresponding RCC), and might possibly be regarded as a molecularly predisposed area surrounding the tumor. A DNA methylation field effect in RCC could potentially be used to predict prognosis, as an accumulation of DNA methylation in histologically normal tissue could indicate a potential risk of local recurrence or metastases, which is seen in up to 20% of patients curatively treated with (partial) nephrectomy[21-28]. In addition, it might be used to screen for precancerous or recurrent lesions, or act as an indicator to evaluate the surgical resection margins[4-6,19].

In addition to potential clinical implications, the occurrence of a DNA methylation field effect in RCC might also influence the evaluation of biomarker performance. In RCC biomarker studies, pathologically normally appearing tissues adjacent to the tumor tissue are often used as control samples[29]. However, the occurrence of a DNA methylation field effect would imply that this tissue, despite its histologically normal appearance, could

actually be aberrant at a molecular level. Depending on the biomarkers' purpose, AN tissue might therefore be inappropriate as biomarker control tissue.
Here, we aimed to evaluate a DNA methylation field effect in RCC, and illustrate the impact of the field effect and choice of control tissues in research on potential biomarkers.

METHODS

Sample collection and preparation

The first population was a hospital-based RCC series containing tumor tissue (RCC; n=86), matched adjacent, normally appearing, kidney tissue from the same patient (AN; n=32), and tissue obtained from healthy kidneys of patients without cancer (autopsy samples, NK; n=63). These samples were derived from the archives of the Department of Histopathology, University Hospital of Leuven, Belgium and Department of Pathology, Maastricht University Medical Center, the Netherlands, and were collected between 1995 and 2008.

The second study population consisted of a pathological consecutive series of 11 RCC patients that underwent a nephrectomy at Maastricht University Medical Center in the period 2019-2020. Per patient, five formalin-fixed, paraffin-embedded (FFPE) tissue blocks were obtained. Next to a sample of the tumor (C0), a sample of the transition from tumor to normal tissue (based on hematoxylin and eosin (H&E) stainings; C1), and 3 more samples each spaced 1 cm apart, starting from C1 were taken (C2-C3-C4) (Figure 1). In addition, H&E stainings were performed on these samples.

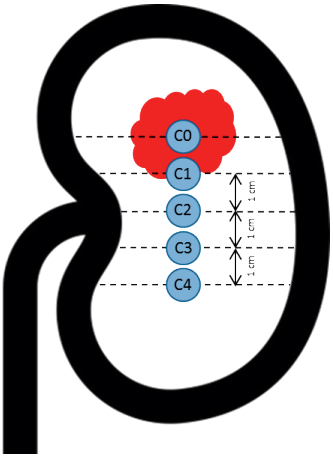

Figure 1. Experimental setup to evaluate the field effect in renal cell carcinoma (RCC). C0 is sampled from cancerous tissue, C1 is sampled from the transition of cancerous to normally appearing tissue, C2-C4 are sampled from normally appearing tissue, spaced 1 cm apart starting from C1.

DNA from FFPE blocks of both series was isolated and bisulfite converted using the Qiagen Epitect fast FFPE bisulfite kit. This study was approved by the Medical Ethical Committee of Maastricht University Medical Center (METC 08-4-030 and METC 20-2371).

Quantitative methylation-specific PCR

DNA methylation of six genes previously identified as potential diagnostic biomarkers for RCC (*Gene 1-6*) was determined by quantitative methylation-specific PCR (qMSP) with hydrolysis probe[30]. Reactions were performed in a final volume of 25µl, consisting of 4.0µl bisulfite converted DNA, 15.3 µl sterile H2O, 2.5µl Magic Buffer (consisting of 0.13 µl 3.2M (NH4)2SO4, 0.84 µl 2.0M Tris pH 8.8, 0.17 µl 1.0M MgCl2, 1.35µl MiliQ), 1.0 µl dNTPs (6.25mM; BIO-39026; Bioline Meridian Bioscience), 0.75 µl forward primer (10µM), 0.75 µl reverse primer (10µM), 0.5 µl probe (5µM) and 0.2 µl HS Taq (5U/µl; BIO-21112; Bioline Meridian Bioscience). The product was amplified using a real-time thermal cycler (Bio-Rad), and the results were normalized using ALU repeat elements as reference. The PCR program was initiated with 3 minutes at 95°C, followed by 50 cycles of 15 seconds at 95°C, 1 minute at 60°C and 1 minute at 72°C. A serially diluted plasmid (Life Technologies) containing the target and reference amplicons was used as a standard curve (4.00E+07 copies/µl, 4.00E+06 copies/µl, 4.00E+05 copies/µl, 4.00E+04 copies/µl, 4.00E+03 copies/µl, 4.00E+02 copies/µl, 4.00E+01 copies/µl). In case ALU was not amplified, the sample could not be analyzed for that particular gene, and was therefore excluded. All assays were performed using commercially available fully unmethylated and fully methylated DNA (Qiagen), and a no-template control as control samples. Primers and probes were obtained from Integrated DNA Technologies.

Data analysis

Quantification of DNA methylation was analyzed by interpolating the Ct value of the unknown sample to the corresponding plasmid copy using Bio-Rad CFX manager 2.0 software (Bio-Rad), which resulted in starting quantity (SQ) values for each sample. qMSP data was consequently analyzed by calculating the percentage methylated reference (PMR) value: $\%PMR = \frac{(\text{SQ marker/SQ ALU})sample}{(\text{SQ marker/SQ ALU})M\ control} \times 100$[31-34]. Receiver operating characteristics (ROC) curves were created to determine the optimal cutoff PMR and the area under curve (AUC) for each marker. Optimal cutoff values were based on the highest likelihood ratio (LR), with corresponding sensitivity and specificity. Based on these cutoff values, samples were classified as methylated or unmethylated.

To evaluate the impact of the field effect on biomarker performance, samples in the hospital-based series were analyzed with ROC curve analysis using the RCCs as cases and AN as controls, or using the RCCs as cases and the NK as controls. ROC-curves, sensitivity, specificity, LRs and corresponding cutoff values were obtained using Graphpad Prism.

RESULTS

Characteristics of the hospital-based study population are shown in Table 1. Patients in the RCC and NK tissue samples population were similar in terms of gender, and differed slightly in age (Table 1). The ccRCC and pRCC samples comprised >90% of the RCCs included, and more than half of the samples were stage 1 (Table 1). Only one stage 4 RCC, and predominantly Fuhrman grade 2 and 3 were included (Table 1).

Characteristics of the pathological consecutive study population are shown in Table 2. Of eleven patients, 8 were male (73%) and 3 were female (27%). Mean age at diagnosis was 71.6 (range 43-85), and only ccRCC and pRCC type 1 samples were studied (Table 2). Tumor stage varied from 1a to 3a, and ISUP grades 2-4 were studied. Mean tumor diameter was 6.0 cm (range 2.4-11.5; Table 2).

Table 1. Clinicopathological features of the hospital-based study population containing renal cell carcinoma (RCC), matched adjacent normal (AN) and normal kidney (NK) tissue samples.

Patient demographics		RCC tissue (n=86) n (%)	NK tissue (n=63) n (%)
Sex			
	Male	53 (62.6)	41 (65.1)
	Female	33 (38.4)	22 (34.9)
Age (years)			
	Mean age (±SD)	58.05 (±12.8)	65.78 (±15.12)
RCC subtype			
	ccRCC	63 (73.3)	
	pRCC	15 (17.4)	
	chRCC	8 (9.3)	
Cancer stage			
	Stage 1	49 (57.0)	
	Stage 2	15 (17.4)	
	Stage 3	21 (24.4)	
	Stage 4	1 (1.2)	
Fuhrman grade			
	Fuhrman grade 1	3 (3.5)	
	Fuhrman grade 2	37 (43.0)	
	Fuhrman grade 3	39 (45.3)	
	Fuhrman grade 4	7 (8.1)	

ccRCC; clear cell renal cell carcinoma, chRCC; chromophobe renal cell carcinoma, pRCC; papillary renal cell carcinoma, RCC; renal cell carcinoma, SD; standard deviation

Table 2. Clinicopathological features of the renal cell carcinoma (RCC) patients in the pathological consecutive study population.

	Gender	Age at diagnosis	RCC subtype	Tumor stage	ISUP grade	Diameter (cm)
Patient 1	Male	62	ccRCC	1a	3	2.4
Patient 2	Male	70	ccRCC	3a	3	5.2
Patient 3	Female	75	pRCC type 1	3a	3	8.6
Patient 4	Male	73	ccRCC	3a	3	5.1
Patient 5	Female	69	ccRCC	1a	3	3.5
Patient 6	Male	77	pRCC type 1	3a	3	5.5
Patient 7	Male	73	pRCC type 1	1b	3	5.5
Patient 8	Male	43	ccRCC	1b	2	5.2
Patient 9	Female	77	ccRCC	3a	4	11.5
Patient 10	Male	84	ccRCC	1a	2	4.0
Patient 11	Male	85	ccRCC	3a	4	9.8

ccRCC; clear cell renal cell carcinoma, cm; centimeter, ISUP; International Society of Urological Pathology, pRCC; papillary renal cell carcinoma, RCC; renal cell carcinoma

Evaluation of a DNA methylation field effect in RCC

After qMSP analysis of *Gene 1, Gene 2, Gene 3, Gene 4, Gene 5* and *Gene 6* for all samples in the hospital-based series, ROC curve analysis was performed using RCC and NK as control samples, and individual cutoffs for all genes were determined according to the highest LR. Based on these cutoffs, samples were labeled as either methylated or unmethylated. As shown in Figure 2A-D, the percentage of methylation of the six analyzed genes varied from 34-56% in RCC samples, and from 13-59% in AN samples. Methylation was highly gene-dependent and methylation in AN did not always correspond to methylation in the RCC; all AN samples (100%) methylated for *Gene 4* corresponded to methylation in the RCC, whereas this holds true for only 0-55.6% for the other studied genes. The lowest number of methylated AN samples was observed for *Gene 1*, only four cases (13%) were methylated in the AN, and none of these samples corresponded with methylation in the tumor. For the other genes however, methylation in AN samples was much higher, ranging from 20% in *Gene 4* to 59% in *Gene 2* (Figure 2D). For these genes, methylation in the AN samples also corresponded more often with methylation in the tumor: 33.3% for *Gene 6* (2/6 methylated AN), 44.4% for *Gene 2* and *Gene 3* (8/18 and 4/9 respectively) and 55.6% for *Gene 5* (5/9). For *Gene 4*, all patients with methylation in the AN sample also showed methylation in the tumor sample (5/5; 100%). Methylation in the normal kidney samples was much lower, 2% for each tested biomarker, corresponding to one out of 63 samples (Figure 2A-B).

A

RCC						
Sample	*Gene 1*	*Gene 2*	*Gene 3*	*Gene 4*	*Gene 5*	*Gene 6*
55T	M	M	M	U	M	M
56T	U	M	U	M	U	U
57T	M	M	U	U	M	U
58T	U	U	M	U	U	U
59T	M	M	U	M	U	M
60T	M	M	M	U	M	U
61T	U	M	M	U	U	U
62T	U	U	M	U	U	M
63T	M	M	U	M	M	U
64T	M	M	U	M	M	M
65T	U	M	U	M	U	M
66T	U	M	U	U	U	U
67T	M	U	M	U	M	U
68T	M	M	U	M	M	U
69T	U	M	U	M	U	U
70T	M	M	M	M	M	M
71T	M	U	U	X	X	U
72T	U	U	U	X	X	U
73T	U	U	U	M	M	U
74T	U	U	U	U	M	U
75T	U	M	U	U	U	U
76T	M	M	U	M	U	M
77T	U	U	U	U	U	U
78T	U	U	M	U	U	U
79T	X	X	X	U	M	X
80T	U	U	U	M	U	M
81T	U	U	U	M	M	U
82T	U	U	M	U	M	U
83T	U	M	U	M	U	M
84T	U	U	M	U	M	U
85T	U	U	M	U	U	M
86T	M	M	M	U	M	U

B

Adjacent Normals						
Sample	*Gene 1*	*Gene 2*	*Gene 3*	*Gene 4*	*Gene 5*	*Gene 6*
55AN	U	U	U	U	M	U
56AN	M	U	U	U	U	U
57AN	U	U	U	U	M	U
58AN	U	M	U	U	U	U
59AN	U	U	M	U	U	U
60AN	U	M	U	U	U	U
61AN	U	M	U	U	M	M
62AN	U	M	M	U	M	U
63AN	U	U	U	U	U	U
64AN	U	U	U	U	U	M
65AN	U	M	U	M	U	M
66AN	U	M	U	U	U	M
67AN	U	M	U	U	U	U
68AN	U	U	U	M	M	U
69AN	U	M	U	M	M	U
70AN	U	M	U	U	U	U
71AN	U	U	M	M	U	U
72AN	U	U	U	X	X	U
73AN	U	M	U	X	X	U
74AN	U	M	M	U	M	U
75AN	U	U	U	U	M	M
76AN	U	U	U	U	U	U
77AN	U	U	M	U	U	U
78AN	M	M	U	U	U	U
79AN	M	M	U	U	U	M
80AN	U	U	M	M	U	U
81AN	U	M	U	U	U	U
82AN	U	M	U	U	U	U
83AN	U	M	U	M	U	U
84AN	U	M	M	U	U	M
85AN	M	M	M	U	U	U
86AN	U	M	M	U	M	U

C

Normal Kidneys						
Sample	*Gene 1*	*Gene 2*	*Gene 3*	*Gene 4*	*Gene 5*	*Gene 6*
NK1	U	U	U	U	U	U
NK2	U	U	U	U	U	U
NK3	U	U	U	U	U	U
NK4	U	U	U	U	U	U
NK5	U	U	U	U	U	U
NK6	U	U	U	U	M	U
NK7	U	U	U	U	U	U
NK8	U	U	U	U	U	U
NK9	U	U	U	U	U	U
NK10	U	U	U	U	U	U
NK11	U	U	U	U	U	U
NK12	U	U	U	U	U	U
NK13	U	U	U	U	U	U
NK14	U	U	U	U	U	U
NK15	U	U	U	U	U	U
NK16	U	U	U	U	U	U
NK17	U	U	U	U	U	U
NK18	U	U	U	U	U	U
NK19	U	U	U	U	U	U
NK20	U	U	U	U	U	U
NK21	U	U	U	U	U	U
NK22	U	U	U	U	U	U
NK23	U	U	U	U	U	U
NK24	U	U	U	U	U	U
NK25	U	U	U	U	U	U
NK26	U	U	U	U	U	U
NK27	U	U	U	U	U	U
NK28	U	U	U	U	U	U
NK29	U	U	M	U	U	U
NK30	U	U	U	U	U	U
NK31	U	U	U	U	U	U
NK32	U	U	U	U	U	U
NK33	U	U	U	U	U	U
NK34	U	U	U	U	U	U
NK35	U	U	U	U	U	U
NK36	M	U	U	U	U	U
NK37	U	U	U	U	U	U
NK38	U	U	U	U	U	U
NK39	U	U	U	U	U	U
NK40	U	U	U	U	U	U
NK41	U	U	U	U	U	U
NK42	U	U	U	U	U	U
NK43	U	U	U	U	U	M
NK44	U	U	U	U	U	U
NK45	U	U	U	U	U	U
NK46	U	U	U	U	U	U
NK47	U	U	U	U	U	U
NK48	U	U	U	U	U	U
NK49	U	U	U	U	U	U
NK50	U	M	U	U	U	U
NK51	U	U	U	U	U	U
NK52	U	U	U	U	U	U
NK53	U	U	U	U	U	U
NK54	U	U	U	U	U	U
NK55	U	U	U	U	U	U
NK56	U	U	U	U	U	U
NK57	U	U	U	U	U	U
NK58	U	U	U	U	U	U
NK59	U	U	U	U	U	U
NK60	U	U	U	U	U	U
NK61	U	U	U	U	U	U
NK62	U	U	U	U	U	U
NK63	U	U	U	M	U	U

D

	Gene 1	*Gene 2*	*Gene 3*	*Gene 4*	*Gene 5*	*Gene 6*
RCC	36%	56%	38%	36%	52%	34%
AN	13%	59%	28%	20%	30%	22%
NK	2%	2%	2%	2%	2%	2%

Figure 2. qMSP results of *Gene 1, Gene 2, Gene 3, Gene 4, Gene 5* and *Gene 6* in the hospital-based series, based on cutoff values after receiver operating characteristic (ROC) curve analysis using renal cell carcinoma (RCC) and normal kidney (NK) as control samples. A) RCC samples and B) matched adjacent normal (AN) samples; C) NK samples; D) percentage of methylated samples per sample category and gene based on all RCC samples (n = 86; see supplementary figure 1), matched AN samples (n = 32) and NK samples (n = 63). M: methylated, U: unmethylated, X: non-analyzable due to lack of ALU amplification.

Next, to evaluate whether methylation shows a gradual decline in tissue located further away from the tumor, qMSPs for *Gene 1, Gene 2, Gene 3, Gene 4, Gene 5* and *Gene 6* were performed on bisulfite converted DNA from each of the samples from the pathological consecutive series. H&E slides of all samples were evaluated by two experienced pathologists (I.S. and A.z.H.) and all tissue was marked as either RCC or benign kidney tissue (representative H&E slides are shown in Figure 3).

In sample 2.C4, no DNA was measured and therefore, no %PMR could be calculated. In general, methylation of *Gene 4* was relatively high in the tumors (mean %PMR 84.87%) and relatively low outside the tumors (mean %PMR 1.20%). The other five genes showed a mean %PMR of 32.67% in the RCC (range 20.89% in *Gene 6* to 69.85% in *Gene 5*), and a mean %PMR of 8.15% outside the tumors (range 2.53% in *Gene 2* to 16.85% in *Gene 5* respectively; Supplementary table 1).

Even though methylation throughout the sampled areas was patient- and gene dependent, three groups of patients could be distinguished. Group A consisted of patients without methylation outside the malignant core C0 (<1% PMR), suggesting no field effect (Patient 4 and 11; Supplementary figure 2A). Group B consisted of patients with methylation in the malignant core C0 and in the transition of tumor to normal tissue C1, but not outside the malignant tissue (Patient 6 and 9; Supplementary figure 2B). Patients in group C also showed methylation outside the tumor core and transition to normal tissue, and in C2-C4 for some of the studied genes, suggesting the possible existence of a methylation field effect (Patient 1, 2, 3, 5, 7, 8 and 10; Supplementary figure 2C; Supplementary table 1).

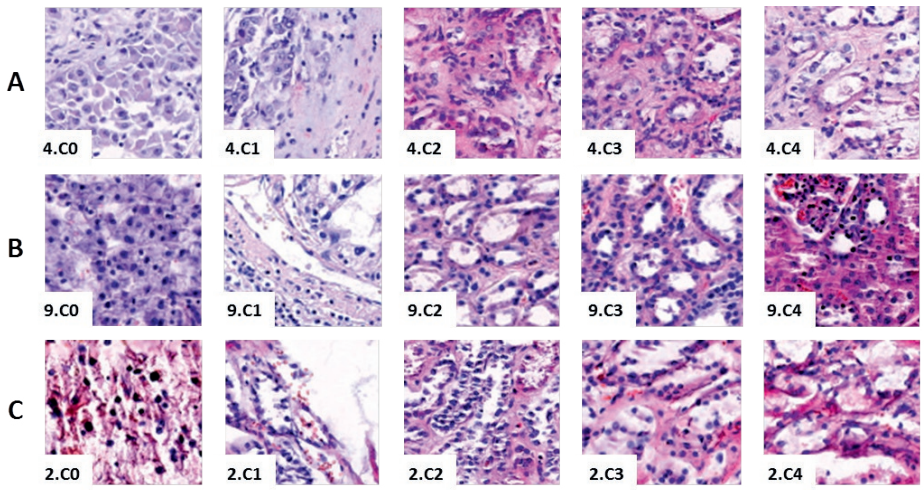

Figure 3. Representative H&E slides of C0-C4 (5x magnification). A) Patient 4, representing group A; B) Patient 9, representing group B; C) Patient 2, representing group C.

Methylation outside C0 and C1 was found throughout all RCC stages, grades, diameters and subtypes, indicating this methylation was not correlated to any of the analyzed pathological features in this small patient group. In addition, methylation outside C0 and C1 was measured across all genes. When evaluating the mean %PMR in all sampled areas of all patients, a gradual decline in methylation was found when moving from the malignant core (C0; mean %PMR 41.37%) towards C4 (mean %PMR 0.24% (Figure 4; Supplementary table 1)).

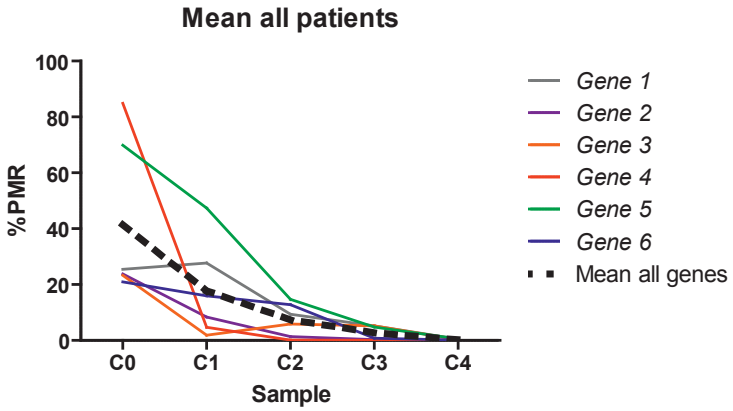

Figure 4. Visual presentation of the mean percentage of methylated reference (%PMR) of *Gene 1, Gene 2, Gene 3, Gene 4, Gene 5* and *Gene 6* in all sampled areas (C0-C4) of all 11 patients.

The impact of control tissues on diagnostic biomarker performance

To assess the impact of a possible field effect on the evaluation of biomarker performance, this performance was calculated with the %PMR of RCC as cases and the %PMR of either AN or NK as control tissues. As a result, the cutoff to determine whether a sample was methylated or unmethylated for an individual gene shifted and subsequently the biomarker performance differed between both methods of analyzing. As illustrated in Figure 5, analyzing biomarker performance using RCC as cases and AN as control tissues yielded lower sensitivities (ranging from 7.10% in *Gene 3* to 37.7% in *Gene 2*) as compared to the sensitivities calculated using RCC as cases and NK as control tissues (ranging from 34.1% in *Gene 6* to 56.5% in *Gene 2*). The greatest difference in sensitivity between both methods of analyzing biomarker performance was shown for *Gene 3* (30.6 percentage points), and the smallest difference in sensitivity of 16.9 percentage points was shown for *Gene 5*. Specificities were similar in both analyses in all genes, except for *Gene 2* (71.9% in AN vs. RCC, 98.4% in NK vs. RCC). Although slight gene-specific differences between both methods of analyzing biomarker performance were noticeable, the choice of appropriate control tissues and its corresponding performance difference seems to be a robust phenomenon throughout genes.

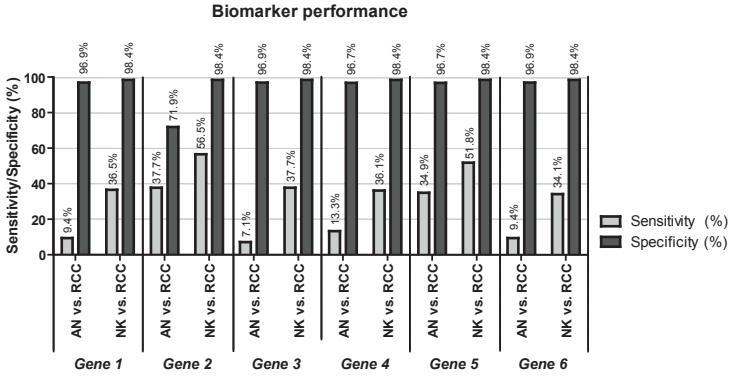

Figure 5. Individual biomarker performance when evaluating receiver operating characteristic (ROC) analyses comparing matched adjacent normal (AN) vs. renal cell carcinoma (RCC), and normal kidney (NK) vs. RCC.

DISCUSSION

The purpose of this study was to evaluate DNA methylation alterations in RCC and its surrounding tissue, and illustrate the impact of the choice of control tissues on the evaluation of biomarker performance. High methylation was found in RCC tissues and matched AN tissues as compared to NK, suggesting the possible existence of a DNA methylation field effect in RCC. The evaluation of this possible DNA methylation field effect in nephrectomy tissues at different distances from the malignant tumor core revealed that, in this dataset, there are patients with DNA methylation exclusively in the tumor and transitional zone to normal tissue, and patients that also have methylation in histologically normal kidney tissue. In addition, the gradual decline in DNA methylation in tissue located further away from the tumor seems to be highly patient- and gene dependent in this study, and no evident clinicopathological differences between groups could be observed. When using AN to determine the cutoff for test positivity, the sensitivity of the biomarker decreased due to presence of DNA methylation in the AN tissue. The sensitivity increased when using NK tissue, as the absence of methylation in this tissue led to a different cutoff for test positivity. This indicates the importance of using appropriate control tissue when evaluating diagnostic performance of a biomarker. Although these different methods do not change the biomarkers' actual performance, but rather change the threshold to determine whether a sample is methylated or unmethylated, using NK as control tissues might be most representative of the true biomarker performance.

It has been established that premalignant cells harbor genetic alterations in order to become cancerous, with the most well-known example being the Vogelstein model for colorectal cancer development[35]. A similar mechanism where cells also accumulate epi-

genetic changes like DNA methylation has been established more recently[5,36,37]. Several studies have shown that cells adjacent to a particular tumor have more methylation as compared to cells further away from the tumor, indicating a possible premalignant effect of DNA methylation[5,36,37]. This was also observed in another study investigating DNA methylation of 9 genes in RCC, suggesting that next to DNA methylation in the tumor, also high DNA methylation was observed in matched normal tissues compared to tissues obtained from healthy individuals[21]. This phenomenon might also have a clinical implication in the evaluation of surgical margins after curative intended partial nephrectomy or ablation. Even when surgical margins were considered clean by pathologists, approximately 5-20% of RCC patients develop a local recurrence within 5 years after resection[22-28]. Therefore, evaluating surgical margins for premalignant features using molecular approaches, rather than current macro- and microscopic approaches might be able to help indicate whether these surgical margins are wide enough to minimize the chance of local recurrences and distant metastases[4,19]. Taken together, this encourages to further hypothesize and investigate the occurrence of this possible DNA methylation field effect in RCC.

In addition to the clinical implications, a field effect could influence biomarker research and the evaluation of biomarker performance in diagnostic studies. In a previous systematic review, we found that two-thirds of all studies evaluating diagnostic biomarker performance in kidney tissue used AN control tissues, and only one-third used normal control tissue from non-cancerous patients[29]. Given the data generated in this study, this might have led to an underestimation of the true biomarker potential, as suboptimal control tissue was used for the biomarkers' (diagnostic) purpose.

Noteworthy, the pathological consecutive series used to evaluate a DNA methylation field effect in RCC only consisted of eleven patients, therefore these results should be considered as hypothesis-generating rather than conclusive. The samples from this series were obtained from radical nephrectomies, enabling us to analyze tissue further away from the tumor core and reducing the risk of sampling bias. Usually, radical nephrectomies would be expected to be performed for relatively large tumors. However, in this series, also small and less aggressive tumors were included, which were radically resected based on co-morbidities or surgical complications. Furthermore, because the samples in this study were recent RCC cases, no follow-up data about developing recurrences or metastases after nephrectomy were available at this time. This prevented us from evaluating possible correlation between future recurrences or metastases and observed DNA methylation patterns in the normal tissue surrounding the tumor.

Future research should be focused on further evaluating a potential DNA methylation field effect in RCC by increasing the sample size and performing genome-wide methylation studies using a similar study-setup as the current study. As only six genes have been evaluated in this study, extending these analyses with e.g. bisulfite sequencing to enable full epigenome coverage might be able to pinpoint key players in a possible DNA

methylation RCC field effect. These key players could subsequently be evaluated for their potential role in monitoring patients during post-nephrectomy follow-up through liquid biopsies. Such liquid biopsy samples like blood or urine are known to represent (epi)genetic features of the tumor, and could therefore indicate recurrence of the disease even before masses can be detected by imaging techniques[38-41] .

Here, we have shown that DNA methylation of six genes in normally appearing tissues surrounding RCC was evident in part of the patients, indicating that a DNA methylation field effect in RCC might exist. In order to further elucidate this field effect, genome-wide studies could pinpoint key players in this possible effect. In addition, we illustrated that choosing appropriate control tissues affected biomarker performance estimation. Therefore, we would advocate to use normal tissue of non-cancerous patients as controls rather than AN in diagnostic biomarker studies, to avoid underestimation of biomarker performance.

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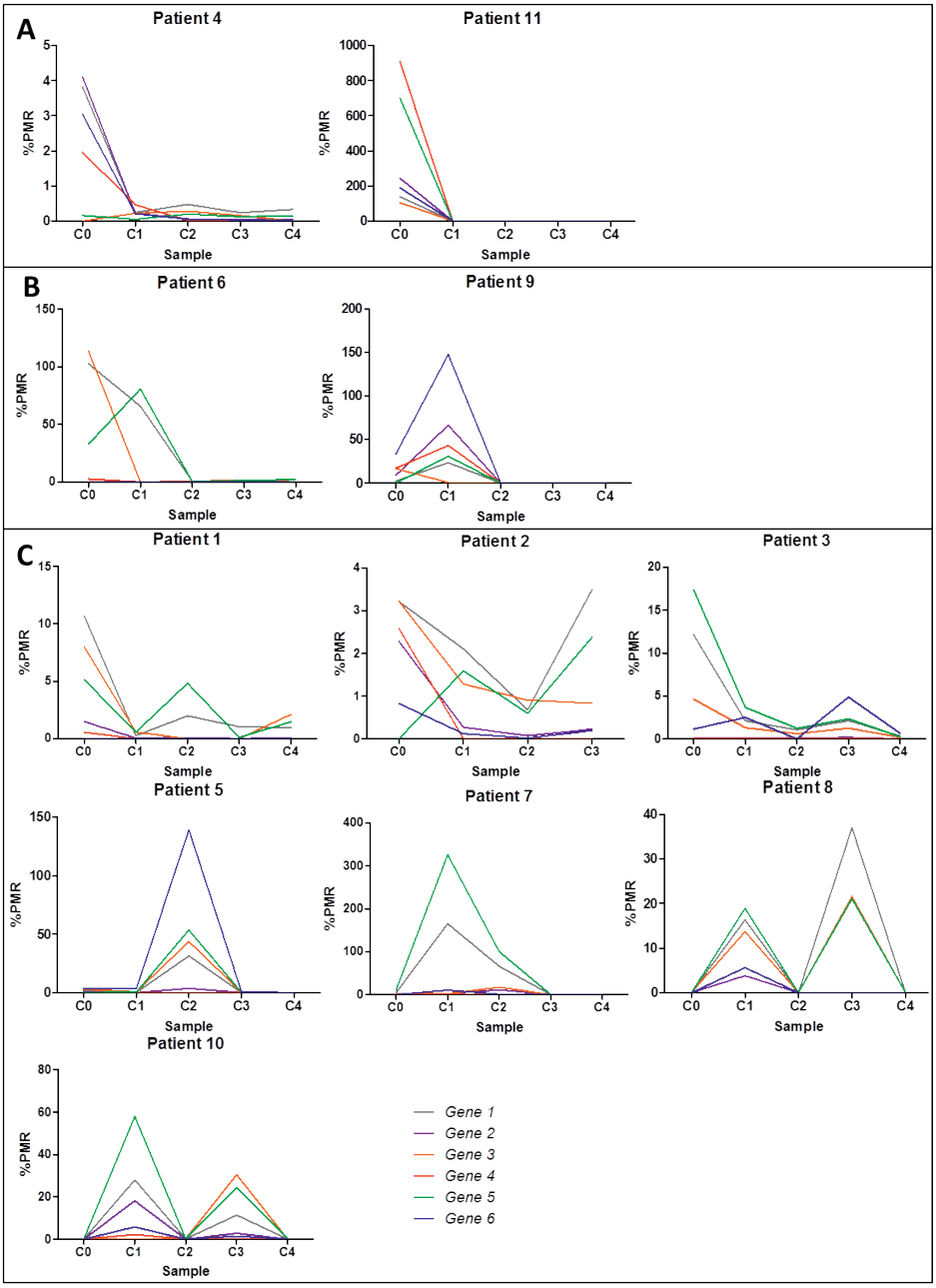
SUPPLEMENTARY MATERIAL

	RCC					
Sample	*Gene 1*	*Gene 2*	*Gene 3*	*Gene 4*	*Gene 5*	*Gene 6*
1T	U	U	U	U	U	M
2T	U	M	U	X	X	M
3T	U	U	U	M	M	U
4T	U	M	U	U	M	U
5T	U	M	M	M	M	U
6T	U	M	U	M	U	M
7T	M	M	M	M	M	U
8T	M	U	M	U	U	U
9T	M	M	U	M	U	U
10T	U	M	U	U	M	U
11T	U	U	M	U	U	M
12T	U	U	U	M	U	U
13T	U	M	U	M	U	U
14T	M	M	U	U	U	U
15T	U	M	U	U	U	U
16T	U	U	U	U	U	U
17T	U	U	M	U	U	M
18T	U	M	U	M	M	U
19T	U	U	U	U	U	U
20T	M	M	U	U	M	U
21T	U	U	U	M	U	U
22T	U	M	U	U	M	U
23T	M	U	M	U	M	U
24T	U	U	U	U	U	U
25T	U	M	U	M	M	M
26T	M	M	U	U	M	U
27T	M	U	U	U	M	U
28T	U	M	U	U	M	U
29T	M	M	M	U	U	M
30T	M	U	U	U	M	U
31T	U	M	M	U	U	U
32T	U	U	U	U	U	U
33T	M	M	M	U	M	M
34T	M	U	M	U	M	M
35T	U	U	M	U	U	M
36T	M	M	M	U	U	M
37T	U	M	M	U	U	U
38T	M	M	U	U	M	U
39T	M	M	U	M	U	M
40T	U	U	M	U	M	M
41T	U	M	U	M	U	U
42T	U	M	M	M	M	M
43T	U	U	M	U	U	M
44T	M	M	U	U	M	U
45T	U	M	U	U	U	M
46T	U	U	U	U	M	U
47T	U	U	U	U	U	M
48T	M	M	U	M	M	U
49T	U	U	M	U	M	M
50T	U	M	U	M	M	U
51T	U	M	M	M	M	M
52T	U	M	M	U	M	U
53T	M	U	M	U	M	U
54T	M	U	U	M	M	U

Supplementary figure 1. Visual presentation of qMSP results of *Gene 1, Gene 2, Gene 3, Gene 4, Gene 5* and *Gene 6* of the renal cell carcinoma (RCC) samples without matched adjacent normal (AN) samples in the hospital-based series, based on cutoff values after receiver operating characteristic (ROC) curve analysis using RCC and normal kidney (NK) as control samples.



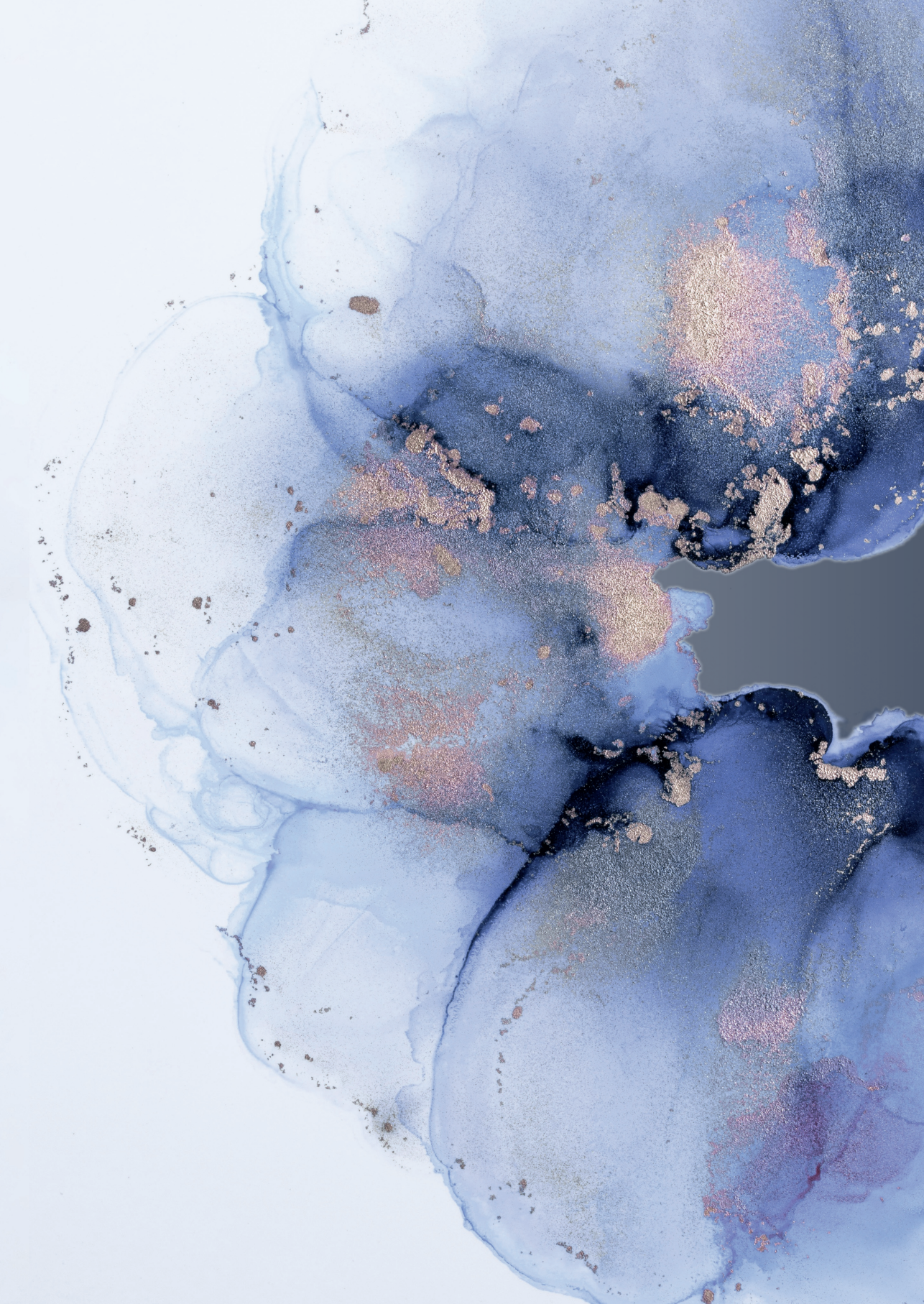
Supplementary figure 2. Visual presentation of the percentage of methylated reference (%PMR) of *Gene 1*, *Gene 2*, *Gene 3*, *Gene 4*, *Gene 5* and *Gene 6* in all sampled areas (C0-C4) per patient. A) Patients without methylation (<1% PMR) outside the malignant core (C0); B) Patients with methylation on transition from tumor to normal (C1), and no methylation outside the malignant tissue (C2-C4); C) Patients with methylation in- and outside the malignant tissue. Note: Y-axis values differ between patients.

Supplementary table 1. Percentage of methylated reference (%PMR) of *Gene 1*, *Gene 2*, *Gene 3*, *Gene 4*, *Gene 5* and *Gene 6* in all sampled areas (C0-C4) per patient.

	%PMR					
Sample	***Gene 1***	***Gene 2***	***Gene 3***	***Gene 4***	***Gene 5***	***Gene 6***
1.C0	10.71%	1.50%	8.01%	0.56%	5.15%	0.00%
1.C1	0.27%	0.06%	0.59%	0.00%	0.57%	0.00%
1.C2	1.99%	0.11%	0.00%	0.00%	4.83%	0.00%
1.C3	1.04%	0.03%	0.00%	0.00%	0.12%	0.04%
1.C4	0.98%	0.10%	2.11%	0.01%	1.47%	0.00%
2.C0	3.22%	2.28%	3.22%	2.59%	0.00%	0.83%
2.C1	2.11%	0.27%	1.29%	0.01%	1.59%	0.13%
2.C2	0.68%	0.08%	0.91%	0.00%	0.60%	0.03%
2.C3	3.49%	0.23%	0.84%	0.00%	2.38%	0.20%
2.C4	-	-	-	-	-	-
3.C0	12.13%	0.12%	4.65%	0.00%	17.35%	1.15%
3.C1	2.11%	0.17%	1.31%	0.01%	3.67%	2.51%
3.C2	1.05%	0.13%	0.62%	0.02%	1.24%	0.00%
3.C3	2.13%	0.19%	1.26%	0.00%	2.35%	4.87%
3.C4	0.34%	0.02%	0.21%	0.00%	0.26%	0.67%
4.C0	3.81%	4.10%	0.00%	1.96%	0.16%	3.03%
4.C1	0.25%	0.23%	0.22%	0.47%	0.05%	0.21%
4.C2	0.47%	0.06%	0.28%	0.01%	0.20%	0.05%
4.C3	0.24%	0.06%	0.16%	0.01%	0.12%	0.03%
4.C4	0.33%	0.05%	0.00%	0.02%	0.15%	0.03%
5.C0	2.07%	0.63%	2.91%	0.04%	0.22%	3.74%
5.C1	0.17%	0.10%	0.77%	0.00%	0.57%	3.37%
5.C2	31.44%	3.65%	43.68%	0.00%	53.53%	139.31%
5.C3	0.11%	0.00%	0.36%	0.00%	0.35%	0.96%
5.C4	0.01%	0.00%	0.00%	0.00%	0.03%	0.11%
6.C0	102.57%	0.21%	113.47%	2.62%	33.02%	0.01%
6.C1	65.69%	0.20%	0.00%	0.00%	80.81%	0.00%
6.C2	0.56%	0.04%	0.70%	0.00%	0.44%	0.03%
6.C3	1.00%	0.00%	1.71%	0.01%	1.19%	0.05%
6.C4	2.16%	0.24%	0.00%	0.00%	2.20%	0.07%
7.C0	4.64%	0.57%	2.00%	0.03%	10.69%	0.42%
7.C1	165.64%	2.96%	2.18%	0.00%	326.00%	10.39%
7.C2	66.19%	11.06%	17.59%	0.98%	100.33%	1.10%
7.C3	0.04%	0.02%	0.14%	0.00%	0.23%	0.01%
7.C4	0.23%	0.02%	0.00%	0.00%	0.21%	0.00%
8.C0	0.03%	0.01%	0.06%	0.00%	0.00%	0.02%
8.C1	16.36%	3.81%	13.72%	5.66%	18.95%	5.61%

Supplementary table 1. Percentage of methylated reference (%PMR) of *Gene 1*, *Gene 2*, *Gene 3*, *Gene 4*, *Gene 5* and *Gene 6* in all sampled areas (C0-C4) per patient. (continued)

	%PMR					
Sample	***Gene 1***	***Gene 2***	***Gene 3***	***Gene 4***	***Gene 5***	***Gene 6***
8.C2	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%
8.C3	37.05%	0.00%	21.66%	0.00%	21.03%	0.00%
8.C4	0.03%	0.00%	0.02%	0.00%	0.01%	0.00%
9.C0	2.26%	8.78%	16.54%	17.15%	0.11%	33.13%
9.C1	23.49%	66.47%	0.76%	43.07%	30.79%	147.54%
9.C2	0.03%	0.01%	0.16%	0.00%	0.12%	0.50%
9.C3	0.05%	0.00%	0.15%	0.00%	0.09%	0.60%
9.C4	0.03%	0.00%	0.10%	0.00%	0.06%	0.23%
10.C0	0.03%	0.02%	0.20%	0.00%	0.18%	0.00%
10.C1	27.95%	18.14%	0.00%	2.24%	57.98%	5.81%
10.C2	0.15%	0.03%	0.30%	0.00%	0.31%	0.03%
10.C3	11.38%	2.80%	30.47%	0.04%	24.34%	1.46%
10.C4	0.03%	0.01%	0.11%	0.00%	0.19%	0.00%
11.C0	138.63%	243.63%	104.93%	908.59%	701.45%	188.45%
11.C1	0.42%	0.03%	0.15%	0.14%	0.58%	0.03%
11.C2	0.23%	0.00%	0.18%	0.00%	0.23%	0.00%
11.C3	0.46%	0.07%	0.14%	0.01%	0.56%	0.05%
11.C4	0.50%	0.08%	0.47%	0.02%	0.48%	0.06%
Mean C0	25.46%	23.80%	23.27%	84.87%	69.85%	20.98%
Mean C1	27.68%	8.40%	1.91%	4.69%	47.41%	15.96%
Mean C2	9.35%	1.38%	5.86%	0.09%	14.71%	12.82%
Mean C3	5.18%	0.31%	5.17%	0.01%	4.80%	0.75%
Mean C4	0.46%	0.05%	0.30%	0.00%	0.51%	0.12%



CHAPTER 7

GENERAL DISCUSSION

GENERAL DISCUSSION

Renal cell carcinoma (RCC) is the most prevalent kidney cancer, responsible for 90-95% of all cases, and the incidence rates have been rising with 2% per year over the past two decades[1,2]. As early diagnosis often allows favorable prognosis and low disease burden, this has become a main focus in cancer research[3]. Due to the increased utilization of imaging procedures, nowadays many RCC patients are diagnosed after the coincidental finding of a renal mass; an incidentaloma[4]. Even though large renal masses are generally correctly diagnosed as malignant RCCs, only 50-70% of all small renal masses (SRM) (<4 cm in diameter) can be accurately categorized as benign or malignant based on imaging techniques[2,5-7]. The increasing number of detected SRMs and the challenge to diagnose these masses accurately based on imaging and biopsies, emphasize the need for improvement in diagnosing early-stage RCC. As a result, research on molecular markers for the early detection of RCC (including DNA methylation) gained interest over the past years. Nevertheless, no diagnostic RCC DNA methylation marker has reached clinical care yet. This lack of translation has previously also been described for cancer biomarkers in general; less than 1% of all published biomarkers are observed to reach clinical care. This indicates a substantial amount of research waste in the biomarker field[8-10]. This biomarker research waste has already been acknowledged and addressed by several researchers, and some suggestions to address it have been proposed, however this has not led to significant improvements in the field[11,12].

The current biomarker development process has previously been described as 'a tortuous series of linearly connected pipes' with several phases; biomarker discovery, validation, translation, evaluation and implementation[11]. However, a circular process with a central focus on clinical application may yield better results[11]. All parts of this biomarker process harbor their own issues and require specific attention in order for them to eventually contribute to the clinical application of the biomarker. In this thesis, we have summarized and discussed the main phases of biomarker development and the current challenges, and attempted to provide handles to overcome these issues.

PREDEFINING STUDY RATIONALE

The foundation of translational research is to build upon existing knowledge, thereby closing the gap from laboratory bench to patient bedside. According to Chalmers and Glasziou, new research should solely be executed when its research question cannot be adequately answered with existing evidence[13]. A predefined rationale for the clinical applicability and development of a biomarker is therefore crucial. Researchers must identify the clinical knowledge gaps they would like to fill (e.g. by performing systematic

reviews or meta-analyses) and determine which specific research is needed to advance the state of the research field[14]. To create an overview of the current evidence for all published diagnostic DNA methylation biomarkers for RCC, we systematically reviewed the available literature in **Chapter 2**. We concluded that, at this moment, none of the studied biomarkers are suitable for clinical use. Performing a meta-analysis rather than a systematic review was unfeasible, because a head-to-head comparison was impeded by the fact that for one single biomarker, a wide variety of different techniques, sample types, assays and genomic locations were used in the different studies. This lack of similarity of individual research methodology is a commonly observed phenomenon, which hinders comparison of studies[13]. An example of the need for a predefined study rationale is the fact that many candidate biomarkers for RCC are still solely validated using tissue samples. Although this research is relevant to gain knowledge on the biomarkers' biological background, a diagnostic tissue-based test will however have little additional clinical value, as pathological evaluation can already accurately diagnose RCC with available tissue. To improve current early detection of RCC, biomarkers in liquid biopsies are preferred, and biomarker studies should be designed considering liquid biopsies properties to serve the future purpose of the biomarker.

Before biomarkers could be considered for clinical implementation, they have to reach Level of Evidence (LoE) I or II, indicating consistent promising results from relevant randomized controlled trials or prospective cohort studies, preferably summarized in a systematic review or meta-analysis[15]. Of all studied biomarkers in **Chapter 2**, none exceeded Level of Evidence (LoE) III, which means these markers cannot be considered for use in clinical practice yet. Moreover, we concluded that after initial publication, subsequent studies often did not substantially add to the LoE. Up until now, no studies have been published to independently validate the markers studied in the initial studies of **Chapter 2**. Next to the fact that validation is often not attempted or published, some biomarkers in **Chapter 2** were already independently validated, but initial biomarker performance could not be replicated in subsequent studies. Despite unpromising results, the same biomarkers tend to be studied repeatedly, as also seen for DNA methylation markers for diagnosing colorectal cancer[16]. At some point, acknowledging that, with the current evidence, such markers cannot serve as biomarkers and are not worth further investigation, might be the right thing to do. Systematically identifying biomarkers through discovery experiments might raise the odds to successfully develop a biomarker. As demonstrated in **Chapter 2**, the majority of published diagnostic DNA methylation biomarkers for RCC were well-known tumor-suppressor genes, known to be involved in other cancer types. Even though their importance in cancer had been established, these genes were not specific to RCC, and could therefore not be appropriate for RCC diagnosis. Instead, we suggest to empirically identify potential biomarkers in a systematic manner, for example by performing identification experiments or by utilizing

publicly available databases such as The Cancer Genome Atlas (TCGA). To demonstrate, the biomarkers identified through discovery experiments or publicly available databases in **Chapter 2** showed superior performance compared to the biomarkers that were solely selected for further evaluation due to their involvement in other cancer types. In addition, considering the biology of a particular candidate biomarker can further indicate its potential, even though not all good biomarkers are strongly linked to biology.

PERFORMING BIOMARKER EXPERIMENTS

Assay design

Designing an appropriate assay to measure biomarkers is essential and seems obvious, as suboptimal assay design might result in either false positive or false negative results. In **Chapter 3**, we evaluated genomic locations and primer- and probe quality of diagnostic DNA methylation liquid biopsy biomarkers for colorectal cancer. The importance of genomic location in the evaluation of DNA methylation biomarkers was previously demonstrated by our group[9,17,18]. However, no specific guidelines for determining the optimal genomic location for diagnostic DNA methylation biomarkers have yet been described. Koch *et al.* previously proposed a method to use TCGA data to identify the location that best represents the most clinically relevant methylation sites[9]. In **Chapter 3**, we utilized this method to identify the optimal genomic locations of the studied DNA methylation markers, and compared that to the genomic locations used in the individual studies included in the article. The majority of studied assays did not include one of the genomic locations we considered optimal, which might have contributed to limited diagnostic performance. As TCGA data is based on Infinium 450K microarrays, this public source of information is limited to the covered CpGs and included probes do therefore not necessarily cover the most relevant CpGs. In order to overcome this, sequencing approaches are required to best study single CpGs and allow full coverage of all CpGs. This has not always been feasible for small research groups with limited funding, but recent advances in sequencing have decreased costs and increased accessibility. This increased accessibility of sequencing and availability of publicly available sequencing data provide opportunities to better identify the most clinically relevant genomic location for DNA methylation marker assays.

Next to genomic location, assay type and corresponding assay design are major factors in order to measure valid and robust biomarker performance. In **Chapter 3**, we assessed the quality of the primers and probes used in studies to measure DNA methylation biomarkers. Even though the most important criteria that allow discrimination of methylated from unmethylated DNA were covered in most assays, there is room for

improvement in primer- and probe design. A comprehensive overview of guidelines on how to design such assays, (**Chapter 3**), should gain more awareness.

Assay design and the choice of sample type are strongly related. The component of liquid biopsies most relevant to epigenetic biomarker research is cell-free DNA (cfDNA), including circulating tumor DNA (ctDNA). As a result of apoptosis and necrosis, cfDNA is released into the blood stream. Small cfDNA fragments of up to around 100 base pairs will pass the glomerular filtration barrier and also end up in urine[19]. The size of cfDNA is therefore crucial to consider when designing a biomarker assay for liquid biopsies, which adds another layer of complexity to adequately designing such an assay. Assays amplicon size should be as small as possible, as working around the limit may already miss part of the cfDNA.

Sample type and selection

Already in 2008, the prospective-specimen-collection, retrospective-blinded-evaluation (PRoBE) study design guidelines have been proposed by Pepe *et al*[20]. The PRoBE study design components regard clinical context and outcomes, criteria for measuring biomarker performance, the biomarker test itself, and the size of the study in context of selecting appropriate samples for the study[20]. Following these guidelines, biological specimens should be prospectively collected from a cohort representative of the target population for the clinical application of the biomarker. Subsequently, the biomarker is assayed in a blinded manner on randomly selected case and control samples within the study cohort[20].

Many liquid biopsies that could be used for biomarker research are collected during routine clinical care, but they are not commonly processed or stored for future research purposes. With increasing interest in liquid biopsies, efforts to establish large liquid biopsy biobanks have been made, and funding has become available. As all liquid biopsy types have their own difficulties in terms of collection, handling, processing and storage (**Chapter 4**)[21-23], it has however proven to be difficult to standardize protocols across biobanks and samples. Standardization is especially hard given the various future purposes a particular sample could have. General biobanks are often collected population-wide, and are therefore suitable for broad research questions. For more specific research questions or rare diseases, a more specialized biobanking approach is necessary to ensure that selected samples suit the research questions and that sufficient samples are available to ensure statistical power. Efforts have been made to split liquid biopsy samples and to process and store them in several ways to ensure samples are available to fit the majority of research questions. For example, PCR inhibitors are a big problem for PCR based approaches such as (q)MSP, as these can disrupt the PCR process at any step, affecting the amplification efficiency and thus resulting in a suboptimal assay. Removing these PCR inhibitors from the samples will yield more reliable and reproducible results,

but also add another processing step[24,25]. Avoiding DNA degradation using DNAse inhibitors can pose new problems, as these can act as a PCR inhibitors in case the wrong concentration is applied[25]. In addition, inter-biobank heterogeneity hinders pooling of samples and prevents the comparison of results[26]. Large liquid-biopsy biobanks could facilitate relatively fast validation on a large scale, thereby facilitating clinical translation of non-invasive biomarkers for early detection of cancer.

In addition to the availability of well-annotated and high-quality samples, selecting the appropriate samples to answer the central research question is crucial. For cancer diagnostic purposes, the sample set should consist of patients with the disease, and include a wide variety of baseline characteristics. More specifically, for early detection biomarkers, including sufficient low-stage cases is crucial. Although it is important for diagnostic biomarkers to be measurable in all stages and grades, the inclusion of a large number of late stage and/or high grade tumors may distort the performance of an early-detection biomarker, as these tumor characteristics are associated with invasion and metastasis[27]. This could be corrected for by performing specific, sufficiently powered, subgroup analyses to ensure that the biomarker performs throughout all disease states. For example, all RCC subtypes originate from distinct biological pathways, so analyzing all RCC subtypes in one group may conceal the diagnostic performance of a particular biomarker, as it may not act as a biomarker in all subgroups, stages and grades. On the other hand, a diagnostic test should preferably be able to accurately detect all RCC subtypes, which advocates for combining multiple biomarkers in a test.

In **Chapter 5**, we put the lessons learned into practice, and identified novel DNA methylation biomarkers for diagnosing RCC in urine. Even though the initial results were promising, it was difficult to replicate the results obtained in tissue samples in urine samples. Abovementioned issues such as amplicon size that passes the glomerular filtration (~100 base pairs) and DNA degradation are likely to have affected the results. It was impossible to design smaller qMSP amplicons due to technical primer and probe design limitations. Therefore, it would be worthwhile to explore more sensitive techniques for measuring DNA methylation such as droplet digital PCR or Discrimination of Rare EpiAlleles by Melt (DREAMing)[28,29]. In addition, the urine series used in **Chapter 5** was collected and stored without addition of a DNAse inhibitor, which means DNA degradation could not have been avoided. To overcome the challenge of finding a urine series that fits our requirements and ensure sufficient appropriately collected and stored samples for future RCC biomarker research, we have established a prospective urine biobank in the Maastricht University Medical Center. In this Maastricht Urine Biobank, urine of patients presenting with suspicion of a renal or bladder malignancy will be included. To allow pooling samples with existing series, but also have access to DNAse deprived samples, this urine is split into two parts after collection, and further processed with and without the addition of the DNAse inhibitor EDTA. In the future, this series of

prospectively collected urines could facilitate the development of novel biomarkers for early detection and prediction of disease progression of urological cancers.

Here, we have solely focused on DNA methylation biomarkers, selecting just a fraction of all potential biomarkers for RCC. Therefore, the addition of other types of biomarkers, such as gene expression or protein markers could be of interest to diagnose RCC in urine. For example, Kidney Injury Molecule 1 (KIM-1) is a protein whose expression is very low in healthy kidney cells, but becomes upregulated when renal tubule cells are damaged[30-33]. The ectodomain of KIM-1 is cleaved and released into the bloodstream, where it can subsequently pass the glomerular filtration barrier and end up in urine. KIM-1 has already been identified as a liquid-biopsy based diagnostic biomarker for RCC; KIM-1 was measured in urine of RCC patients and not in urine of patients with benign renal tumors and in healthy persons' urine[30-33]. Moreover, KIM-1 expression decreased after surgical resection of the RCC[30]. Even though KIM-1 is specific to kidney damage rather than kidney cancer, it may complement the DNA methylation markers identified in **Chapter 5**. A diagnostic test including multiple types of biomarkers that complement each other can increase the diagnostic value of a test, as evident by the successful implementation of e.g. Cologuard® as a screening test for colorectal cancer.

In addition to selecting appropriate patient samples, selecting appropriate control samples is perhaps equally or even more important; something that is often overlooked in biomarker research. In **Chapter 6**, we studied the biomarkers identified in **Chapter 5** in a patient population of matched RCC and normal cases, and healthy normal kidney tissue, to illustrate the impact of the choice of control samples. In this study we observed that the identified markers detect DNA methylation in the matched normal samples (taken from normally appearing kidney tissue adjacent to the RCC), whereas this was not observed in the healthy normal kidney tissue, which indicates a so-called field effect. It was previously suggested that this normally appearing adjacent tissue might be molecularly predisposed to become malignant, and therefore might not be an appropriate control[34]. In addition, DNA methylation observed in matched adjacent normal tissue did not always correspond to DNA methylation in the tumor. As a result of shifted cutoffs for test positivity, the biomarkers studied in **Chapter 6** seemed to perform better in terms of sensitivity when analyzing its performance using healthy normal kidney tissue compared to matched adjacent normal tissue. This emphasizes that not only selecting cases, but also selecting appropriate control samples needs specific consideration.

Practical performance of biomarker studies

Diagnostic tests (including biomarker tests) are most often defined based on their sensitivity and specificity; the ability to correctly classify patients with and without the disease[35]. Ideally, a diagnostic biomarker would have both a high sensitivity and specificity, which is however hard to achieve[36]. Sensitivity and specificity are inversely

related to each other, and it is therefore important to find the correct balance between the two. Receiver operating characteristic (ROC) curve analysis is often used to display the sensitivity and specificity across all observed test outcomes, and therefore represents this balance[35,37]. For continuous test outcomes, depending on the clinical setting the diagnostic biomarker will be used in, several methods to determine a cutoff value to separate diseased and healthy persons have previously been postulated. The likelihood ratio (LR) represents the likelihood of a positive test outcome to be a true positive; the higher the LR, the better the biomarker predicts the presence of the disease[35,38-40]. Therefore, we used this data-driven LR cutoff method to determine the individual biomarker cutoffs in **Chapter 5**, as this method is based on the biomarker outcome data, rather than a clinical application. Alternatively, depending on the clinical application of the biomarker, a cutoff value corresponding to a fixed sensitivity or specificity could be decided upon (a decision-driven cutoff method). For example in a population-wide screening setting, high specificities of 90-95% are required to avoid unnecessary follow-up diagnostic procedures, thereby confining both screening burden on the health care system and costs[41,42]. For the discrimination of benign from malignant renal masses, high specificity is most important to spare patients without RCC from surgery, thereby avoiding overtreatment[43]. After combining the individual biomarkers into a panel, we therefore defined that when ≥3 individual markers are methylated, the test is considered positive. This cutoff corresponded to both the highest LR and a high (98%) specificity (**Chapter 5**).

Reporting and validation

A lack of adequate reporting hampers reproducibility of the study and thereby independent validation of a biomarker. International initiatives, such as the EQUATOR network aim to improve the reliability and reproducibility of health-related literature by recommending reporting guidelines for specific study designs[44,45]. Partly due to such initiatives, The Standards for Reporting Diagnostic Accuracy (STARD) for diagnostic[46], and Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) for prognostic[47] biomarkers have gained attention over the years, and several journals have started to demand original studies to adhere to these guidelines[48]. As touched upon in **Chapter 2**, the STARD guidelines were not specifically developed for diagnostic (DNA methylation) biomarker studies, and do therefore not fully apply to and meet the need for these studies. This might partly explain why few studies adhere to these guidelines, and adapted versions of such guidelines for specific types of research might allow better reporting[48]. On the other hand, researchers might not be aware of the existence of such guidelines, or realize they should adhere to these.

The availability of adequately designed, executed and reported biomarker studies will allow reproducibility and thereby independent validation of the studied biomark-

ers. Independent validation is a broadly interpreted term; even though studying partially overlapping or extended study populations by (partly) the same researchers as the original study is a common practice, this is highly susceptible to overestimated results[11]. Proper independent validation means that unrelated or affiliated researchers from the original study validate results in an unrelated study population[11]. The majority of biomarker studies are never independently replicated at all, and most often the original researchers are involved when independent validation is performed[11]. When actual external independent validation in an independent study population is performed, the biomarker performance often cannot be replicated[11]. In addition, smaller studies tend to show promising biomarker performance, compared to larger studies. Often, these small studies with optimistic results are highly cited (citation bias), driving misleading interest and expectations of a particular biomarker[49]. Therefore, a crucial role in the biomarker process lies in meta-research, in the form of systematic reviews or meta-analyses, summarizing all available evidence[50].

TOWARDS A CHANGE IN BIOMARKER RESEARCH

Research has mainly been centered around novel discoveries. In biomarker research, this means that identifying novel biomarkers, rather than validating previously proposed biomarkers is most popular for several reasons. First of all, it is in a researchers' nature to aspire groundbreaking research, instead of repeating and validating others' work. The same holds true for funders; novelty of the proposed research is often highly appreciated and a main component in the decision to grant research funding. However, as replication of previous results is crucial for the development and clinical implementation of biomarkers, providing funding for validation studies could encourage researchers to validate existing knowledge within an academic setting. Forming large biomarker consortia that agree upon and receive funding for validation of each other's work, in addition to identifying novel biomarkers, could allow both novel discoveries and validation of previous work.

We have to start filling knowledge gaps, rather than creating even more of them. Many research groups have been advocating a change in multiple aspects of biomarker research for years, but little has happened so far. In order to convince the biomarker research community to change, a shift in mentality is necessary. Medical journals could have a crucial role in this shift by introducing mandatory adherence to specific (reporting) guidelines. After *the Lancet* published the series "Increasing value: reducing waste" in 2014, several journals have strengthened the journals requirements. For example, *the Lancet* now mandates a 'research into context' section in all submitted articles. Initiatives like the International Committee of Medical Journal Editors and the Institute of Medicine

have established reducing research waste as a pillar of their agendas[51]. In addition, if we would shift focus from mainly rewarding high impact factors and H-indexes (which are not reliable measures of research quality[52]) towards also rewarding valid and reproducible research, (especially junior) researchers might be more eager and have more time to perform research with the highest rigor, without publication pressure[13,52-54]. In order to accomplish this, universities, governments, journals and funding organizations should educate, facilitate and acknowledge appropriate research methodology, desiring quality over quantity. Not publishing (or not being able to publish) null findings, could cause other researchers to perform similar experiments over and over again[13,54]. Recently, several journals have normalized and encouraged submitting and publishing null results, and stimulated not to overestimate research results. Making study findings appear more favorable than the results justify, so-called "spin", is common practice. An estimated 80% of published papers in the field of biomedicine harbors at least one form of spin[55]. Spin may lead to unjustified optimism in the interpretation of study results and to false claims which will eventually lead to ineffective medical interventions[55,56]. To limit or even avoid spin, appropriate academic writing should become an even more important aspect of research-focused university education. Next to experimental study design, the magnitude of both methodological study design and reporting education by universities should increase drastically. For instance, research methodology courses should become mandatory curricular activities in all research-oriented study programs.

To change scientific mentality, researchers, peer reviewers, journals, editors, universities and funders should all work together. Reducing research waste starts with many considerations upfront, rather than merely in the lab. This should go hand-in-hand with a framework that forces researchers to think about rationale of the study upfront, and in which guidelines for every part of both the discovery and the validation studies are established. Rewarding research proposals that have considered the existing knowledge gaps (e.g. by performing systematic reviews or meta-analyses) upfront, and designed an appropriate approach to fill these gaps might reduce research waste in the end[14,57]. Funding agencies play a crucial role by educating their reviewers about appropriate research design. Adequately designed, executed and reported biomarker research is reproducible, can therefore be validated and subsequently translated into clinical care.

CONCLUSIONS

In conclusion, this thesis summarized the current status of diagnostic DNA methylation biomarkers for RCC, and identified several issues that could explain the lack of clinical translation of any of these. We analyzed and summarized several technical considerations of PCR-based assay design, and emphasized the importance of considering both assay- and sample type, especially in liquid biopsy approaches. Considering all of the above, we identified and validated novel candidate DNA methylation markers for non-invasively diagnosing RCC in urine. Last, we illustrated the impact of appropriate control samples on biomarker performance. With plenty of research advocating a change in biomarker research, and little impact so far, it is now time to constructively change the (biomarker) research environment and mentality. All parties involved should acknowledge this shared responsibility, act upon that responsibility and co-operate in creating a research environment in which quality is promoted and favored over quantity. Eventually, this will contribute to reducing research waste and increase the clinical translation rate of biomarkers.

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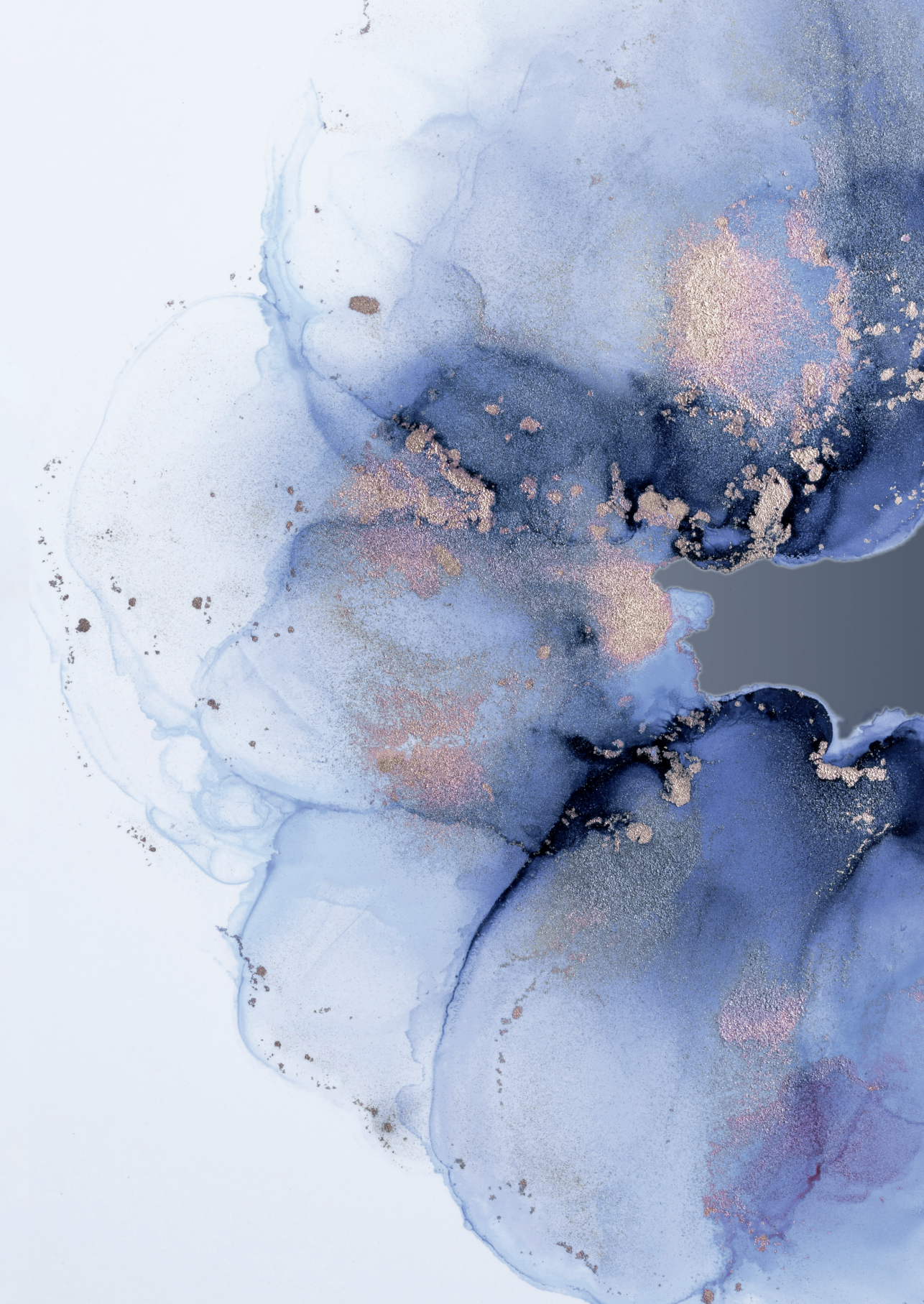

IMPACT PARAGRAPH

IMPACT PARAGRAPH

In 2020, approximately 430.000 new cases of kidney cancer were reported globally, representing 2.2% of all cancers diagnosed[1]. In that same year, an estimated 180.000 deaths could be attributed to kidney cancer[1,2]. Renal cell carcinoma (RCC) is the most prevalent type of kidney cancer, responsible for 90-95% of all cases, and the incidence rates have been rising with 2% per year over the past two decades[2,3]. The 5-year survival rate of RCC confined to the kidney is 93%, whereas the 5-year survival of distant metastasized RCC is decreased to 13%[4]. Globally, 3.3 million disability-adjusted life-years were estimated for RCC in 2017, and the disease burden has not decreased over the past 30 years despite advancements in RCC management[5,6]. With the increasing use of imaging techniques, a substantial proportion of patients are diagnosed after a coincidental finding during an unrelated procedure[7,8]. The rising incidence, partly caused by the increasing amount of incidental findings, together with the aging population will continue to increase both disease and economic burden because of RCC.

The increasing amount of incidentally diagnosed small renal masses (SRMs), and the fact that only 50-70% of these SRMs are correctly being diagnosed as benign or malignant based on medical imaging, goes hand-in-hand with overtreatment of many patients[2,4,7,8]. Whenever the nature of an SRM is uncertain, partial or radical nephrectomy can be decided upon. Patients will undergo burdensome surgeries to remove (part of) a kidney, never without risks of complications, which after pathological evaluation turn out to have been unnecessary in 25% of cases[9]. In addition, patients experience current diagnostic procedures like computed tomography (CT) scans and magnetic resonance imaging (MRI) scans as unpleasant because of scan duration, loud noise and space- and motion restrictions[10,11]. Next to that, these imaging procedures are considered time-consuming, not only because of the duration of the scan itself, but also because patients have to travel to and from the hospital[10]. From a hospital- and societal burden perspective, imaging techniques are also burdensome in terms of costs, time, effort and the need for specialized staff. Taken together, this emphasizes the importance of improving the current regime in accurately diagnosing RCC in early stages. To limit and potentially substitute part of burdening diagnostic imaging procedures, more accurately diagnose SRMs and avoid overtreatment, studies have been aiming to improve RCC diagnostics by focusing on non-invasive molecular markers in liquid biopsies. These patient-friendly diagnostic biomarkers for RCC could reduce both health- and economic burden not only by directly replacing costly and unpleasant imaging procedures, but also by avoiding unnecessary surgeries and subsequent medical follow-up.

Despite the great interest and research invested in molecular markers to replace invasive procedures, only <1% of all published biomarkers have reached clinical care[12,13]. This is in line with the fact that a large proportion of research investments being avoidably

wasted at this moment[14]. Unfortunately, the same holds true for DNA methylation biomarkers specifically, illustrated by the fact that not a single DNA methylation biomarker for diagnosing RCC has reached clinical care.

After initial publication, the vast majority of biomarkers never reach the stage of validation. In this thesis, we have addressed several reasons for the lack of success and validation and illustrated the effect of assay type, assay design, sample type, sample selection and control selection on biomarker development in the individual chapters of this thesis. In addition, we discussed to adequately address all these problems when performing a biomarker study. As argued in **Chapter 7**, validation studies are considered less innovative as compared to identification of novel biomarkers, and therefore also less of interest to scientific journals and funding agencies, leading to publication bias and an imbalance in funding streams[15]. Changing this scientific mentality is a shared responsibility of the academic community, including researchers, peer reviewers, journals, editors, universities and funding organizations. Filling knowledge gaps, rather than creating even more of them needs to be supported, facilitated and enforced by all parties. Accordingly, reducing research waste starts with many considerations upfront, rather than merely in the lab.

Suggestions provided in this thesis strive towards more efficient use of biomarker research funding, thereby not only reducing research waste, but also increasing the chance of developing clinically useful biomarkers for the accurate and early diagnosis of RCC. In that way, the results and perspectives from this thesis have both scientific and economic impact, as changing the biomarker research mentality will encourage decent and reproducible research, thereby facilitating validation of biomarkers, and subsequently allowing clinical utility of these biomarkers. The clinical applicability of these biomarkers will in turn lead to a reduction in health- and economic burden, by replacing and sparing imaging procedures, surgeries and medical follow-up.

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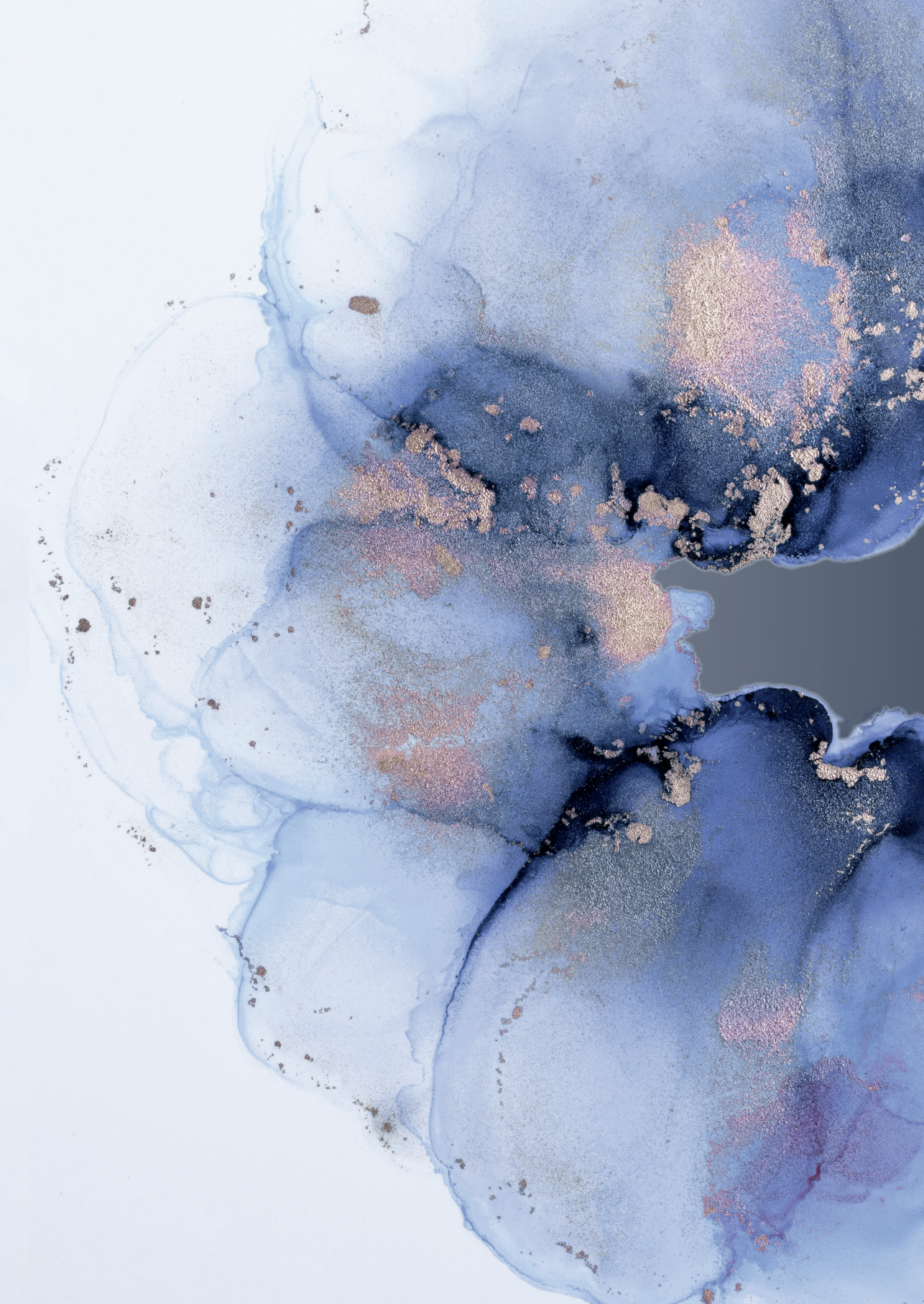


SUMMARY

SUMMARY

Renal cell carcinoma (RCC) is the most common type (90-95%) of kidney cancer, representing 2.2% of all diagnosed cancers globally. As early diagnosis is associated with favorable prognosis and low disease burden, it has become a main focus in cancer management. Because of the increasing use of cross-sectional imaging techniques, renal masses are also frequently detected coincidently during unrelated procedures; these masses are called incidentalomas and can be either benign or malignant. Partly due to this increase in incidental detection of renal masses, the incidence of RCC has been rising over the past decades. Large renal masses are often correctly diagnosed as being malignant RCCs, however only 50-70% of the small renal masses (SRM) <4 cm in diameter can be adequately diagnosed as being benign or malignant based on these imaging procedures. The increasing amount of detected SRMs and the challenge to diagnose these masses accurately based on imaging emphasizes the room for improvement in diagnosing early-stage RCC. Consequently, molecular markers for the early detection of RCC gained interest over the past years.

Despite the great interest in molecular markers, not a single molecular marker for diagnostic purposes has reached clinical care for RCC yet. Previously, it was described that less than 1% of all published biomarkers has successfully been implemented into clinical care, which also holds true for DNA methylation cancer biomarkers. The fact that the vast majority of research activities do not lead to clinical translation indicates that a large proportion of research investments are wasted.

The **aim** of this thesis was to identify and evaluate the utility of DNA methylation markers for the non-invasive early diagnosis of RCC. In addition, we evaluated reasons for the lack of clinical translation of diagnostic DNA methylation markers and discussed how to overcome these.

In **Chapter 2**, we systematically reviewed and summarized all literature regarding diagnostic DNA methylation biomarkers for RCC. We provided an overview of these biomarkers and summarized their current Level of Evidence (LoE). We found that 44 DNA methylation biomarkers and 11 multi-marker panels were described for diagnostic purposes in RCC; however, only 15 of these biomarkers were independently validated. None of the reported biomarkers exceeded LoE III, indicating that these biomarkers have limited clinical relevance at this moment. After initial publication, subsequent studies often do not considerably add to the LoE, indicating inadequate study design to facilitate validation. Additionally, we identified multiple issues that may hamper increasing LoE and thus clinical translation of these biomarkers, including methodological and technical heterogeneity between studies. Moreover, by evaluating The Standards for Reporting Diagnostic Accuracy criteria, we identified that study reproducibility and further development of these biomarkers is greatly hampered by inadequate reporting.

After identifying the issues described in **Chapter 2**, we further evaluated several technical considerations in PCR-based assay design for diagnostic DNA methylation markers in-depth in **Chapter 3**. As it was previously described that the exact genomic location of an assay could influence the biomarkers' diagnostic performance, we studied these genomic locations of all included biomarkers. We identified the optimal genomic locations of the studied biomarkers according to a previously proposed method utilizing TCGA data and compared that to the genomic locations used in the individual studies. The limited diagnostic performance of the included biomarkers might have partially been caused by the fact that the majority of the studied assays did not include an extracted optimal location. In addition, we assessed the primer and probe quality of all assays according to criteria based on both literature and expert opinion. Even though the most important criteria that allow discrimination of methylated from unmethylated DNA were covered in most assays, there is room for improvement in primer- and probe design. Therefore, we assembled a set of guidelines on how to adequately design PCR-based DNA methylation assays for diagnostic cancer biomarkers.

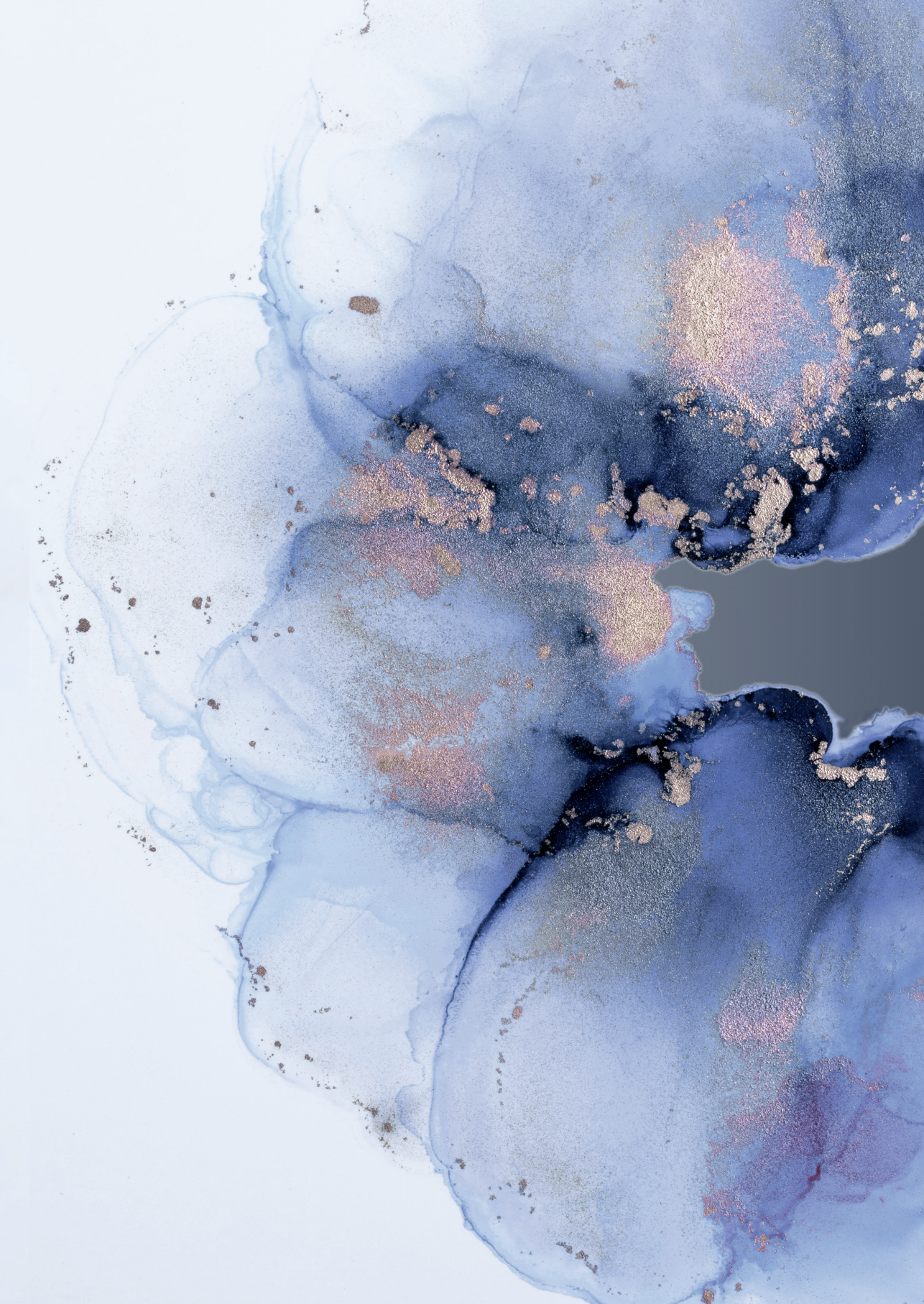
The availability of large study cohorts of appropriate samples, complemented by extensive and well-annotated clinical and pathological patient data is crucial for adequate and relatively fast validation of biomarkers. With increasing interest in liquid biopsies, efforts to establish large liquid biopsy biobanks are being made. Therefore, in **Chapter 4** we elaborated on considerations for establishing new biobanks, as well as for using existing biobanks, both in general and specific for certain specimen types, in order to develop optimal conditions for future validation of diagnostic cancer biomarkers in liquid biopsies.

Taking together all findings and recommendations made in **Chapter 2, 3,** and **4**, we used a novel *in silico* approach to identify diagnostic DNA methylation markers for RCC, and evaluated their diagnostic potential in both tissue and urine samples in **Chapter 5**. After evaluating nine DNA methylation markers in RCC and normal kidney tissue samples, the six most promising biomarkers were selected for further evaluation in urine samples. After evaluating these markers in RCC patients' and healthy persons urine, the final diagnostic model consisted of 4 biomarkers (*Gene 1, Gene 2, Gene 5* and *Gene 6*), sex and age, with an optimism-corrected AUC of 0.84. This DNA methylation marker panel for diagnosing RCC in urine showed to be a robust model in the sample set studied. Therefore, it serves as a promising starting point for further validation and extension by addition of other types of biomarkers, to further improve this model.

In addition to the previously discussed technical and methodological issues in biomarker research, the choice of appropriate control samples is an often overlooked factor. The fact that normally appearing tissues adjacent to the tumor might be molecularly predisposed to become malignant (called a field effect), emphasizes the importance of carefully selecting appropriate control tissues in biomarker studies. Nevertheless,

normally appearing tissue adjacent to the tumor is frequently used as control tissue in biomarker studies. In **Chapter 6**, we therefore evaluated DNA methylation alterations in ascending distances from the malignant RCCs, and demonstrated the impact of these alterations on biomarker identification and development. High methylation rates were present in RCC tissues (ranging from 34-56%) and matched adjacent normal (AN) tissues (ranging from 13-59%) as compared to NK (2% in all genes). All AN samples (100%) that were methylated for *Gene 4* corresponded to methylation in the RCC, whereas this holds true for only 0-55.6% of the other studied genes. A gradual decline in the percentage of methylated reference (%PMR) was found when moving from the tumor (41.4%) towards the area furthest away from the tumor (0.2%). Analyzing biomarker performance using AN rather than NK as control tissues yielded different cutoffs for test positivity. When using AN to determine the cutoff for test positivity, the sensitivity of the biomarker decreased due to presence of methylation in the AN tissue. The sensitivity increased when using NK tissue, as the absence of methylation in this tissue led to a different cutoff for test positivity. This indicates the importance of using appropriate control tissue when evaluating diagnostic performance of a biomarker. Although these different methods do not change the biomarkers' actual performance, using NK as control tissues might be most representative of the true biomarker performance.

In the general discussion of **Chapter 7**, the findings from this thesis were summarized and reflected upon. Along with discussing several reasons for the lack of clinical translation of DNA methylation biomarkers, we reflected on biomarker research in general. Several pitfalls have been identified and acknowledged in biomarkers research, and we here proposed recommendations to overcome these problems in DNA methylation based biomarkers. With plenty of research advocating a change in biomarker research in order to decrease research waste, and little impact so far, it is now time to constructively change the (biomarker) research environment and mentality. Co-operation of all involved parties to create a quality-based, rather than a quantity-based, research environment will eventually contribute to reducing research waste. Taken together, we have provided future perspectives and recommendations relevant to the development of clinically useful diagnostic DNA methylation biomarkers for cancer management.



ACKNOWLEDGEMENTS / DANKWOORD

DANK JULLIE ALLEMAAL!!!

Nouuuuuu daar issie dan!
Mijn (!!!) proefschrift
AAAAAAAAHHHHH!!!
!!!
!!!

Dit is het laatste dat ik van dit hele proefschrift schrijf, maar zeker ook wel een van de belangrijkste delen! Wat een ontzettend mooie, leuke en leerzame tijd is dit PhD traject geweest. Ik ga proberen m'n enthousiasme te beheersen en dit kort te houden…

De allergrootste dank is natuurlijk aan mijn promotieteam:

Prof. dr. van Engeland, beste Manon, ik vergeet nooit dat jij zei 'mensen die zeggen dat biomarker onderzoek saai is, doen het niet goed!'. Jouw aanstekelijke passie en enthousiasme zorgde er tijdens mijn eerste stage op de afdeling pathologie al voor dat ik ook als een blok voor biomarkers viel. Bedankt ook voor alle leuke en interessante 'behind the scenes' momenten tijdens mijn periode als PhD representative voor GROW. Daarnaast wil ik je natuurlijk enorm bedankt dat jij het vertrouwen in me hebt om mijn academische carrière voort te zetten als postdoc in jouw onderzoeksgroep!

Dr. Schouten, beste Leo, al vanaf het begin van mijn PhD project (en zelfs al daarvoor als stagiair) ben jij nauw betrokken geweest bij mijn onderzoek naar nierkanker, wat immers ook jouw specialisme is! In eerste instantie voornamelijk via het maandelijkse 'nieroverleg', maar gaandeweg ook steeds meer daarbuiten! Erg blij was ik dan ook toen jij officieel werd toegevoegd aan promotieteam! Bedankt voor de altijd enorm scherpe blik en opmerkingen, vooral in de details die niemand anders zag!

Dr. Smits, beste Kim, als iemand de meeste woorden in dit dankwoord verdiend dan ben jij het zeker weten! Waar moet ik beginnen… Toen jij terug kwam naar Pathologie, had je een leuk project, maar nog geen mensen om dit in te vullen. Toevallig zocht ik een afstudeerstage in de biomarkers en zo werden wij een match: team Kim en Kim! Naast de bestaande prognostische nierkanker onderzoekslijn wilde jij je ook gaan focussen op diagnostische biomarkers voor nierkanker. Gelukkig was men dit met je eens en kon jij een promovendus aannemen; ik! YESSSSS! Bedankt dat jij het vertrouwen in mij had om samen met jou aan deze nieuwe diagnostische nierkanker lijn te gaan werken, en kijk wat we in die 4 jaar al bereikt hebben! Naast dit mooie proefschrift en nog wat artikelen in het verschiet, hebben wij samen ook de Maastrichtse Urine Biobank opgezet, wat toch

ook wel wat voeten in de aarde had… ☺ Bedankt dat je mij zo vrij hebt gelaten, en voor alles dat ik van jou heb mogen leren, zowel over biomarkers en nierkanker als over hoe deze hele academische wereld in elkaar steekt! Ik kon (kan) altijd bij jou binnenlopen voor vragen, advies, frustraties of om gewoon even te kletsen. Bedankt voor de snelle feedback en het feit dat ik altijd op je kon terugvallen; ik heb altijd geweldig met jou samengewerkt, mede omdat jij net zo gestructureerd werkt als ik haha, heerlijk! Ik hoop in de loop van mijn carrière net zo'n goede mentor te worden, als dat jij altijd voor mij bent geweest en bent! Heel blij ben ik dan ook dat we tijdens mijn postdoc nog veel zullen samenwerken!

Naast dit promotieteam zijn er natuurlijk nog een hoop mensen die een bedankje verdienen, dus here we go!

Veerle, hoewel jij formeel geen deel uitmaakt van mijn promotieteam voelt het toch een beetje zo! Je was altijd geïnteresseerd in mijn onderzoek alsook daarbuiten. Bedankt voor jouw scherpe blik en opmerkingen tijdens presentaties en op manuscripten, maar ook voor alle wijze lessen over deze academische wereld! Jouw meest wijze les in de academie (kinderen krijgen tijdens je PhD) heb ik toch maar niet opgevolgd, sorry not sorry haha! Ik kan jouw heerlijke flapuit-heid altijd erg waarderen, ik kan m'n mond zelf natuurlijk ook niet zo goed houden… ☺ Thanks voor alles Veerle!

I would like to express my gratitude towards the assessment committee of this PhD thesis; Prof. dr. John Heesakkers, Prof. dr. Isabelle Boutron, Dr. Ludy Lutgens and Dr. Iris van Vlodrop, I greatly appreciate your effort to critically evaluate this PhD thesis. The same holds true for the members of the corona; thank you for taking the time to read my thesis and participate in my PhD defense!

Ook wil ik graag nog alle co-auteurs bedanken voor de waardevolle input, toevoegingen, scherpe opmerkingen en de prettige samenwerking! Mede dankzij jullie is dit mooie resultaat tot stand gekomen!! In het bijzonder wil ik hier ook het urologie team van het Radboudumc noemen, dankzij jullie konden wij een eerste validatie in urine uitvoeren!

Een groot deel van bovengenoemde co-auteurs brachten wij maandelijks samen in het 'nieroverleg', naast Manon, Leo, Kim en ik, waren ook Jeroen, Selena, Maureen, Joep, Tom, en Iryna hier vaste deelnemers. Deze overleggen waren altijd erg leuk, informeel en vooral ook erg leerzaam omdat we hier alle disciplines die aan nierkanker werken binnen het MUMC samenbrachten. Dank jullie allemaal voor de discussies en waardevolle bijdrage aan mijn onderzoek!

Dan is het natuurlijk tijd voor mijn directe collega's bij pathologie!

Allereerst mijn liefste collega's, die naast collega's ook echt vriendinnen zijn geworden ♥ Maartje, Nikkie, Simone, Jaleesa, Imke en Laura: Thanks voor alle koffietjes, uitjes, feestjes, weekendjes, en rages met de deur dicht!

Maart, girllllll jij bent toch wel mijn sister from another mister. Wat zijn wij hetzelfde, maar toch ook verschillend! Zelfde mindset, alleen ben jij vet chaotisch en ik vet georganiseerd ☺ Grote waardering heb ik voor hoe jij jouw geneeskunde studie en jouw PhD hebt gecombineerd, en kijk jou eens even shinen als AIOS interne geneeskunde, én bijna gepromoveerd op je 26e, trots op jou! Er was dan ook geen twijfel over mogelijk dat jij een van mijn paranimfen moest zijn, thanks girl for everything!

Nik, toch wel mijn meest die-hard koffie maatje. En look-a-like schijnt, gezien wij al een heel aantal keren voor elkaar werden aangezien haha. Thanks voor alle hulp in het lab, maar ook de technische discussies over onze qPCRs die weer eens niet werkten zoals we wilden haha… Heel leuk vind ik het ook dat wij tijdens mijn postdoc nog veel gaan samenwerken! En natuurlijk ook een dikke dankje dat je mijn paranimf wil zijn!

Simon, thanks dat jij altijd de kalmte bracht in ons kantoor. Jij was altijd de boei als Maartje en ik weer een verhitte discussie aan het voeren, of frustraties aan het uiten waren met de deur dicht. Wij waren vaak samen degene die van alles organiseerden en regelden binnen de groep, gezien wij hier nou eenmaal de organisatietalenten (lees; regelteven) waren ;) Ook besloten Maartje, jij en ik spontaan een instagram account te maken, en daarop ons leventje als jonge vrouwen in de wetenschap te delen, wat ons al heel wat leuks gebracht heeft. Ook delen wij een guilty pleasure, namelijk 'het foute uur', heerlijk no-shame mee blehren op kantoor en het lab! Succes met de afronding van jouw PhD traject, you got this girl!

Jalees, thanks voor al jouw hulp in het lab, en het feit dat jij ook altijd alles netjes en opgeruimd wilde hebben in dat lab! Samen hebben we heel wat gevloekt op alle rotzooi, en die vervolgens ook weggegooid haha. Ook ging ik jou tijdens jouw zwangerschap helpen met het grote BBMRI project, om dat allemaal snel en efficiënt af te ronden. Tijdens die tijd hebben we enorm gelachen, en ook weer gevloekt trouwens (vooral op de Symphony)… Hmmm ik zie een trend haha.

Im, voordat wij collega's werden op pathologie waren wij al soort van collega's bij moleculaire celbiologie, waar wij beiden als stagiair rondliepen. Het was dan ook heel leuk dat wij op pathologie op hetzelfde moment aan onze PhD startte! Nu dit traject voor jou

ook ten einde loopt, kan ik je alleen maar heel veel succes wensen met de afronding. Leuk dat ook jij nog een tijdje als postdoc blijft!!

Lau, jou ken ik natuurlijk al vanaf de bachelor, maar we zijn pas echt goede maatjes geworden tijdens de master, en al helemaal toen wij beiden op pathologie gingen afstuderen! Lachen, gieren, brullen in het studentenhok, en daarna had ook jij toch maar mooi een PhD gefixt op de afdeling. Klein uitstapje naar Lyon voor jou werd ook en weekendje Lyon voor ons allemaal. Na een dramatische heenreis waren we allemaal herenigd en hebben we de gehuild van het lachen (nu nog, de details kan ik hier helaas niet onthullen, sorry iedereen). Veel succes met de afronding van jouw PhD, en heeeeel veel succes met je opleiding tot KMBP'er!

Daarnaast wil ik natuurlijk mijn andere collega's ook allemaal hartelijk bedanken.

Selena, naast kantoorgenoot ook mede-nierkanker-PhD student bij Kim! Dankjewel voor alle gezellige gesprekken, wetenschappelijke discussies, delen van frustraties, en natuurlijk koffietjes. Voor jou is het einde ook alweer in zicht, veel succes met de afronding van jouw PhD!

Nathalie, de ondertussen niet meer weg te denken postdoc! Jij hoort ondertussen bij het interieur van de afdeling hehe. Bedankt voor jouw bijdrage aan mijn systematisch review, maar ook voor het altijd meedenken bij presentaties etc. In de laatste maanden was het voor mij ook erg fijn om nog iemand op de afdeling te hebben die dit hele promoveren traject vrij recent nog heeft gedaan! Thanks voor al jouw adviezen en voorbeelden!!

Kim W, ja, de derde Kim in dit verhaal. Of moet ik zeggen de eerste, jij bent er immers als het langst ☺. Jij hebt mij ontzettend veel geleerd in het lab, vooral op het gebied van DNA, bisulfiet, methylering, primers, probes en (q)PCRs. Jij was altijd bereid om mee te kijken, te brainstormen en te helpen waar nodig. Bedankt voor al jouw waardevolle hulp tijdens mijn hele traject!

Audrey, wat zouden we zonder jou toch moeten… Alle keren dat ik aan je bureau stond voor administratie, verlof en formulieren, alle vergaderruimtes die je voor me geregeld hebt. Jij bent echt een topper, maar echt! Je bent een erg waardevolle steunpilaar van onze onderzoeksgroep, besef je dat goed!

Mijn overige pathologie collega's Meike, Amy, Musa, Werend, Marion, Mat, Gregorio, Jacques en Lisa, maar ook zeker alle ex collega's Muriel, Sophie, Alexander, Glenn,

Zheng, Tim, Lindsay, Kathleen, Barry, Peter, Edith: Bedankt voor al jullie hulp, adviezen en een luisterend oor waar nodig! Bedankt ook aan alle andere collega's van de afdeling pathologie!

Johan, enorm bedankt voor al jouw expertise die je op mij hebt overgebracht. Ik heb ook van jou heel veel geleerd, vooral over primers, probes, qPCR en sequencing! Ook redde jij ons van slechte primers en probes wanneer ik vreemde resultaten kreeg, we zijn toen maar snel geswitcht!

Anja Dullens, heel erg bedankt dat jij je zo enorm hebt ingezet om de inclusie van onze Maastrichtse Urine Biobank zo soepel te laten verlopen! Ook een bedankje voor de dames bij het patiëntenzorgsecretariaat, voor het opvangen van deze urines!

De dames bij GROW; Brigitte, Judith, Christel en Lina, bedankt voor al jullie hulp en enthousiasme! Vooral ook tijdens mijn periode als PhD representative!

Familie van Koeverden, bedankt voor het mogelijk maken van dit project. Uw enthousiasme, halfjaarlijkse bezoekjes en oprechte interesse in ons project zijn een grote drijfveer geweest. Mede dankzij u is dit mooie proefschrift tot stand gekomen, hartelijk bedankt voor uw vertrouwen!

Kankeronderzoekfonds Limburg, in het bijzonder Ellen, Ineke, Marcel en Philip. Bedankt voor alles! Onder andere jullie interesse, enthousiasme en waardering hebben er voor gezorgd dat dit mooie project vorm heeft gekregen. Daarnaast waren alle extra activiteiten altijd erg leuk om aan deel te nemen!

Last but not least, kan ik natuurlijk al mijn vrienden en familie niet vergeten te bedanken. Als eerste ElAnPiKi: Elmie, Anne en Pien; thanks voor jullie luisterend oor en interesse in mijn onderzoek de afgelopen jaren. Heerlijk om samen met jullie te lunchen, spelletjes te doen, te zuipen, of simpelweg te chillen in trainingsbroek op de bank onder het genot van een borrelplank en een drankje! Altijd een hele fijne manier om af te schakelen!

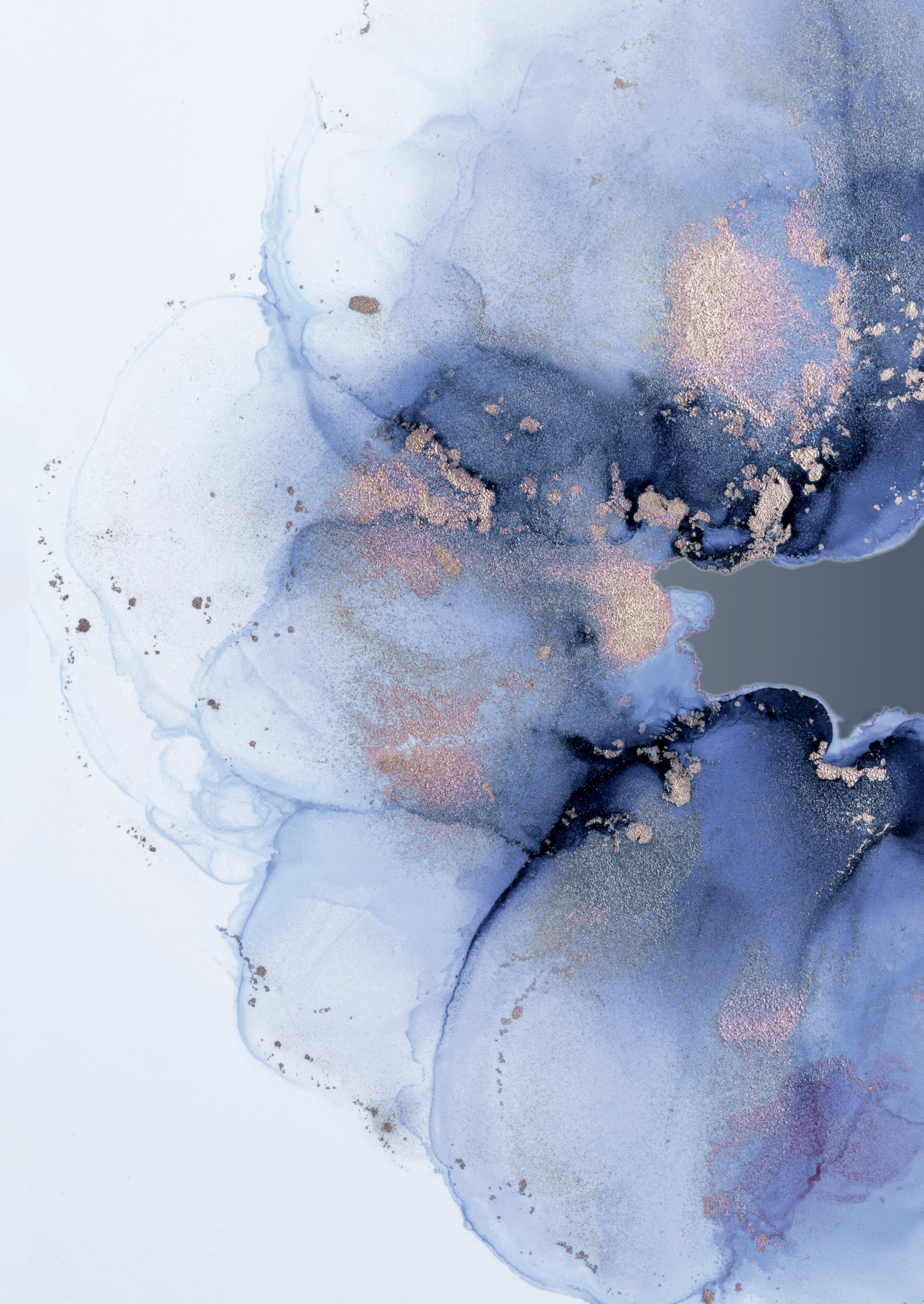
Ook de vriendengroep uit Meijel; dank jullie allemaal voor jullie interesse in mijn onderzoek, en ook jullie dankjewel om als uitlaatklep te dienen in het weekend (jawel ook weer onder het genot van een drankje, zijn jullie vrij goed in ☺)!

Harrie, Cobie, Marit, Renie; lieve schoonfamilie, voor jullie was dit toch een herkenbaar proces omdat Marit mij voor ging! Heel erg bedankt voor jullie support de afgelopen jaren!

Pap en mam, volgens mij hadden jullie niet echt een goed idee van wat ik nou eigenlijk allemaal aan het doen was hier in Maastricht. Nou, dit dus. Thanks dat jullie mij nooit hebben tegen gehouden, en me altijd hebben laten doen wat ik per se wilde (ook al vertelde ze me op school wat anders ☺).
En inderdaad pap, klaage bàt neet!

Linda & Jules, geschikte datums vinden waarop we konden afspreken bleek nogal eens lastig met zowel een piloot als een militair om rekening mee te houden… Gelukkig is het toch regelmatig gelukt, thanks voor de grappen en grollen, de hotpot-en-speciaalbier avondjes en jullie interesse in mijn onderzoek.

Miel, sjnoepfel, jij bent de beste uitlaatklep die ik me kan wensen. Jouw werk als militair en alles wat daarbij komt kijken is echt niet altijd gemakkelijk.. Op de momenten dat je gewoon thuis bent genieten we dan ook extra hard. Vrijdagavond is dan ook vaak onze avond waarop we samen in trainingsbroek op de bank serie kijken, onder het genot van (speciaal)bier en lekkere kaas. Vaak wordt na het nodige speciaalbier de serie verruild voor keiharde muziek en meezingen, meestal van Queen, maar ook Rammstein, Frank Sinatra en Vieze Jack komen regelmatig voorbij… Heerlijk! Deze momenten hebben er echt voor gezorgd dat ik kon afschakelen, gelukken delen we deze liefde voor lekker bier, kaas (überhaupt lekker eten trouwens) en goeie (of slechte hehe) muziek. Thanks dat je probeert mijn werk wereldje te snappen, dat probeer ik ook bij die van jou (lukt niet altijd even goed ☺). Kussie ♥



ABOUT THE AUTHOR

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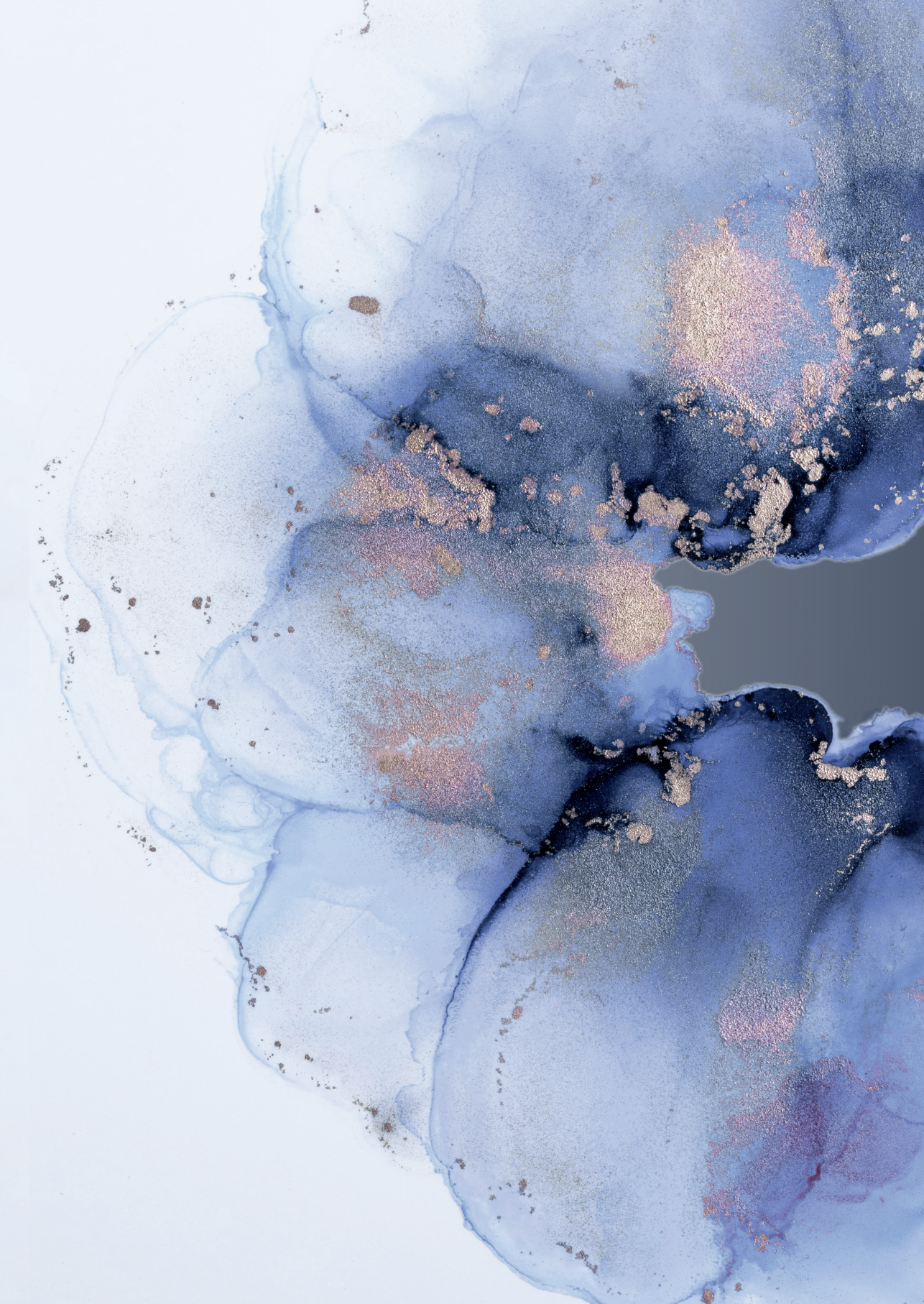
Kim Lommen was born on February 23rd, 1994 in Helden, The Netherlands. She completed secondary school (VWO) following the profiles Nature & Health and Nature & Technology at the Bouwens van der Boijecollege in Panningen in 2013. In September 2013, she started to study Health Sciences at Maastricht University, and specialized in Biology and Health. After completing her bachelor internship at the department of Molecular Cell Biology (supervised by Dr. Ton Hopman), she obtained her Bachelor of Science degree in July 2016. Subsequently in September 2016, she started to study the master in Biomedical Sciences (specialized in Oncology and Developmental Biology) at the Transnational University of Limburg (collaboration of Maastricht University and Hasselt University). During her first year, she performed an internship within the Department of Pathology of the Maastricht University Medical Center (MUMC+) under supervision of Dr. Muriel Draht. The second year involved writing a research proposal, and the subsequent execution of this research proposal in a 9-month internship. This internship was performed under supervision of Dr. Kim Smits at the Departments of Pathology and Medical Oncology of the MUMC+, and Kim obtained her Master of Science degree in July 2018.

After obtaining her master's degree, Kim continued her work as a PhD student in the research group of Dr. Kim Smits and Prof. Dr. Manon van Engeland, where she studied diagnostic DNA methylation biomarkers for renal cell carcinoma, and biomarker methodology in general. After finishing her PhD, Kim will continue pursuing her scientific career as a postdoctoral researcher in the research group of Prof. Dr. Manon van Engeland and Dr. Kim Smits, studying prognostic biomarkers for melanoma.



LIST OF PUBLICATIONS

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Lommen, K., Odeh, S., de Theije, C.C., Smits, K.M. Biobanking in molecular biomarker research for the early detection of cancer. *Cancers* 2020, *12*, 776.

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Lommen, K., Samarska I.V., zur Hausen, A., Marcelissen, T., van Roermund J.G., van der Meer, J., Aarts M.J., Kerkhofs, T., Schouten, L.J., van Engeland, M., Smits, K.M. Exploring a DNA methylation field effect in renal cell carcinoma and its implications for biomarker research. *Manuscript in preparation.*