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# **TOWARD A NEW ERA OF CANCER DETECTION**

**PATIENT-FRIENDLY  
SOLUTIONS**

**BIRGIT M.M. WEVER**



# **TOWARD A NEW ERA OF CANCER DETECTION**

**PATIENT-FRIENDLY  
SOLUTIONS**

**BIRGIT M.M. WEVER**

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VRIJE UNIVERSITEIT

**TOWARD A NEW ERA OF CANCER DETECTION:  
PATIENT-FRIENDLY SOLUTIONS**

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'What is now proved was once, only imagined'

*William Blake*



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# CHAPTER 1

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## GENERAL INTRODUCTION

**GENERAL INTRODUCTION**

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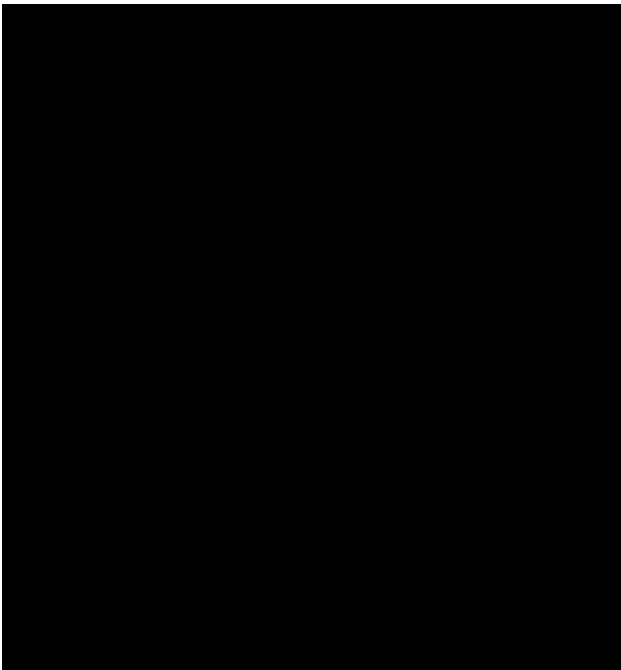
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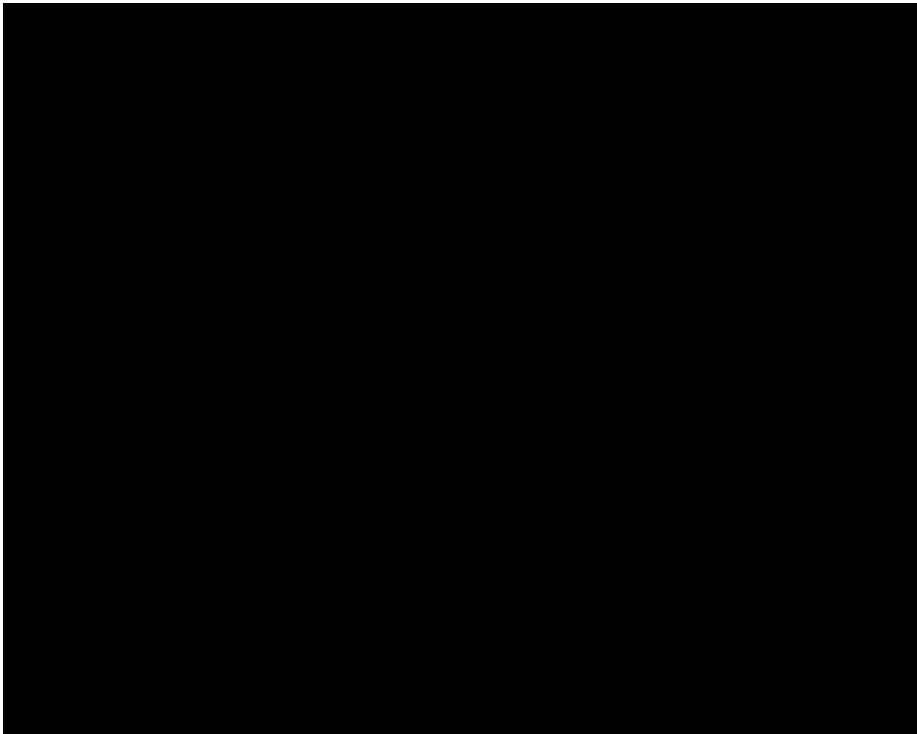
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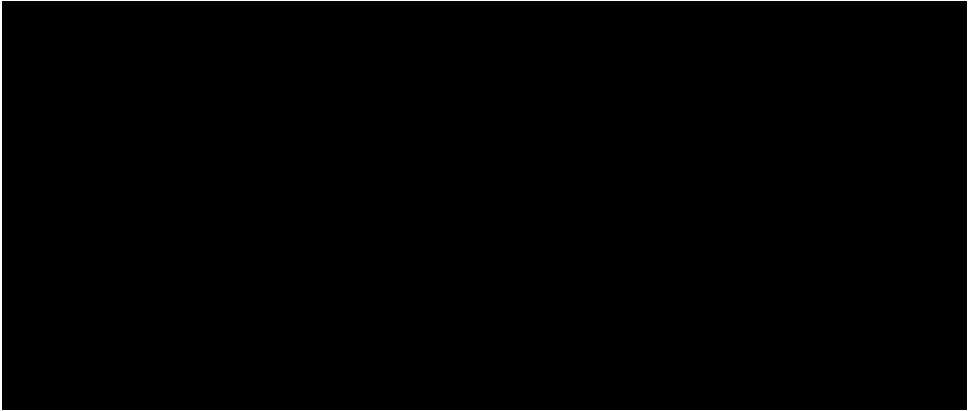
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1.6 Thesis rationale and outline

The overall objective of this thesis is to develop patient-friendly cancer detection methods to advance cancer diagnostics by focusing on DNA methylation analysis in urine for the detection of endometrial, ovarian, and lung cancer. For endometrial and ovarian cancer, also alternative patient-friendly sampling methods are assessed, including self-collected cervicovaginal samples and clinician-taken cervical scrapes (Figure 5).

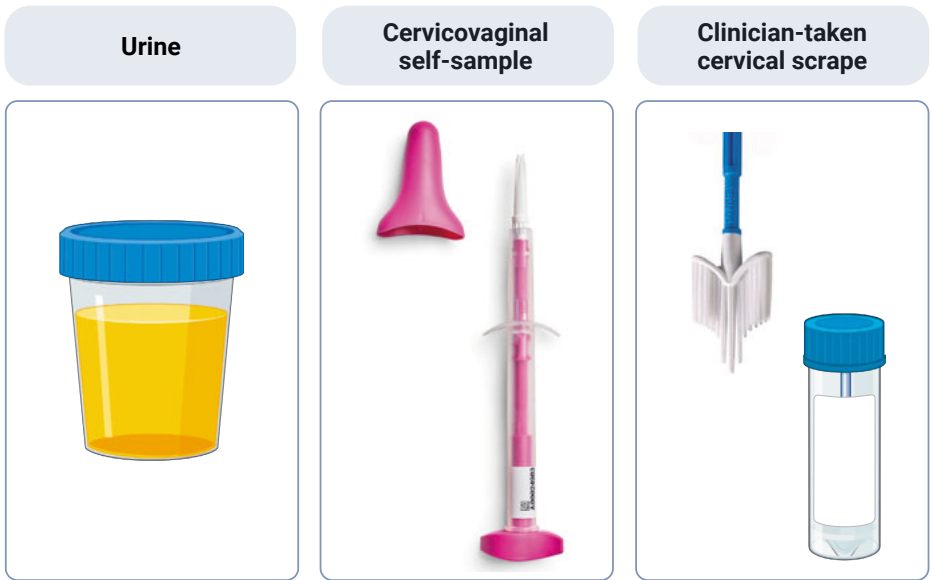


Figure 5: Patient-friendly sample types investigated in this thesis: urine, cervicovaginal self-samples, and clinician-taken cervical scrapes. Created with BioRender.com.

In **Part 1** of this thesis, the potential of detecting endometrial and ovarian cancer in patient-friendly samples is described. In **Chapter 2**, the feasibility of endometrial cancer detection in urine is evaluated by testing three methylation markers in different urine fractions. In **Chapter 3**, a systematic review of the literature is performed to select which methylation markers for endometrial cancer detection in patient-friendly sample types deserve further development. In **Chapter 4**, nine methylation markers, retrieved from Chapters 2 and 3, are tested for endometrial cancer detection in paired urine, cervicovaginal self-samples, and clinician-taken cervical scrapes to comprehensively determine and compare their performance in different patient-friendly sample types. In **Chapter 5**, the use of patient-friendly samples for ovarian cancer detection is explored using different molecular analyses. Nine methylation markers are analyzed in urine, cervicovaginal self-samples, and clinician-taken cervical scrapes. Additionally, copy number aberrations and cfDNA fragmentation patterns are analyzed in the urine of ovarian cancer patients.

In **Part 2** of this thesis, the applicability of urine for the detection of non-small cell lung cancer (NSCLC) is evaluated. In **Chapter 6**, three methylation markers are tested to explore the use of urine for the detection of non-metastatic primary and recurrent NSCLC. For successful clinical implementation, it is essential to explore the day-to-day and within-days variation in urine cfDNA measurements to fully comprehend its potential as a diagnostic tool. Therefore, in **Chapter 7**, the dynamics of methylated cfDNA in patients with advanced stage NSCLC are investigated to determine whether a preferred collection time and frequency exists.

The outcomes of this thesis contribute to a new era of patient-friendly solutions for cancer detection that can be widely implemented in future clinical practice.

Category	Sub-category	Percentage
U.S. should take action	Total	80%
	U.S.-born	85%
U.S. should not take action	Total	19%
	U.S.-born	15%

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# PART 1

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## ENDOMETRIAL AND OVARIAN CANCER DETECTION IN PATIENT-FRIENDLY SAMPLES



# CHAPTER 2

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## NON-INVASIVE DETECTION OF ENDOMETRIAL CANCER BY DNA METHYLATION ANALYSIS IN URINE

Published as:  
van den Helder, R., Wever, B.M.M., van Trommel, N.E., van Splunter, A.P., Bleeker, M.C.G., Steenbergen, R.D.M. (2020). Non-Invasive Detection of Endometrial Cancer by Methylation Analysis in Urine. *Clinical Epigenetics*. 12, 1, p. 165.

## ABSTRACT

### Background

The incidence of endometrial cancer is rising, and current diagnostics often require invasive biopsy procedures. Urine may offer an alternative sample type, which is easily accessible and allows repetitive self-sampling at home. Here, we set out to investigate the feasibility of endometrial cancer detection in urine using DNA methylation analysis.

### Results

Urine samples of endometrial cancer patients ( $n = 42$ ) and healthy controls ( $n = 46$ ) were separated into three fractions (full void urine, urine sediment, and urine supernatant) and tested for three DNA methylation markers (*GHSR*, *SST*, *ZIC1*). Strong to very strong correlations ( $r = 0.77 - 0.92$ ) were found amongst the different urine fractions. All DNA methylation markers showed increased methylation levels in patients as compared to controls, in all urine fractions. The highest diagnostic potential for endometrial cancer detection in urine was found in full void urine, with area under the receiver operating characteristic curve values ranging from 0.86 to 0.95.

### Conclusions

This feasibility study demonstrates, for the first time, that DNA methylation analysis in urine could provide a non-invasive alternative for the detection of endometrial cancer. Further investigation is warranted to validate its clinical usefulness. Potential applications of this diagnostic approach include the screening of asymptomatic women, triaging women with postmenopausal bleeding symptoms, and monitoring women with increased endometrial cancer risk.

## BACKGROUND

Endometrial cancer (EC) is the most common gynecological cancer in developed countries and the sixth most common cancer worldwide (1). Its incidence is rising globally (2) with over 380,000 new cases and 89,929 deaths reported in 2018 (3). The increasing incidence of EC is partly attributable to the rise in the prevalence of risk factors associated with EC development, like obesity (4, 5).

Despite the rising incidence of EC and proven value of early diagnosis, no screening program for EC exists (6, 7). In addition, if EC is suspected, invasive biopsy procedures remain necessary in routine clinical practice to detect EC in symptomatic women. Besides, the opportunity to detect EC in asymptomatic women by cytological evaluation of cervical scrapes during cervical cancer screening programs will be missed by the transition toward a primary high-risk human papillomavirus screening approach in many countries.

Hence, there is a need to detect EC using less invasive sampling methods, combined with the analysis of cancer-specific markers (6). One of the emerging biomarkers for early cancer detection is DNA methylation, which involves the addition of a methyl group to a cytosine-guanine dinucleotide (CpG). Altered DNA methylation is a common epigenetic event that occurs during the early stages of carcinogenesis of many cancer types, including EC, and has been linked to gene silencing of tumor suppressor genes. Testing for elevated DNA methylation levels of specific genes is promising in early cancer detection (8).

Previous studies have shown that aberrant EC-specific DNA methylation signatures can be measured in various minimally-invasive sample types, including cervical scrapes (9-12), endometrial brushes (13), vaginal swabs (14, 15) and vaginal tampons (16, 17). The ability to detect EC in cervicovaginal samples implicates shedding of endometrial cells and cell fragments into the lower genital tract, and, potentially, also into the urine. Apart from cellular tumor DNA, tumor-derived DNA can be released into the bloodstream as cell free DNA (cfDNA) and pass to the urine by filtration through transrenal excretion (18, 19). The suitability of EC detection in urine has been supported by the presence of EC-specific micro-RNAs in urine (20, 21). The measurement of DNA methylation markers in urine, has been proven useful for the detection of cervical cancer (22, 23), as well as other cancers, including bladder (24-27), lung (28), and prostate cancer (29-32). However, to the best of our knowledge, no such approach has been investigated for the detection of EC.

The majority of DNA methylation markers that hold promise for EC detection have been derived from studies on EC, but also markers developed for cervical cancer detection showed potential diagnostic relevance for EC detection (33). We considered the markers *GHSR*, *SST* and *ZIC1* as interesting candidates to evaluate the detection of EC in the urine by DNA methylation marker testing, based on our previous studies on urinary methylation markers and their diagnostic marker potential for different cancer types (22, 23, 25, 34).

This study investigates the feasibility of DNA methylation analysis in different urine fractions for the detection of EC. DNA methylation of genes *GHSR*, *SST*, and *ZIC1* was analyzed in full void urine, urine sediment and urine supernatant samples of women with various types, histological grades and stages of EC and a healthy control group to determine the most optimal urine fraction and applicability of these genes for the detection of EC in the urine.

## METHODS

### Study population

A total of 88 urine samples were used in this study, consecutively collected from women with EC (n = 42) and healthy female controls (n = 46). EC patients were recruited within the SOLUTION1 study which involved the collection of cervicovaginal and urine samples of women diagnosed with gynecological cancer. Samples from healthy female controls were collected through the Urine Controls (URIC) Biobank. Informed consent was acquired from each participating individual before urine collection. Ethical approval was obtained by the Medical Ethical Committee of the VU University Medical Center for both the SOLUTION1 study (no 2016.213) and the use of the URIC biobank (no 2017.112).

Enrolled patients included women with histologically proven EC of any stage before receiving primary treatment. The revised American Joint Committee on Cancer/ Union for International Cancer Control Tumor-Node-Metastasis (TNM) Cancer Staging classification was used to determine tumor stage (35). Other patient characteristics that were documented included age, histological grade and EC type. Control urine samples were retrieved from the URIC biobank (n = 36), including healthy volunteers without any cancer diagnosis in the past 15 years, and from our previously published healthy control cohort (n = 10) (22).

### Urine collection and processing

Both patients and controls collected urine at home in three 30 mL collection tubes, containing 2 mL 0.6 M Ethylenediaminetetraacetic acid (EDTA) as a preservative agent (final concentration of 40 mM). Urine samples were shipped to the Pathology department of Amsterdam UMC, VU University Medical Center, by regular mail and processed within 24 – 72 hours after collection. 15 mL of full void urine was centrifuged at 3000 x g for 15 minutes to separate the urine sample into two fractions: the sediment and the supernatant. The urine sediment, urine supernatant, and remaining full void urine were stored at -20 °C. This collection and storage protocol has previously been validated for reliable DNA methylation detection in urine (36).

### DNA extraction and bisulfite modification

DNA was extracted and modified from full void urine, urine sediment, and urine supernatant as described before (22, 23). Briefly, DNA was isolated from full void urine (15 mL) and urine supernatant (15 mL) using the Quick DNA urine kit (Zymo Research, Irvine, CA, US). DNA was isolated from the urine sediment (15 mL original volume) using the DNA mini and blood mini kit (Qiagen, Hilden, Germany). DNA concentration and DNA quality were measured using a NanoDrop 1000 (ThermoFisher Scientific, Waltham, MA, US). Purified DNA was subjected to bisulfite conversion using the EZ DNA Methylation Kit (Zymo Research). All procedures were carried out according to the manufacturer's guidelines.

### DNA methylation analysis by quantitative methylation-specific PCR (qMSP)

DNA methylation analysis of *GHSR*, *SST*, and *ZIC1* was executed by multiplex qMSP, including *ACTB*, using 50 ng modified DNA input on an ABI-7500 real-time PCR-system (Applied Biosystems, Waltham, MA, US), as described previously (22, 37). *ACTB* was used as a reference gene for quantification and quality assessment. Sample quality was ensured by excluding samples with a quantification cycle (Cq) value exceeding 32 from methylation analysis.

### Data analysis

The DNA quality of each urine fraction of both patients and controls, of which all paired fractions were available, was examined by comparing their median *ACTB* Cq values using the Friedman Test, followed by the non-parametric Wilcoxon signed-rank test. In addition, the number of samples tested invalid (*i.e.* excluded due to an *ACTB* Cq value  $\geq 32$ ) was documented per urine fraction.

The correlation between Cq ratios of each DNA methylation marker between paired urine fractions of both patients and controls was assessed using Spearman's rank

correlation. Correlation coefficient  $r$  was defined as moderate ( $r = 0.40 - 0.59$ ), strong ( $r = 0.60 - 0.79$ ), or very strong ( $r = 0.80 - 1.00$ ).

Differences in DNA methylation levels amongst each urine fraction (*i.e.* full void urine, urine sediment, and urine supernatant), and between patients and controls were evaluated by comparing the log2-transformed Cq ratios. Cq ratios were computed by normalizing the methylation levels of all markers according to the reference gene *ACTB* using the comparative Cq method ( $2^{-\Delta Cq} \times 100$ ). Methylation levels of all urine fractions of both patients and controls were displayed in boxplots and tested for statistical significance using the non-parametric Mann-Whitney U test.

The diagnostic potential of *GHSR*, *SST*, and *ZIC1* for distinguishing patients and controls were evaluated by computing receiver operating characteristic (ROC) curves of all methylation markers, and results were quantified by the area under the curve (AUC).

Statistical analysis was performed in IBM SPSS 26, and graphs were created using GraphPad Prism 8.

## RESULTS

### Patient characteristics

A total of 42 EC patients and 46 healthy controls were enrolled in this study. An overview of clinical characteristics is displayed in Table 1.

### DNA quality of urine fractions

To select the most suitable urine fraction for DNA methylation analysis, the quality of DNA isolated from paired full void urine, urine sediment, and urine supernatant samples was first assessed by comparing the quantification cycle (Cq) values of the reference gene *ACTB* (Table 2). While the Cq values of *ACTB* were nearly identical in full void urine samples (24.7) and urine sediments (24.8), they were significantly higher ( $p < 0.001$ ) in urine supernatant samples (26.1). Of note, amongst the different fractions, none of the samples tested invalid in urine sediment, as compared to two in both full void urine and urine supernatant samples.

**Table 1:** Patient characteristics.

Healthy controls			
n		46	
Age: median		56	
Age: min - max		45 - 82	
Endometrial cancer cases			
n		42	
Age: median		66	
Age: min - max		40 – 86	
Histology	n	%	
Endometrioid	23	54.8	
<i>Grade 1</i>	8		
<i>Grade 2</i>	7		
<i>Grade 3</i>	8		
Serous	11	26.2	
Carcinosarcoma	4	9.5	
Clear cell	1	2.4	
Mixed*	3	7.1	
FIGO stage	n	%	
I	27	64.3	
II	3	7.1	
III	7	16.7	
IV	5	11.9	

\*Patients with endometrial carcinomas of mixed subtypes included two mixed clear cell and endometrioid carcinomas, and one mixed serous and carcinosarcoma.

**Table 2:** DNA quality characteristics of paired urine fractions of controls and EC patients (n = 76).

	<b>Full void urine</b>		<b>Urine sediment</b>		<b>Urine supernatant</b>	
	Median Cq	Invalid (%)	Median Cq	Invalid (%)	Median Cq	Invalid (%)
ACTB	24.7	2 (2.6)	24.8	0 (0.0)	26.1	2 (2.6)

EC: endometrial cancer.

Invalid (%): invalid for methylation analysis based on a Cq value for ACTB  $\geq$  32.

### Comparison of DNA methylation analysis in different urine fractions

Subsequently, the DNA methylation levels of *GHSR*, *SST*, and *ZIC1* were compared among paired urine fractions to determine the correlation between the different urine components. For all markers, a strong to very strong ( $r \geq 0.77 - 0.92$ ) correlation was found between different urine fractions of women with EC (Table 3).

**Table 3:** Correlation of methylation markers between paired urine fractions from EC patients (n = 40).

	Full void urine versus urine sediment	Full void urine versus urine supernatant	Urine sediment versus urine supernatant
<i>GHSR</i>	0.85	0.92	0.89
<i>SST</i>	0.78	0.91	0.74
<i>ZIC1</i>	0.87	0.90	0.77

EC: endometrial cancer.

Spearman's rank correlation coefficient ( $r$ ) was calculated based on the log2-transformed Cq ratio's.  $r = 0.40 - 0.59$  moderate correlation,  $r = 0.60 - 0.79$  strong correlation,  $r = 0.80 - 1.00$  very strong correlation.

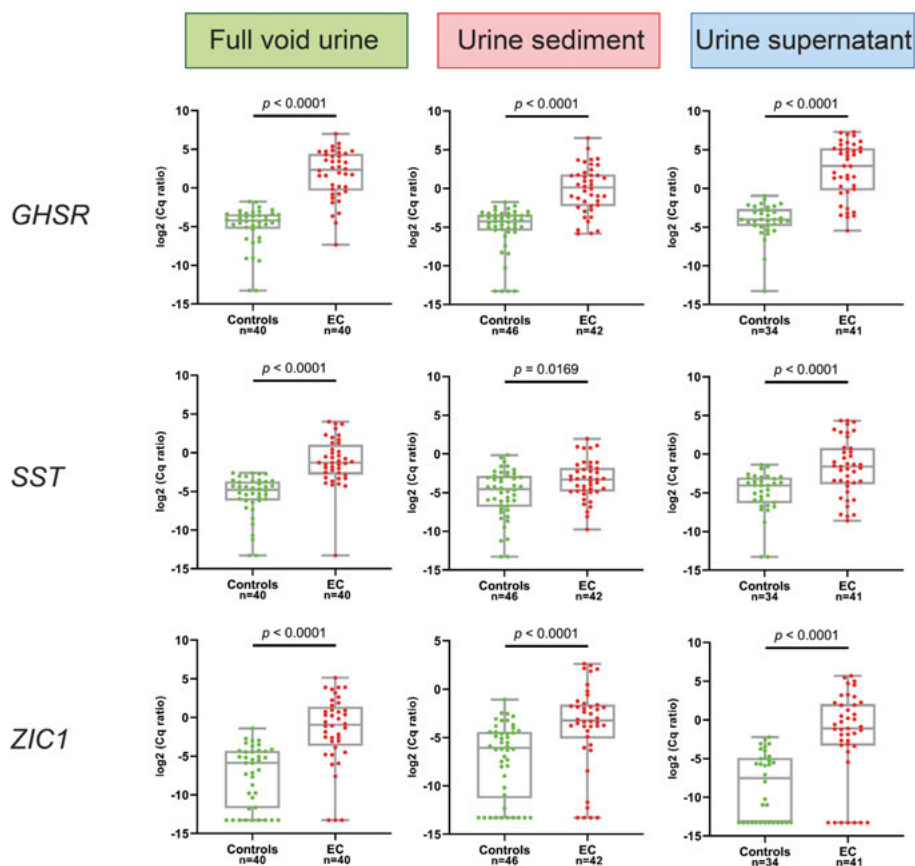
### DNA methylation as diagnostic marker for EC detection in each urine fraction

All DNA methylation markers showed highly increased methylation levels in patients as compared to controls, resulting in  $p$  values  $< 0.001$  for *GHSR* and *ZIC1* in all urine fractions, and for *SST* in full void urine and urine supernatant (Figure 1). The diagnostic potential of each urine fraction was determined by computing ROC curves (Supplementary Figure 1) and quantifying AUCs of all markers (Table 4). Full void urine samples showed the highest discriminatory power for distinguishing patients from controls, with AUCs of 0.95, 0.92, and 0.86 for *GHSR*, *SST*, and *ZIC1*, respectively.

**Table 4:** The AUC (95%-CI) of *GHSR*, *SST* and *ZIC1* in urine fractions for EC detection.

	Full void urine	Urine sediment	Urine supernatant
<i>GHSR</i>	0.95 (0.90 – 1.00)	0.89 (0.81 – 0.96)	0.92 (0.86 – 0.98)
<i>SST</i>	0.92 (0.86 – 0.98)	0.65 (0.53 – 0.76)	0.76 (0.65 – 0.87)
<i>ZIC1</i>	0.86 (0.77 – 0.94)	0.76 (0.66 – 0.87)	0.84 (0.74 – 0.93)

AUC: area under the ROC curve; 95%-CI: 95% confidence interval; EC: endometrial cancer.



**Figure 1:** DNA methylation levels of *GHSR*, *SST*, and *ZIC1* in full void urine, urine sediment and urine supernatant from healthy female controls and women with endometrial cancer (EC).

## DISCUSSION

Urine is a promising alternative for the non-invasive detection of EC. The results of this feasibility study are the first to demonstrate that EC can be detected in urine by DNA methylation analysis with high diagnostic accuracy. A systematic comparison of different urine fractions demonstrated that full void urine is most optimal for EC detection. DNA methylation analysis of *GHSR*, *SST*, and *ZIC1* in full void urine all showed an excellent discriminatory power for EC detection (AUC 0.86 – 0.95).

Detecting EC in urine represents an accessible method for cancer diagnosis. The collection of urine can be done in an outpatient setting or by self-sampling at home, and can easily be performed repeatedly. Moreover, urine appears to be a stable medium for

the preservation of genetic material, when handled correctly (36, 38, 39). This enables delivery to a testing laboratory per mail.

Urine consists of a heterogeneous collection of cell components. We evaluated three urine fractions (full void, sediment, and supernatant) to determine the most optimal source of DNA for EC detection by methylation analysis, assuming that the urine supernatant mainly contains cell-free DNA fragments, and the urine sediment largely consists of cellular DNA (19). Despite this supposed varying origin of DNA in the different urine components, DNA methylation analysis showed significantly increased methylation levels of all markers in all urine fractions of EC patients as compared to controls. Different urine fractions showed strong to very strong correlations ( $r \geq 0.77 - 0.92$ ). Similar findings have been described for the detection of cervical cancer (22, 23) and bladder cancer (25) in different urine fractions. When comparing the AUC values of all fractions, full void urine shows the highest potential for EC detection. An advantage of using full void urine, instead of urine sediment or urine supernatant, is that this fraction does not require pre-processing of the urine sample.

Current routine EC diagnostics are facing several challenges and limitations for which urine could offer a potential solution. Transvaginal sonography remains insufficient in distinguishing benign and malignant endometrial lesions, with a specificity that ranges from 36 to 68% among symptomatic women (40). Apart from its limited specificity, not all endometrial malignancies present with thickened endometrium (41, 42), and the optimal cut-off of endometrial thickness that demands further examination is still under debate (43-45). As a result, many women undergo invasive endometrial tissue sampling. This biopsy procedure can be hampered by conditions that hinder access to the uterus (e.g. cervical stenosis or discomfort) or may yield insufficient tissue for diagnosis (46).

Urine testing could not only reduce the need of performing invasive biopsies, but also has potential in screening of asymptomatic women or to triage women presenting with postmenopausal bleeding symptoms. Additionally, accurate DNA methylation marker testing in urine could be useful to monitor women with increased EC risk (e.g. women with Lynch syndrome). Among women at risk of developing EC, serial sampling of urine may offer an alternative for repeated invasive testing. Urine sampling for EC detection may also be valuable in developing countries with limited access to effective screening programs and early detection methods.

These encouraging results warrant further research to determine whether DNA methylation testing in urine meets the requirements for consideration as a diagnostic tool applicable to clinical practice in the management of EC. Currently, our sample

size is being extended, together with paired cervicovaginal self-samples and clinician collected cervical scrapes to compare the diagnostic potential of DNA methylation analysis for EC detection in different sample types. We expect that a combination of present methylation markers with EC-specific markers could improve urine-based EC detection even further (33). Since EC is more common in older women with abnormal bleeding symptoms, it is important to note that the control subjects used in this study were slightly younger and information concerning abnormal bleeding symptoms was not documented. Therefore, the specificity of this approach remains to be determined in larger source populations that also include symptomatic and asymptomatic women at risk of EC, and women with benign endometrial lesions.

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## CONCLUSIONS

Our study demonstrates the feasibility of urine as a promising non-invasive specimen for EC detection. DNA methylation testing in urine could provide an attractive strategy for non-invasive EC detection for initial diagnosis during screening of asymptomatic women, to distinguish the minority of women presenting with postmenopausal bleeding symptoms due to underlying malignancy from those without EC, and to monitor women with an increased EC risk.

### Acknowledgements

The authors would like to thank Birgit I. Lissenberg-Witte for her assistance in conducting the statistical analyses.

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### Ethics approval and consent to participate

Ethical approval was obtained by the Medical Ethical Committee of the VU University Medical Center for both the SOLUTION1 study (no 2016.213) and the URIC biobank (no 2017.112). Each participant gave written informed consent upon recruitment.

### Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

### **Competing interests**

RDMS has a minority share in Self-screen B.V., a spin-off company of Amsterdam UMC, location VUmc. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

### **Author's contributions**

RvdH, NEvT, MCGB and RDMS designed the study. RvdH and BMMW drafted the manuscript. RvdH performed the data analysis and statistics. RvdH and AvS carried out the lab work. RvdH, NEvT, JCK, and CHM guided and provided the collection of clinical samples. NEvT, MCGB, and RDMS supervised the study. All authors commented on the final manuscript. All authors read and approved the final manuscript.

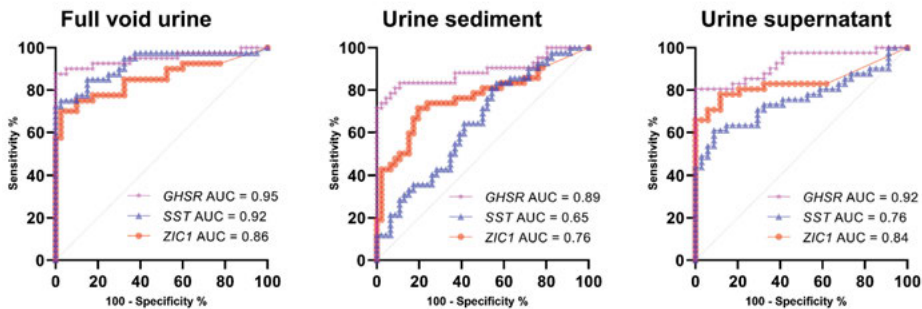
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# SUPPLEMENTARY MATERIALS



**Supplementary Figure 1:** Receiver operating characteristic (ROC) curves of DNA methylation markers *GHSR*, *SST*, and *ZIC1* in full void urine, urine sediment, and urine supernatant. Results are quantified for all markers by an area under the curve (AUC) value.





# CHAPTER 3

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## DNA METHYLATION MARKERS FOR ENDOMETRIAL CANCER DETECTION IN MINIMALLY INVASIVE SAMPLES: A SYSTEMATIC REVIEW

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## **ABSTRACT**

### **Aim**

DNA methylation testing for endometrial cancer detection in minimally invasive specimens is a promising tool to improve screening and diagnostic procedures. Available literature was systematically reviewed to assess the potential of this approach and define methylation markers deserving further development.

### **Methods**

A systematic search up to March 31 2020 was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines.

### **Results**

15 methylation markers with an area under the curve value of  $\geq 0.80$  for endometrial cancer detection in cytological specimens were selected from nine studies.

### **Conclusion**

Detection of methylation markers in cytological samples indicate the feasibility of minimally invasive testing methods, potentially guiding diagnosis and detection of endometrial cancer in high-risk women and in cancer screening programs.

## INTRODUCTION

Endometrial cancer (EC) is the most commonly diagnosed gynecological cancer in developed countries (1). Incidence is rising worldwide with around 382,000 new cases and 89,929 deaths reported in 2018 (2). This is partly driven by the increased global prevalence of risk factors, such as obesity and aging (3).

Conventionally, ECs are categorized into two groups based on tumor grade. Low-grade estrogen-related endometrioid carcinomas (also called type I tumors) are most common. The more aggressive high-grade tumors, like serous or clear cell carcinomas (also called type II tumors), are less common (3, 4). Various risk factors, such as obesity, prolonged estrogen exposure, and Lynch syndrome, contribute to the heterogeneous presentation of ECs (5).

Over the last decade, molecular efforts revealed an objective molecular stratification of ECs that reflect their biological and clinical heterogeneity (6). The four prognostic molecular subtypes include ultramutated DNA polymerase epsilon, hypermutated microsatellite instable, copy number low and copy number high carcinomas. Though not yet clinically implemented, this genomic classification emphasizes the additional value of molecular markers during diagnostics (7).

Although the majority of EC cases are preceded by postmenopausal bleeding symptoms, women presenting with these symptoms pose a diagnostic dilemma since only 9% has an underlying malignancy like EC (8). Diagnostic evaluation of women with suspected EC involves the measurement of endometrial thickness by transvaginal ultrasonography (TVS) (9). In case thickened endometrium is observed by TVS, endometrial pipelle sampling is performed to aspirate endometrial tissue and diagnose endometrial pathology (10). Absence of endometrial thickening does not exclude EC, since especially high-grade ECs can be present without endometrial thickening (11, 12).

Since the gold standard for EC diagnosis remains histological examination, invasive procedures to obtain endometrial tissue are still essential to determine the presence of an endometrial malignancy (13). Considering the low disease prevalence and the fact that TVS might miss EC, many women without cancer undergo unnecessary painful biopsy procedures (14). Detecting and excluding EC in high-risk women, like women with Lynch syndrome for which screening is recommended (15), and women presenting with abnormal bleeding using minimally invasive tools could prevent redundant clinical interventions.

A subset of ECs were previously found at cytological evaluation of cervical scrapes taken during cervical cancer screening programs. Malignant endometrial cells can be detected by cervical cytology of cervical scrapes with an overall efficacy of nearly 40% for EC detection (16). However, as most cervical cancer screening programs are moving toward a primary high-risk human papillomavirus (HPV) testing approach, the opportunity to detect EC during cervical cancer screening is often missed nowadays.

The urgent need to improve EC diagnostics has led to the development of novel diagnostic approaches, combining the use of minimally invasive cytological specimen collection with the detection of epigenetic alterations (14). The analysis of methylated DNA in tampons for the purpose of EC detection was already pioneered in 2004 (17). DNA methylation is a common epigenetic change in cancer, which involves the addition of a methyl group to the cytosine base at regions of cytosine-guanine bonds (CpG). Promoter hypermethylation-induced silencing of tumor suppressor genes is known to occur during the early stages of carcinogenesis (18) and has therefore widely been appreciated as a biomarker for cancer detection (18-20). Notably, the above-mentioned DNA methylation events in tumor suppressor genes differ from promoter hypermethylation associated with the inactivation of the *MLH1*, *MSH2*, *MSH6* or *PMS2* mismatch repair genes in Lynch syndrome (21).

Despite the identification of EC-specific DNA methylation markers with high diagnostic potential (14), they are not yet being implemented in a clinical setting. Determining which marker deserves further development is challenging and a comprehensive overview of the available literature is lacking. Here, we conducted a systematic review to summarize current evidence on the clinical utility of DNA methylation markers for minimally invasive EC detection. Additionally, we critically comment on methodological aspects of the selected studies, aiming to identify the most promising DNA methylation markers for improved EC detection in high-risk women and during cancer screening programs.

## METHODS

### Review format

This systematic review was executed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (22, 23), where applicable. The full PRISMA checklist can be found in the Supplementary Material. No review protocol on this topic was previously published.

## Search strategy

PubMed, EMBASE.com and Web of Science were searched on March 31, 2020 for relevant publications by two reviewers (RvdH and JAvT) with support of a medical information specialist (JCFK). The search terms included both words as keywords as well as free text terms for 'endometrial carcinoma' and 'methylation'. Furthermore, the bibliography of identified articles was checked for other relevant publications. Google Scholar was also searched on March 31, 2020 for additional references. Duplicates were excluded. The full search strategy is outlined in the Supplementary Material.

## Eligibility criteria & study selection

Two reviewers (RvdH and JAvT) independently selected studies based on title, abstract, and full text in particular cases, including only Dutch and English language articles. Any disagreement between reviewers was resolved by discussion with a molecular biologist specialized in epigenetics (RDMS). Articles were regarded as eligible to be included in this review when DNA methylation biomarkers were explored or evaluated for EC detection, using minimally invasive sample collection methods. In this review, minimally invasive sampling is defined as cytological sample (*i.e.* cell specimen) collection with minimal discomfort to the patient without local or general anesthesia. All studies on the detection of EC-specific DNA methylation markers in minimally invasive samples (*i.e.* cervical scrapings, endometrial brushes, vaginal swabs, and vaginal tampons) were included, regardless of the methodology used for DNA methylation detection. Moreover, studies that used liquid biopsies (*i.e.* blood or urine) for EC detection using DNA methylation markers were included. Both individual DNA methylation markers and DNA methylation marker panels were included. DNA methylation in this review covers CpG island methylation positioned in the promoter region of a gene as well as at other CpG-rich locations of the genome.

To discover DNA methylation markers that allow detection of all histological subtypes of EC, the inclusion of articles was not restricted to specific subtypes of EC. Articles that merely used tissue samples, only focused on prognosis, therapeutic use or methylation markers related to Lynch syndrome (*i.e.* *MLH1*, *MSH2*, *MSH6* or *PMS2* promoter hypermethylation (21)), were excluded. Studies without healthy control subjects were also excluded.

## Methylation marker selection

Diagnostic biomarker performance is usually evaluated by plotting sensitivity against 1 – specificity in a receiver operating characteristic (ROC) curve. The area under the ROC curve (AUC) summarizes the overall diagnostic accuracy of the biomarker or diagnostic test, with a value ranging from 0 to 1. An AUC of 1 represents an excellent

diagnostic test and an AUC of 0.5 implicates the absence of diagnostic potential. Both the individual DNA methylation markers and marker panels investigated in the included studies were summarized, irrespective of their diagnostic accuracy. Individual DNA methylation markers achieving an AUC value of  $\geq 0.80$  were considered highly valuable for the minimally invasive detection of EC and selected from eligible studies.

### **Data extraction**

Data from selected studies were extracted from the full text by two reviewers (RvdH and JAvT). Collected data were processed using a standardized data registration form reporting the following information: first author and research group, year of publication, journal and belonging impact factor, marker identification methods (*e.g.* genome-wide screen, a targeted approach or literature analyses), study design (*e.g.* discovery, test or validation set), study population (*i.e.* the total number of cases and controls, and tumor subtypes included), patient characteristics (*i.e.* presented with symptoms or detected during a screening), sample type, DNA methylation markers studied, assay used for DNA methylation detection, outcome measures (*i.e.* percentage methylated in cases and controls) and marker performance (*i.e.* AUC value, sensitivity, specificity and belonging 95% confidence interval [CI]).

### **Risk of bias assessment**

The risk of bias was independently assessed as low, high or unclear according to the Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) guidelines by two reviewers (RvdH and BMMW). Scoring was piloted in two independent studies (*i.e.* not included in this review) to ensure reproducibility. Disagreements between the two reviewers were resolved by discussion with a specialist (RDMS). The risk of bias assessment scores were merely used to determine the quality of selected studies and not to exclude articles from the review. In case a study performed marker discovery as well as marker validation, only the latter was assessed for bias. Furthermore, quality assessment was only focused on the validation of markers analyzed in minimally invasive collected cytological specimens (*i.e.* cervical scrapes, endometrial brushes, vaginal swabs and vaginal tampons). A figure summarizing the risk of bias scores per study was constructed using Review Manager 5.3 software.

## **RESULTS**

### **Search results**

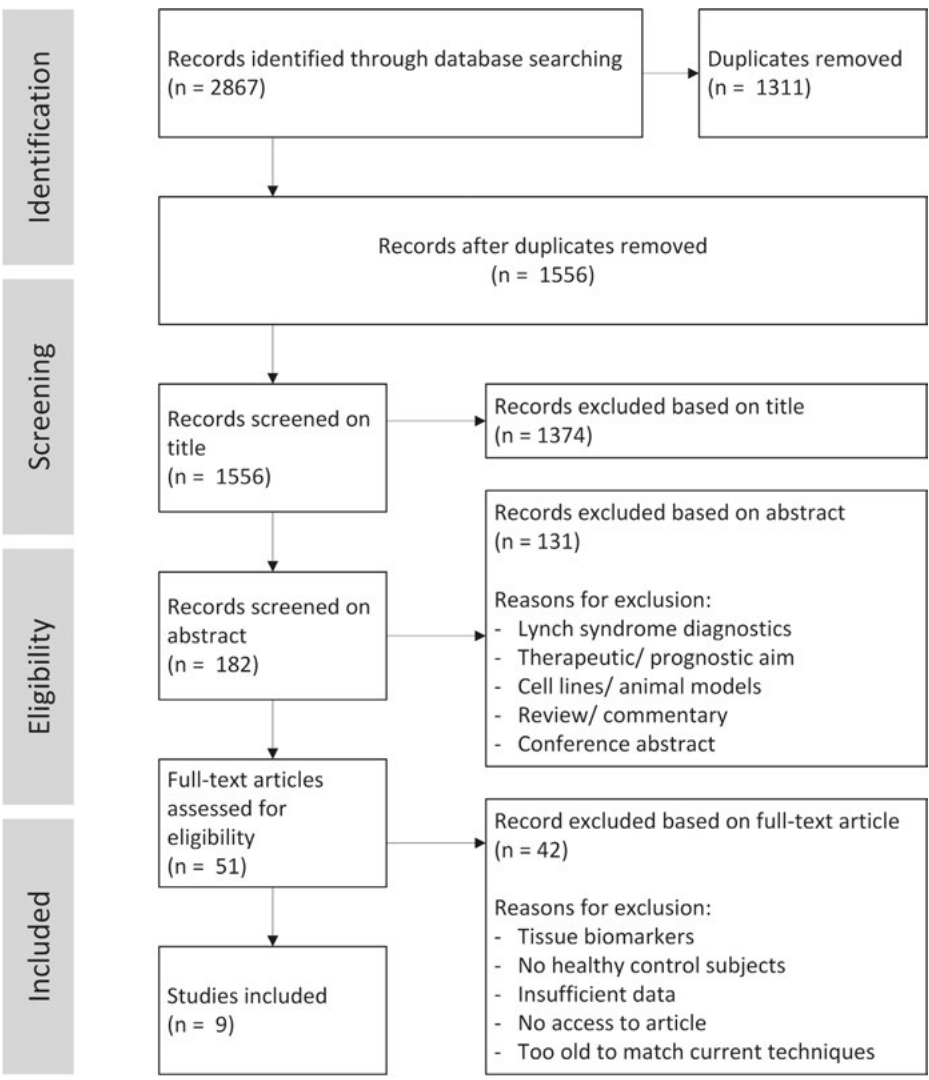
The literature search and selection process are outlined in Figure 1. A total of 1556 potentially relevant articles were retrieved from PubMed, Embase.com, Web of Science

and Google Scholar. After a series of selections, 1547 articles were excluded on the basis of their title (n=1374), abstract (n=131) and after reading the full text for not meeting the predefined inclusion criteria (n=42). This selection procedure resulted in nine articles that were included in this review.

### Study characteristics

The characteristics of the studies included in this systematic review are summarized in Table 1. Of the nine included studies, three were carried out in Taiwan (13, 16, 24), three in the USA (25-27), two in the UK (28, 29) and one in the Netherlands (30). Accordingly, study populations of Asian, American and European origin were used. The nine selected articles were case-control studies and comprised sample sizes varying from 38 to 141 subjects. The number of EC cases and controls ranged from 21 to 50 and 17 to 120, respectively. All studies included cases of both subtypes of EC (*i.e.* I and II) with variable stages (stage I up to stage IV). DNA was extracted from a variety of cytological specimen, comprising cervical scrapes (13, 16, 24, 30), endometrial brushes (25, 27), vaginal swabs (28, 29), and vaginal tampons (25, 26). Studies addressing EC-specific DNA methylation detection in liquid biopsies (*i.e.* blood or urine) were not found. DNA methylation levels were assessed by either pyrosequencing (25-27), quantitative methylation-specific PCR (qMSP) (13, 16, 24, 30) or MethyLight PCR (28, 29), all using bisulfite converted DNA.

Further details on study design and patient selection are provided in Supplementary Table 1. Four studies reported that included patients presented with postmenopausal bleeding (28, 29) or abnormal bleeding (13, 25). One study selected patients retrospectively from a population-based cervical screening cohort (30).



**Figure 1:** Preferred reporting items for systematic reviews and meta-analysis (PRISMA) flowchart for study selection.

AUC values of individual DNA methylation markers for distinguishing EC from benign endometrium could be extracted from seven (13, 16, 24, 25, 27-29) out of nine included studies. Selected markers with AUC values  $\geq 0.80$  originated from six studies (13, 16, 25, 27-29), of which three (16, 24, 28) provided a 95% CI of the reported AUC values. Sensitivity and specificity values of individual markers were reported in only three (16, 24, 26) out of nine included studies. A total of four studies (13, 24, 26, 30) investigated the performance of DNA methylation marker panels, comprising up to three genes.

Sensitivity and specificity could be extracted from all studies reporting on marker panels, of which two (26, 30) also computed a 95% CI of the reported sensitivity and specificity values.

**Table 1:** Characteristics of the included studies.

Study (year)	Cases (n) / controls (n)	Tumor type (n)	Cytological sample type(s)	Methylation analysis	Ref.
Bakkum-Gamez (2015)	38 <sup>†</sup> / 37	E (31), CAH (1), CC (3), PS (3)	Endometrial brushes, vaginal tampons	Pyrosequencing	(25)
Chang (2018)	30 / 30	E (30)	Cervical scrapes	qMSP	(16)
Doufekas (2013)	41 <sup>†</sup> / 38 <sup>†</sup>	E (38), CAH (1), U (2)	Vaginal swabs	MethyLight PCR	(28)
Huang (2017)	50 / 56	E (50)	Cervical scrapes	qMSP	(24)
Jones (2013)	18/13 <sup>††</sup> / 17 <sup>†</sup>	E (18/13)	Vaginal swabs	MethyLight PCR	(29)
Liew (2019)	46 <sup>†</sup> / 38 <sup>†</sup>	E (33), PS (6), O (7)	Cervical scrapes	qMSP	(13)
Sangtani (2020)	38 / 27	E (31), CAH (1) CC (3), PS (3)	Vaginal tampons	Pyrosequencing	(26)
De Strooper (2014)	21 / 120	U	Cervical scrapes	qMSP	(30)
Wentzensen (2014)	37 / 37	E (30), CC (2), PS (2), M (3)	Endometrial brushes	Pyrosequencing	(27)

<sup>†</sup> Women presented with postmenopausal (28, 29) or abnormal bleeding (13, 25).

<sup>††</sup> Stage Ia / Stage Ib/III/III.

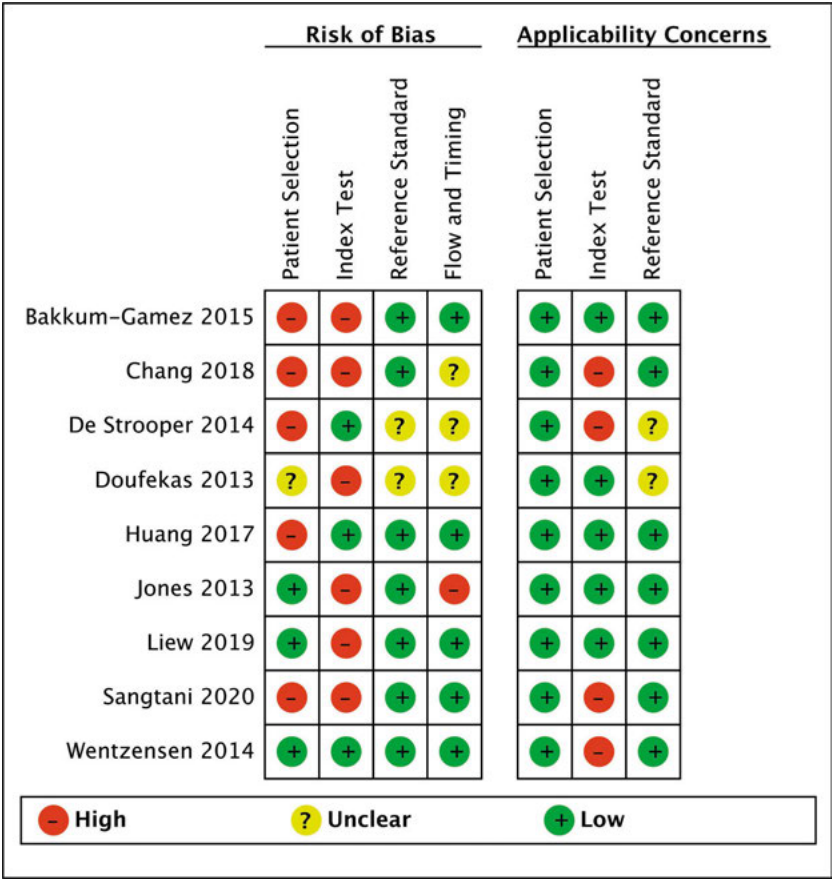
CAH = complex atypical hyperplasia; CC = clear-cell carcinoma; E = endometrioid adenocarcinoma; O = other; M = mixed; PCR = polymerase chain reaction; PS = papillary serous carcinoma; qMSP = quantitative methylation-specific PCR; U = adenocarcinoma, histotype unknown.

### Risk of bias of all included studies

Selected studies underwent quality assessments according to the QUADAS-2 tool, of which the results are presented in Figure 2. In over half of the studies, a high risk of bias was introduced during patient selection, mainly due to their case-control design in which patients were specifically selected based on their confirmed histological status. Additionally, handling DNA methylation levels of the markers without pre-specified thresholds (e.g. based on the most optimal marker performance) introduced a high risk of bias in six of the included studies. In two studies, the risk of bias score for the reference test was unclear. Even though these studies did perform pathological examination of the included samples, they did not specify which cancer histotypes were found or what specimen type was used (e.g. use of a biopsy or a larger surgical specimen) during this examination. Likewise, the risk of bias introduced during the flow and timing were scored unclear when the reference test was not accurately described or

the interval between the index and reference test was not indicated. All studies scored low on applicability concerns regarding patient selection. Applicability concerns for the index test were raised in four studies where, for example, thresholds were specified according to another cancer (*i.e.* cervical cancer) or sample type (*e.g.* tissue). In case the execution of the reference standard was not clearly stated, the applicability concerns were also scored unclear.

The studies with low, or limited, risk of bias concerns were performed by Huang *et al.* (24), Liew *et al.* (13) and Wentzensen *et al.* (27).



**Figure 2:** Risk of bias and applicability concerns summary: review authors’ judgments about each domain for each included study. Overview constructed using Review Manager 5.3 software.

### Methylation marker performance for EC detection

Table 2 provides an overview of individual DNA methylation markers for EC detection in minimally invasive specimens. Markers with a high diagnostic accuracy (*i.e.* AUC  $\geq$  0.80) are marked in bold. Among the 32 genes investigated in the final article selection, 15 individual genes most competent for EC detection were selected, including *ADCYAP1* (25, 27), *ASCL2* (27), *BHLHE22* (13, 24), *CDH13* (25, 27), *CDO1* (13, 24), *CELF4* (24), *GALR1* (28), *HAND2* (29), *HS3ST2* (27), *HTR1B* (25), *MAGI2* (16), *MME* (25, 27), *POU4F3* (16), *RASSF1* (25), and *ZNF662* (24), with AUC values ranging from 0.80 to 0.96.

**Table 2:** Performance of individual DNA methylation markers in minimally invasive specimens for distinguishing endometrial carcinoma from benign endometrium.

Gene	AUC	(95% CI)	Sensitivity %	Specificity %	Cytological sample type	Study (year)	Ref.
<b>ADCYAP1</b>	<b>0.88</b>	(U)	U	U	Endometrial brushes	Bakkum-Gamez (2015)	(25)
<i>ADCYAP1</i>	0.67	(U)	U	U	Vaginal tampons	Bakkum-Gamez (2015)	(25)
<b>ADCYAP1</b>	<b>0.86</b>	(0.76 – 0.96)	U	U	Endometrial brushes	Wentzensen (2014)	(27)
<i>ASCL2</i>	0.76	(U)	U	U	Endometrial brushes	Bakkum-Gamez (2015)	(25)
<i>ASCL2</i>	0.69	(U)	U	U	Vaginal tampons	Bakkum-Gamez (2015)	(25)
<b>ASCL2</b>	<b>0.81</b>	(0.70 – 0.92)	U	U	Endometrial brushes	Wentzensen (2014)	(27)
<b>BHLHE22</b>	<b>0.95</b>	(0.91 – 0.99)	83.7	93.7	Cervical scrapes	Huang (2017)	(24)
<b>BHLHE22</b>	<b>0.88</b>	(U)	U	U	Cervical scrapes	Liew (2019)	(13)
<i>CADM1</i>	U	(U)	U	U	Cervical scrapes	De Strooper (2014)	(30)
<b>CDH13</b>	<b>0.86</b>	(U)	U	U	Endometrial brushes	Bakkum-Gamez (2015)	(25)
<i>CDH13</i>	0.67	(U)	U	U	Vaginal tampons	Bakkum-Gamez (2015)	(25)
<b>CDH13</b>	<b>0.86</b>	(0.76 – 0.96)	U	U	Endometrial brushes	Wentzensen (2014)	(27)
<b>CDO1</b>	<b>0.95</b>	(0.91 – 0.98)	82.0	93.8	Cervical scrapes	Huang (2017)	(24)
<b>CDO1</b>	<b>0.84</b>	(U)	U	U	Cervical scrapes	Liew (2019)	(13)
<b>CELF4</b>	<b>0.94</b>	(0.90 – 0.97)	96.0	78.7	Cervical scrapes	Huang (2017)	(24)
<b>GALR1</b>	<b>0.93</b>	(0.87 – 0.97)	92.7	78.9	Vaginal swabs	Doufekas (2013)	(28)
<i>GTF2A1</i>	0.55	(U)	U	U	Vaginal tampons	Bakkum-Gamez (2015)	(25)

**Table 2:** (Continued)

Gene	AUC	(95% CI)	Sensitivity %	Specificity %	Cytological sample type	Study (year)	Ref.
<i>GTF2A1</i>	0.45	(U)	U	U	Endometrial brushes	Bakkum-Gamez (2015)	(25)
<i>HAAO</i>	0.68	(U)	U	U	Vaginal tampons	Bakkum-Gamez (2015)	(25)
<i>HAAO</i>	0.68	(U)	U	U	Endometrial brushes	Bakkum-Gamez (2015)	(25)
<b>HAND2</b>	<b>0.91</b>	(U)	U	U	Vaginal swabs	Jones (2013)	(29)
<i>HAND2</i>	0.77	(U)	U	U	Cervical scrapes	Liew (2019)	(13)
<i>HOXA9</i>	0.77	(U)	U	U	Vaginal tampons	Bakkum-Gamez (2015)	(25)
<i>HOXA9</i>	0.58	(U)	U	U	Endometrial brushes	Bakkum-Gamez (2015)	(25)
<i>HOXA9</i>	U	(U)	37.8	100	Vaginal tampons	Sangtani (2020)	(26)
<i>HS3ST2</i>	0.75	(U)	U	U	Endometrial brushes	Bakkum-Gamez (2015)	(25)
<i>HS3ST2</i>	0.73	(U)	U	U	Vaginal tampons	Bakkum-Gamez (2015)	(25)
<b>HS3ST2</b>	<b>0.80</b>	(0.69 – 0.90)	U	U	Endometrial brushes	Wentzensen (2014)	(27)
<i>HSP2A</i>	0.68	(U)	U	U	Endometrial brushes	Bakkum-Gamez (2015)	(25)
<i>HSP2A</i>	0.67	(U)	U	U	Vaginal tampons	Bakkum-Gamez (2015)	(25)
<b>HTR1B</b>	<b>0.81</b>	(U)	U	U	Vaginal tampons	Bakkum-Gamez (2015)	(25)
<i>HTR1B</i>	0.67	(U)	U	U	Endometrial brushes	Bakkum-Gamez (2015)	(25)
<i>HTR1B</i>	U	(U)	38.9	100	Vaginal tampons	Sangtani (2020)	(26)
<i>HTR1B</i>	0.68	(0.55 – 0.81)	U	U	Endometrial brushes	Wentzensen (2014)	(27)
<b>MAGI2</b>	<b>0.90</b>	(0.8 – 1.0)	90.0	75.0	Cervical scrapes	Chang (2018)	(16)
<i>MAL</i>	U	(U)	U	U	Cervical scrapes	De Strooper (2014)	(30)
<i>miR124-2</i>	U	(U)	66.7	U	Cervical scrapes	De Strooper (2014)	(30)
<b>MME</b>	<b>0.83</b>	(U)	U	U	Endometrial brushes	Bakkum-Gamez (2015)	(25)
<i>MME</i>	0.69	(U)	U	U	Vaginal tampons	Bakkum-Gamez (2015)	(25)

**Table 2:** (Continued)

Gene	AUC	(95% CI)	Sensitivity %	Specificity %	Cytological sample type	Study (year)	Ref.
<b>MME</b>	<b>0.86</b>	(0.76 – 0.96)	U	U	Endometrial brushes	Wentzensen (2014)	(27)
<i>NPY</i>	0.67	(U)	U	U	Vaginal tampons	Bakkum-Gamez (2015)	(25)
<i>NPY</i>	0.60	(U)	U	U	Endometrial brushes	Bakkum-Gamez (2015)	(25)
<i>NPY</i>	0.76	(0.64 – 0.88)	U	U	Endometrial brushes	Wentzensen (2014)	(27)
<b>POU4F3</b>	<b>0.80</b>	(0.7 – 0.9)	83.0	69.0	Cervical scrapes	Chang (2018)	(16)
<b>RASSF1</b>	<b>0.86</b>	(U)	U	U	Endometrial brushes	Bakkum-Gamez (2015)	(25)
<i>RASSF1</i>	0.79	(U)	U	U	Vaginal tampons	Bakkum-Gamez (2015)	(25)
<i>RASSF1</i>	U	(U)	40.0	100	Vaginal tampons	Sangtani (2020)	(26)
<i>TBX5</i>	0.70	(U)	U	U	Cervical scrapes	Liew (2019)	(13)
<b>ZNF662</b>	<b>0.89</b>	(0.83 – 0.95)	92.0	80.0	Cervical scrapes	Huang (2017)	(24)

†AUC separately calculated for Stage Ia (0.91) and Stage Ib/II/III (0.97).

Markers with high performance (AUC ≥ 0.80) are marked in bold.

AUC = Area under the curve; CI = Confidence interval; U = Unknown.

The performance of DNA methylation marker panels for EC detection in minimally invasive samples is summarized in Table 3. The gene panels investigated include *BHLHE22/CDO1* (13), *BHLHE22/CDO1/CELF4* (24), *BHLHE22/CDO1/HAND2* (13), *BHLHE22/CDO1/TBX5* (13), *CADM1/MAL/miR124-2* (30) and *HOXA9/RASSF1/HTR1B* (26), with reported sensitivity and specificity values ranging from 60.0 - 91.8% and 70 - 100%, respectively.

**Table 3:** DNA methylation marker panels for endometrial cancer detection with combined AUC, sensitivity and specificity values.

Gene panel	AUC	Sensitivity %	Specificity %	Study (year)	Ref.
<i>BHLHE22 / CDO1</i>	U	84.8	88.0	Liew (2019)	(13)
<i>BHLHE22 / CDO1 / CELF4</i>	U	91.8	95.5	Huang (2017)	(24)
<i>BHLHE22 / CDO1 / HAND2</i>	U	87.0	86.0	Liew (2019)	(13)
<i>BHLHE22 / CDO1 / TBX5</i>	U	89.1	80.0	Liew (2019)	(13)
<i>CADM1 / MAL / miR124-2</i>	U	76.2	80.8 <sup>†</sup>	De Strooper (2014)	(30)
<i>HOXA9 / HTR1B / RASSF1</i>	U	60.0	100	Sangtani (2020)	(26)

†Specificity described for human papilloma virus (HPV)-positive and -negative women with low-grade cervical intraepithelial neoplasia (<CIN2).

AUC = area under the curve; U = unknown.

## DISCUSSION

In this review, we provide an overview of the reported DNA methylation markers for the detection of EC in minimally invasive specimens. EC-specific DNA methylation markers could be clinically relevant to guide the detection of EC in women presenting with postmenopausal bleeding, during cancer screening programs, and potentially in women with Lynch syndrome. We selected 15 DNA methylation markers with high potential that require further validation before drawing conclusions on their diagnostic accuracy in a clinical setting.

The findings of studies included in this systematic review indicate the feasibility of EC detection in cytological specimens. Shedding of malignant endometrial cells into the cervical tract was already demonstrated by their detection in cervical scrapes by cytology (14). The use of sensitive DNA methylation markers broadly expands the use of cervical scrapes. Huang *et al.* (24) even reached a sensitivity and specificity of 91.8% and 95.5%, respectively, for EC detection when testing for a three-gene methylation marker panel in cervical scrapes.

In addition to cervical scrapes, also endometrial brushes, vaginal swabs, and vaginal tampons seem promising sources of methylated DNA for EC detection. Wentzensen *et al.* (27) investigated the diagnostic potential of 8 genes frequently hypermethylated in EC tissue in DNA extracted from endometrial brushes. They validated their candidate markers in endometrial brush material with a combined AUC of 0.85. In a follow-up study, Bakkum-Gamez *et al.* (25) showed similar results in DNA extracted from vaginal tampons for all candidate genes, except for *SOX1* due to technical issues. Bakkum-Gamez *et al.* (25) also tested DNA methylation markers for EC detection established by others. Among these, *RASSF1* methylation showed the second-highest AUC value of 0.75 in vaginal tampon specimens as compared to an AUC value of 0.82 found in paired endometrial brushes. Remarkably, for *HTR1B* the opposite effect was found, with an AUC value of 0.68 in endometrial brushes and 0.82 in tampons.

Differences in marker performance may partly be explained by the use of different sample types. While endometrial brush samples are physician-taken and enable sampling of a wide area of the endometrial surface (31), vaginal tampons are self-collected specimens that indirectly obtain endometrial material. The varying presence of methylated background DNA in different sample types may also affect marker performance (32).

The pioneering discovery studies included in this review all employed endometrial tissue samples to discover novel DNA methylation markers, of which the majority also

appeared valuable for the detection of EC in minimally invasive samples (24, 25, 27-29). Yet, it would be interesting to investigate whether a discovery screen using minimally invasive sample types would yield even more accurate markers. This approach has been proven valuable for the discovery of novel diagnostic DNA methylation markers for the early detection of cervical (pre)cancer in self-collected lavage samples (33).

The methylation status of the extracted DNA can be measured by several techniques (34) which may also contribute to differences in marker performance (35). Studies selected in this systematic review used three different methods to assess methylation status, including MethyLight PCR, pyrosequencing and qMSP. Although it has been shown that the use of diverse assays could lead to differences in clinical decision making (35), this has not fully been elucidated yet for our marker selection. Draht *et al.* (36) emphasized the importance of precise optimization of the chosen assay to measure DNA methylation levels. They showed that the prognostic value of a DNA methylation marker was not affected by using different techniques if the chosen assay was adequately optimized. Reporting information on optimization of the used assay to measure DNA methylation is therefore of high importance. Yet, none of the selected articles in our review discussed the optimization of the used method in sufficient detail. Different sources of DNA were often assessed for DNA methylation using the same conditions and cut-off values, possibly leading to skewed sensitivity and specificity of the assay.

The lack of a standardized cut-off for the assessment of methylation levels makes DNA methylation research challenging. Marketed DNA methylation assays use standardized cut-off values and often rely on a biomarker panel instead of a single gene (37). Similarly, the included studies in this review indicated increased sensitivity of markers when combined in a marker panel. For example, the sensitivity of the individual DNA methylation markers *HOXA9*, *HTR1B*, and *RASSF1* described by Sangtani *et al.* (26) ranged from 37.8 to 40% but increased to 60% when combined in a marker panel without any impact on specificity. Combining DNA methylation markers with genetic alterations, such as DNA mutations or copy number alterations, seems another attractive approach to strengthen minimally invasive cancer detection (13, 26, 38, 39). Genetic alterations provide a binary readout and may complement DNA methylation markers.

A strategy for completely non-invasive EC detection could be the use of urine as an alternative DNA source, which has already been proven feasible for cervical cancer detection (40, 41). Considering the anatomical proximity of the uterus and cervix, urine could also be a valuable specimen for EC detection. Notably, the presence of EC-specific microRNAs in urine has been demonstrated previously (42, 43).

Although the identified DNA methylation markers appear promising for EC detection in minimally invasive specimens, individual studies show various discordances, making it difficult to compare study outcomes. The studies selected in this review vary substantially in population selection, characteristics of case and control groups, sample type, sample preparation, DNA methylation detection techniques, and the cut-off values used to handle DNA methylation levels.

Included studies investigated small sample sizes of varying types of cases and controls. Besides normal endometrium, control groups contained different types of benign endometrial and ovarian lesions, and in some cases atypical endometrial hyperplasia (EH) and low-grade cervical intraepithelial neoplasia (CIN1). This varying selection of controls may have influenced the resulting AUC for distinguishing EC from benign endometrium of the corresponding marker, and therefore also our ultimate marker selection. None of the studies included a separate EH group and case or control groups contained only limited numbers of women with EH. DNA methylation markers that enable detection of EH with a high risk of cancer progression could, therefore, have been overlooked.

The source populations of which the study participants were enrolled also showed major differences among the selected studies. For instance, De Strooper *et al.* (30) recruited participants from a population-based cervical screening cohort, whereas others included participants that presented with abnormal (13, 25) or postmenopausal (28, 29) bleeding symptoms. Interestingly, Nair *et al.* (44) performed a genomic analysis of cancer-specific somatic mutations in uterine lavage samples to detect EC and found that the women with cancer-specific somatic mutations were more likely to be older and postmenopausal. Likewise, the performance of the selected DNA methylations markers may have been affected by the age and postmenopausal status of the study participants.

The majority of cancers in the included studies comprised low-grade endometrioid carcinomas, which is coherent to the fact that this subtype involves more than 80% of EC cases (4). High-grade ECs are known to be more aggressive, as they have a higher risk to metastasize and a worse prognosis (3). DNA methylation of the three-gene panel *BHLHE22/CDO1/CELF4*, described by Huang *et al.* (24), allowed accurate detection of both groups. When used in a screening setting, it is important to ensure that the DNA methylation markers allow the detection of all subtypes.

Our review has several limitations. The final selection of studies in our review is small and originates from only four research groups, underlining the scarcity of publications on this particular topic. Moreover, none of the DNA methylation markers has independently been validated by investigators outside those groups. The lack of

external validation could have implications for the reproducibility of our final marker selection. It is also worth noting that none of the studies performed a separate DNA methylation marker discovery screen on cytological material. Markers were either discovered using endometrial or cervical cancer tissue, or EC tissue data downloaded from The Cancer Genome Atlas Program. Although studies showed variable risk of bias scores, they were not excluded on the basis of their risk of bias. A high risk of bias was frequently introduced during patient selection due to their case-control design. Moreover, cases and controls were often not age-matched. Thresholds used to handle DNA methylation levels were not pre-specified in many studies or specified according to another cancer or sample type, which also introduced bias in the majority of the studies.

## CONCLUSIONS

We selected the individual genes *ADCYAP1*, *ASCL2*, *BHLHE22*, *CDH13*, *CDO1*, *CELF4*, *GALR1*, *HAND2*, *HS3ST2*, *HTR1B*, *MAGI2*, *MME*, *POU4F3*, *RASSF1*, and *ZNF662*, with AUC values ranging from 0.80 to 0.96, as potential DNA methylation markers for the detection of EC using minimally invasive specimens. This approach could potentially guide the detection of EC in women presenting with postmenopausal bleeding, during cancer screening programs and potentially in women with Lynch syndrome. Validation in larger, prospective and unbiased cohorts is warranted to determine their true clinical diagnostic accuracy (45).

## FUTURE PERSPECTIVE

Current literature indicates the feasibility of DNA methylation marker testing in minimally invasive samples for EC detection. Nevertheless, diagnostic methylation marker research is facing several challenges, with heterogeneity of study populations and the lack of standardized methylation assays as the main concerns. Instead of only contributing to the discovery of new diagnostic methylation markers, studies should also focus on the further validation of the methylation markers described so far. The performance of the selected DNA methylation markers with potential clinical value may be improved by combining DNA methylation markers with additional (epi)-genetic markers and using alternative sources of DNA. The most interesting and likely application of DNA methylation markers would be in diagnostics to guide two clinical problems: to discriminate the minority of women with underlying malignancy within the group of women presenting with postmenopausal bleeding symptoms, and to monitor women with increased EC risk.

## EXECUTIVE SUMMARY

### A systematic review of the literature on DNA methylation markers for endometrial cancer detection

- A total of nine studies that investigated DNA methylation markers for the minimally invasive detection of endometrial cancer (EC) were included, resulting in 15 potential DNA methylation markers with area under the curve values ranging from 0.80 to 0.96.

### Promising DNA methylation marker selection

- Comparability of studies was hampered due to differences in population selection, characteristics of case and control groups, sample type, sample preparation, DNA methylation detection techniques and the cut-off values used to handle DNA methylation levels.
- Despite the above-mentioned differences among studies, the individual genes *ADCYAP1*, *ASCL2*, *BHLHE22*, *CDH13*, *CDO1*, *CELF4*, *GALR1*, *HAND2*, *HS3ST2*, *HTR1B*, *MAGI2*, *MME*, *POU4F3*, *RASSF1* and *ZNF662* were considered promising for EC detection according to their area under the curve value of  $\geq 0.80$  for distinguishing benign endometrium from EC.

### Conclusion

- We selected 15 promising DNA methylation markers for the minimally invasive detection of EC using cell specimens.
- DNA methylation markers would be clinically relevant for the detection of EC in women presenting with postmenopausal bleeding, during cancer screening programs and potentially in women with Lynch syndrome. Validation in larger, prospective and unbiased cohorts is warranted to determine their true diagnostic accuracy.
- The performance of selected methylation markers with potential clinical value may be improved by combining DNA methylation markers with additional (epi)genetic markers and using alternative sources of DNA.

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44. Nair N, Camacho-Vanegas O, Rykunov D, Dashkoff M, Camacho SC, Schumacher CA, et al. Genomic Analysis of Uterine Lavage Fluid Detects Early Endometrial Cancers and Reveals a Prevalent Landscape of Driver Mutations in Women without Histopathologic Evidence of Cancer: A Prospective Cross-Sectional Study. *PLoS Med*. 2016;13(12):e1002206.
45. Clarke MA, Long BJ, Sherman ME, Lemens MA, Podratz KC, Hopkins MR, et al. A prospective clinical cohort study of women at increased risk for endometrial cancer. *Gynecol Oncol*. 2020;156(1):169-77.

## SUPPLEMENTARY MATERIALS

### Supplementary Methods

#### PRISMA Checklist

Section/topic	#	Checklist item	Reported on page #
<b>TITLE</b>			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
<b>ABSTRACT</b>			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
<b>INTRODUCTION</b>			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3,4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4,5
<b>METHODS</b>			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	6
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	6
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	6
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	6, supplements
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	6, 7
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	7
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	6/7
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	8
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	7

**PRISMA Checklist** (Continued)

Section/topic	#	Checklist item	Reported on page #
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., $I^2$ ) for each meta-analysis.	N/A
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	N/A
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	N/A
<b>RESULTS</b>			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	8
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	8, 9
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	9, 10
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	N/A
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	N/A
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	N/A
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	N/A
<b>DISCUSSION</b>			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	10, 14
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	11/12/13/14
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	15
<b>FUNDING</b>			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	16

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit: [www.prisma-statement.org](http://www.prisma-statement.org).

**PubMed search strategy (March 31, 2020)**

Search	Query	Items found
#3	#1 AND #2	791
#2	"Methylation"[Mesh] OR dna methyl*[tiab] OR dna methyltransferase*[tiab] OR hypermethylat*[tiab] OR methylat*[tiab]	131529
#1	"Endometrial Neoplasms"[Mesh] OR (("Endometrium"[Mesh] OR endometri*[tiab]) AND (neoplasms[mesh] OR cancer[sb] OR adenoma*[tw] OR anticarcinogen*[tw] OR cancer*[tw] OR carcinogen*[tw] OR carcinom*[tw] OR carcinosarcoma*[tw] OR malignan*[tw] OR metasta*[tw] OR neoplas*[tw] OR oncogen*[tw] OR oncolog*[tw] OR paraneoplastic[tw] OR precancerous[tw] OR teratocarcinoma*[tw] OR tumor*[tw] OR tumour*[tw]))	65114

**Embase.com search strategy (March 31, 2020)**

No.	Query	Results
#4	#3 NOT 'conference abstract'/it	955
#3	#1 AND #2	1373
#2	'methylation'/exp OR 'dna methylation'/exp OR 'dna methylation assay'/exp OR 'dna methyltransferase*':ti,ab,kw OR hypermethylat*':ti,ab,kw OR methylat*':ti,ab,kw	175714
#1	'endometrium cancer'/exp OR (('endometrium'/exp OR endometri*':ti,ab,kw) AND ('neoplasm'/exp OR adenoma*':ti,ab,kw OR anticarcinogen*':ti,ab,kw OR blastoma*':ti,ab,kw OR cancer*':ti,ab,kw OR carcinogen*':ti,ab,kw OR carcinom*':ti,ab,kw OR carcinosarcoma*':ti,ab,kw OR malignan*':ti,ab,kw OR metasta*':ti,ab,kw OR neoplas*':ti,ab,kw OR oncogen*':ti,ab,kw OR oncolog*':ti,ab,kw OR paraneoplastic:ti,ab,kw OR precancerous:ti,ab,kw OR teratocarcinoma*':ti,ab,kw OR tumor*':ti,ab,kw OR tumour*':ti,ab,kw))	86532

**Clarivate Analytics/Web of Science Core Collection search strategy (March 31, 2020)**

Set	Results	Query
#3	1,121	#2 AND #1
#2	166,372	TOPIC: ("dna methyltransferase*" OR "hypermethylat*" OR "methylat*")
#1	51,443	TOPIC: (("endometri*" AND ("adenoma*" OR "anticarcinogen*" OR "cancer*" OR "carcinogen*" OR "carcinom*" OR "carcinosarcoma*" OR "malignan*" OR "metasta*" OR "neoplas*" OR "oncogen*" OR "oncolog*" OR "paraneoplastic" OR "precancerous" OR "teratocarcinoma*" OR "tumor*" OR "tumour*"))

**Google Scholar query**

methyltransferase|hypermethylation|methylation+endometrium|endometrial+cancer|tumor|tumour|adenoma|carcinogenic|carcinoma|carcinosarcoma|malignancy|metastasis|neoplasm|oncogenic|oncology|paraneoplastic|precancerous

## Supplementary Table

**Supplementary Table 1:** Study designs of the selected studies.

Study (year)	Study type (sample type)	Case and control selection	Presenting symptoms	Timing of minimally invasive sampling
Bakkum-Gamez (2015)	Discovery (tissue) and validation (swabs)	Prospective	Abnormal bleeding <sup>†</sup>	Prior to hysterectomy
Chang (2018)	Test (scrapes)	Retrospective	U	Prior to surgery
Doufekas (2013)	Discovery (tissue) and validation (swabs)	Prospective	Postmenopausal bleeding	Prior to surgery
Huang (2017)	Discovery (TCGA data) and validation (scrapes)	U	U	U
Jones (2013)	Discovery (tissue) and validation (swabs)	Prospective	Postmenopausal bleeding	Prior to hysteroscopy, endometrial biopsy or hysterectomy
Liew (2019)	Validation (scrapes)	Retrospective	Abnormal bleeding	Prior to surgery
Sangtani (2020)	Validation (tampons)	Prospective	U	Prior to hysterectomy
De Strooper (2014)	Test (scrapes)	Retrospective	U	During cervical screening program or when attending an outpatient clinic <sup>‡</sup>
Wentzensen (2014)	Discovery (TCGA data) and validation (endometrial brush)	Prospective	U	Prior to hysterectomy

<sup>†</sup>Presenting symptoms only described for cases.

<sup>‡</sup>Leftover material from population-based cervical screening or attending a gynecological outpatient clinic.

TCGA = The Cancer Genome Atlas; U = unknown.





# CHAPTER 4

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## DNA METHYLATION TESTING FOR ENDOMETRIAL CANCER DETECTION IN URINE, CERVICOVAGINAL SELF-SAMPLES AND CERVICAL SCRAPES

Published as:

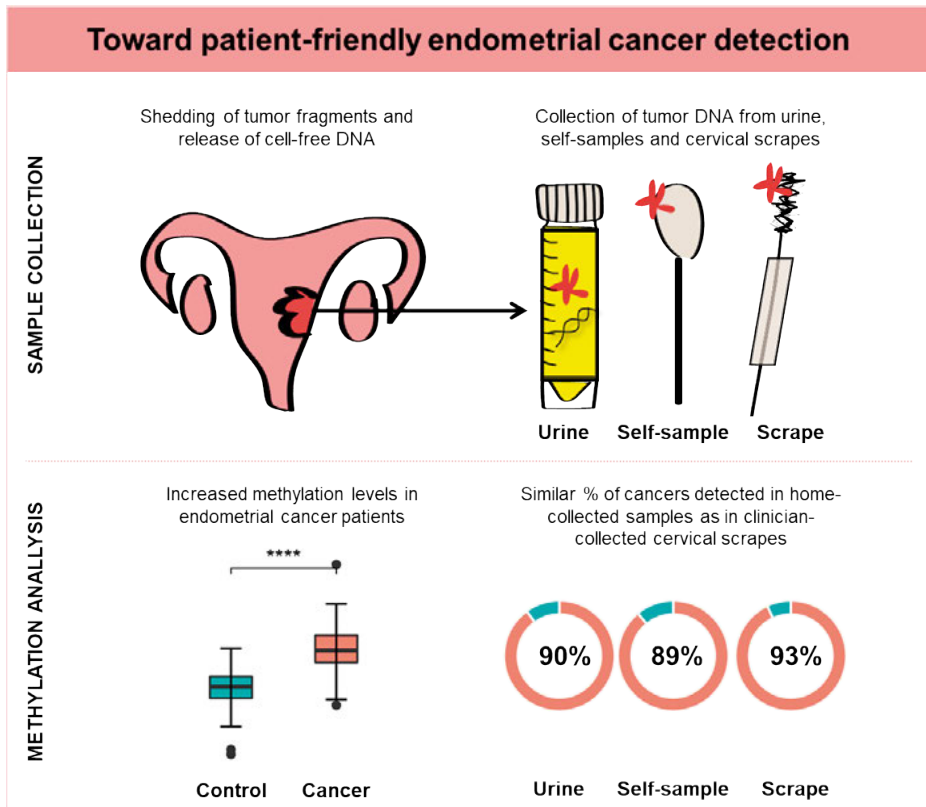
Wever, B.M.M.\* & van den Helder, R.\*; van Splunter, A.P., van Gent, M.D.J.M., Kasius, J.C., Trum, J.W., Verhoeve, H.R., van Baal, W.M., Hulbert, A., Verhoef, L., Heideman, D.A.M., Lissenberg-Witte, B.I., van Trommel, N.E., Steenbergen, R.D.M.\* & Bleeker, M.C.G.\* (2023). DNA methylation testing for endometrial cancer detection in urine, cervicovaginal self-samples, and cervical scrapes. *International Journal of Cancer*. 153(2): 341-351.

\*Equal contribution.

## ABSTRACT

Endometrial cancer incidence is rising and current diagnostics often require invasive biopsy procedures. DNA methylation marker analysis of minimally- and non-invasive sample types could provide an easy-to-apply and patient-friendly alternative to determine cancer risk. Here, we compared the performance of DNA methylation markers to detect endometrial cancer in urine, cervicovaginal self-samples and clinician-taken cervical scrapes. Paired samples were collected from 103 patients diagnosed with stage I to IV endometrial cancer. Urine and self-samples were collected at home. All samples were tested for nine DNA methylation markers using quantitative methylation-specific PCR. Methylation levels measured in endometrial cancer patients were compared to unpaired samples of 317 healthy controls. Diagnostic performances were evaluated by univariable and multivariable logistic regression analysis, followed by leave-one-out cross-validation. Each methylation marker showed significantly higher methylation levels in all sample types of endometrial cancer patients compared to healthy controls ( $P < .01$ ). Optimal three-marker combinations demonstrated excellent diagnostic performances with area under the receiver operating curve values of 0.95 (95% CI: 0.92-0.98), 0.94 (0.90-0.97) and 0.97 (0.96-0.99), for endometrial cancer detection in urine, self-samples and scrapes, respectively. Sensitivities ranged from 89% to 93% at specificities of 90% to 92%. Virtually equal performances were obtained after cross-validation and excellent diagnostic performances were maintained for stage I endometrial cancer detection. Our study shows the value of methylation analysis in patient-friendly sample types for endometrial cancer detection of all stages. This approach has great potential to screen patient populations at risk for endometrial cancer.

## GRAPHICAL ABSTRACT



## NOVELTY AND IMPACT

Endometrial cancer incidence is rising and current diagnostic approaches often require invasive biopsy procedures. Here, the authors compared the diagnostic value of endometrial cancer detection by DNA methylation testing between paired urine samples, cervicovaginal self-samples and clinician-taken cervical scrapes. Endometrial cancer detection in samples collected by home-based methods was excellent and comparable to diagnostic performance in clinician-taken cervical scrapes. The results demonstrated the value of methylation analysis in patient-friendly sample types for detection of endometrial cancer of all stages. The approach has great potential to non-invasively screen patient populations at risk for endometrial cancer.

## INTRODUCTION

Endometrial cancer is the most frequently diagnosed cancer of the female genital tract and the sixth most common cancer in women globally (1). With its rising incidence worldwide, endometrial cancer accounted for 417,000 new diagnoses and over 97,000 deaths in 2020 (2, 3). Early detection is crucial since advanced stage disease has a poor prognosis and a high risk of relapse (4).

In about 90% of cases, postmenopausal bleeding precedes endometrial cancer (5). Consequently, diagnostic procedures that require a referral for specialized care are indicated for all patients presenting with postmenopausal bleeding, causing discomfort and high healthcare costs (6-8). Yet only 5% to 10% of patients with this common alarming symptom have an underlying malignancy (9). Another small subset of asymptomatic endometrial cancer patients is detected via a Pap smear obtained during cytology-based cervical cancer screening or other indications (10-12). As cervical cancer screening programs have shifted from cytology to primary testing for human papillomavirus (HPV) in many countries today, the detection of asymptomatic endometrial cancers via Pap testing is declining (13).

Besides the detection of endometrial cancer in Pap smears, recent cytology research demonstrates that endometrial cancer cells are detectable in vaginal samples (14) and urine (15) by shedding through the cervix into the vaginal debris. An added benefit of using minimally invasive types of diagnostic samples, including urine and cervicovaginal self-samples, is that they can be collected at home, which is rather inexpensive and reduces the burden of health care.

As an alternative to cytology, objective biomarker testing on minimally invasive sample types has demonstrated great potential and would be ideal to triage patients with postmenopausal bleeding. DNA methylation signatures in promoter regions of tumor suppressor genes represent a valuable biomarker for the detection of early-stage disease. In the early stages of cancer development, promoter hypermethylation can lead to gene silencing and loss of their tumor suppressive function (16). Methylation testing does not necessarily require the presence of intact tumor cells for interpretation and is also measurable using tumor-shedded circulating DNA.

Based on our previous studies and literature, nine markers (*ADCYAP1*, *BHLHE22*, *CDH13*, *CDO1*, *GALR1*, *GHSR*, *HAND2*, *SST*, and *ZIC1*) are considered to be suitable for detection of endometrial cancer in minimally invasive sample types (17-25). Our study was initiated to evaluate the diagnostic performance of endometrial cancer detection using DNA

methylation analysis of these markers in paired urine, cervicovaginal self-samples and clinician-taken cervical scrapes.

## METHODS

### Study population

#### *Paired minimally- and non-invasive samples from endometrial cancer patients*

Paired samples from endometrial cancer patients were collected within the SOLUTION1 study, between October 2016 and August 2020. Our study included patients diagnosed with endometrial cancer irrespective of FIGO (2009) stage and histological subtype. From each patient, a urine sample, a cervicovaginal self-sample, and a clinician-taken cervical scrape were collected before primary treatment. A complete urine void was collected at home, irrespective of time of collection and personal hygiene. The cervicovaginal self-sample was also collected at home after urine collection. The clinician-taken cervical scrape was collected in the operating room, prior to surgery. In case the clinician-taken cervical scrape was not collected, the residual cytology sample of the cervical scrape that was taken for clinical diagnostics was used.

#### *Unpaired minimally- and non-invasive samples from healthy women*

For comparison, unpaired urine samples, cervicovaginal self-samples, and clinician-taken cervical scrapes from healthy female controls were included. Urine controls were obtained through the Urine Controls (URIC) biobank. Controls were selected for eligibility based on a questionnaire in which age, sex, and cancer history was documented. Only controls without any cancer history in the past 5 years were included. A subset of urine samples was previously used and published (25). Cervicovaginal self-samples and clinician-taken cervical scrapes were derived from leftover material of the Dutch national cervical cancer screening program coordinated by the Dutch National Institute for Public Health and the Environment (RIVM). Selection was based on age and a negative high-risk HPV test. Selected controls were similar to cases with respect to age.

#### *Tissue samples*

Methylation markers *GHSR*, *SST*, and *ZIC1* were previously discovered for cervical cancer detection (26), but also appeared diagnostically relevant for endometrial cancer detection in urine (25). To verify that increased methylation levels originate from the endometrial tumor, formalin-fixed paraffin-embedded (FFPE) tissue specimens of a subset of endometrial cancer patients from the SOLUTION1 study were also tested. FFPE tissues of normal endometrium were collected from patients with early-stage ovarian cancer without metastases to the endometrium who underwent a surgical

staging procedure. The age of selected controls was within the same age range as the cancer patients.

### ***Ethical approval***

Ethical approval was provided by the Medical Ethical Committee of the VU University Medical Center for the use of samples collected within the SOLUTION1 study (METc 2016.213) (Trial registration ID: NL56664.029.16), samples archived under the URIC biobank (TcB 2018.657) and samples archived under the biobank containing leftover material of the Dutch national cervical cancer screening program (TcB2020.245). Women participating in the screening program were informed that their residual cervical sample could be used for anonymized research and had the opportunity to opt out. Only left-over material from women who did not opt out was used. All women were 18 years or older and signed informed consent. For the FFPE tissue samples of normal endometrium, the Code of Conduct for Responsible Use of Left-over Material of the Dutch Federation of Biomedical Scientific Societies was adhered (27).

### **Sample processing**

Urine of endometrial cancer patients and healthy female controls were collected from home in collection tubes containing 0.6 M ethylenediaminetetraacetic acid (EDTA; final concentration: 40 mM), to maintain DNA quality during transport, following a previously validated storage and collection protocol (28). In a previous feasibility study analyzing different urine fractions for optimal endometrial cancer detection using methylation markers, full void urine was shown to perform best and therefore used in the current study (25). The cervicovaginal self-samples were collected using a dry-brush device (Evalyn Brush, Rovers Medical Devices, Oss, The Netherlands). After collection of the urine and cervicovaginal self-sample, these samples were sent together within 72 hours by regular mail to the Pathology department of Amsterdam UMC, location VU University Medical Center, and processed directly upon arrival. Urine was stored at -20°C, and the dry brush was placed in 1.5 mL ThinPrep PreservCyt medium (Hologic, Marlborough, MA, US), vortexed and stored at 4°C. The clinician-taken cervical scrapes were obtained with a Cervex-Brush (Rovers Medical Devices, Oss, The Netherlands), immediately preserved in 10 mL Thinprep Preservcyt medium and stored at 4°C.

FFPE tissue samples were cut into serial sections, and the first and last sections were Hematoxylin and Eosin (H&E) stained for a histopathological review by a pathologist (MB) to confirm the presence of endometrial cancer or normal endometrium.

### DNA extraction and bisulfite treatment

For DNA isolation of full void urine of cases and controls (30 mL; one-third of the original sample), the Quick DNA urine kit (Zymo Research, Irvine, CA) was used. DNA was isolated from the cervicovaginal self-samples and the clinician-taken cervical scrapes of cases and controls (each one-sixth of the original sample) using the NucleoMag 96 Tissue kit (Machery-Nagel) and a Microlab Star robotic system (Hamilton, Germany). DNA of the tissue samples was isolated using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany). The NanoDrop 1000 (ThermoFisher Scientific, Waltham, MA, US) was used to measure the DNA concentration. Bisulfite conversion of isolated DNA was done using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, US). All procedures were performed according to manufacturer's guidelines.

### DNA methylation analysis using quantitative methylation specific PCR

Promoter hypermethylation of the *ADCYAP1*, *BHLHE22*, *CDH13*, *CDO1*, *GALR1*, *GHSR*, *HAND2*, *SST*, and *ZIC1* genes was tested in three multiplex assays by quantitative methylation-specific PCR (qMSP) using 50 ng of bisulfite-converted DNA. Primer and probe sequences were described before (26, 29), or are available upon request. Each assay also targets the reference gene *ACTB* for quantification and quality control. To ensure sufficient sample quality, samples with a Cycle threshold (Ct) value for *ACTB*  $\geq 32$  were excluded from further analysis. Methylation levels were determined using the comparative Ct method using the following formula:  $2^{-(Ct_{\text{marker}} - Ct_{\text{ACTB}})} \times 100$ . The discriminatory power of the qMSP assays was verified by testing tissue specimens of a subset of endometrial cancer patients included in the SOLUTION1 study and normal endometrial tissue specimens as controls.

### Data analysis

Only complete sample sets with valid DNA methylation test results (*ACTB*  $< 32$ ) from endometrial cancer patients were included (e.g. of cases with an invalid urine sample, also the self-sample and scrape were removed from the analysis). Methylation levels were expressed as 2log-transformed Ct ratios. Differences in DNA methylation levels between endometrial cancer patients and controls were visualized using boxplots and tested for statistical significance using the Mann-Whitney U test. To assess the correlation of DNA methylation levels between paired sample types, the Spearman's rank correlation was used. The Spearman's rank correlation coefficient ( $r$ ) was interpreted as poor ( $r \leq .19$ ), fair ( $r = .20 - .39$ ), moderate ( $r = .40 - .59$ ), strong ( $r = .60 - .79$ ), and very strong ( $r \geq .80$ ) (30). The diagnostic performance of individual methylation markers was evaluated by univariable logistic regression analysis in which the predicted probability was calculated for each sample. The predicted probability (a value ranging from 0 to 1) represents the probability for the presence of endometrial cancer. Optimal

three-marker combinations were formed for each sample type using multivariable logistic regression analysis with backward selection. The performance of the individual markers and optimal three-marker panels was visualized using receiver operating characteristic (ROC) curves, including the area under the ROC curve (AUC) with corresponding 95% confidence intervals (CIs). Sensitivities and specificities were based on the Youden's Index (*J*) threshold. Diagnostic performances of each marker and three-marker panels for the detection of early-stage endometrial cancer was evaluated in a sub-analysis in which only stage IA and IB cancers were taken along in the univariable and multivariable regression analyses which were performed as described above. The diagnostic performances of each marker and the three-marker panels were assessed outside the set by leave-one-out cross-validation (LOOCV). Predicted probabilities of the individual markers and optimal three-marker panels were also plotted individually in a heatmap format to illustrate differences between the sample types, histological subtypes and the potential added value of the marker combination.

Data were collected using Castor EDC (31). Statistical analyses were performed in RStudio (v3.6.1) using the *corrplot* (v0.84), *cowplot* (v1.1.0), *compareGroups* (v4.5.1), *dplyr* (v1.0.2), *ggplot2* (v3.3.5), *MASS* (v7.3-58) and *pROC* (v1.18.0) packages. Reported *P*-values are two-sided and considered statistically significant if  $P < .05$ .

## RESULTS

### Study population and characteristics

A total of 158 patients with histologically confirmed endometrial cancer were included in the SOLUTION1 study. For various reasons, mostly because not all three sample types were available ( $n = 40$ ), cases were excluded, resulting in a final study population of 103 endometrial cancer patients (Supplementary Figure 1). Within this group, a paired urine, cervicovaginal self-sample, and clinician-taken cervical scrape of each case were available for methylation analysis. Unpaired samples of control women ( $n = 317$ ) were used for comparison. Clinical characteristics of endometrial cancer patients and controls with valid qMSP results are depicted in Table 1. Additionally, FFPE tissue was collected from endometrial cancer cases of various histological subtypes ( $n = 33$ ) and healthy endometrium ( $n = 15$ ).

### DNA methylation levels in minimally- and non-invasive samples

Methylation levels of *ADCYAP1*, *BHLHE22*, *CDH13*, *CDO1*, *GALR1*, *GHSR*, *HAND2*, *SST*, and *ZIC1* were significantly higher (Mann-Whitney U test; all  $P < .01$ ) in urine samples, cervicovaginal self-samples and clinician-taken cervical scrapes of endometrial cancer

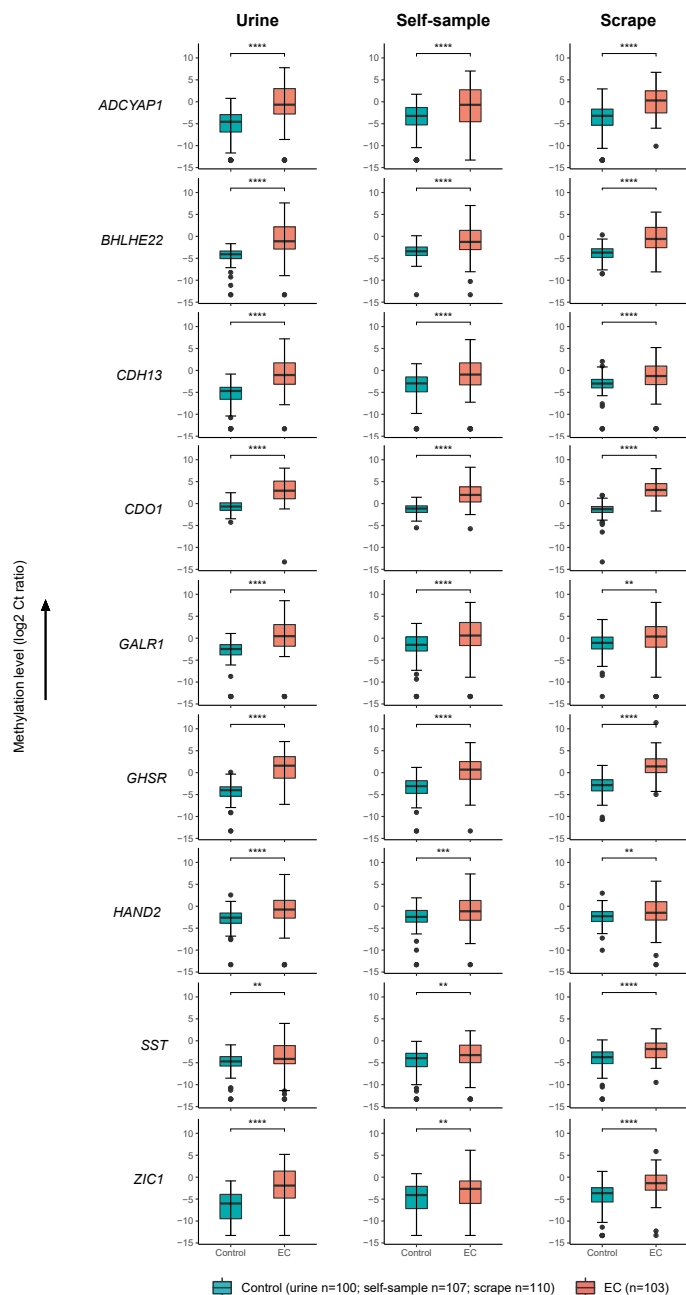
patients as compared to samples of healthy control women (Figure 1). The discriminatory power of all markers was verified at a tissue level by comparing methylation levels of endometrial cancer tissue of various histological subtypes with normal endometrium (Mann–Whitney U test; all  $P < .01$ ; Supplementary Figure 2).

**Table 1:** Characteristics of endometrial cancer patients and controls.

	n	(%)	Age: median (IQR)
<b>Endometrial cancer</b>	103	(100%)	66 (60-71)
Histology			
Endometrioid carcinoma	52	(51%)	
<i>Grade 1</i>	20	(19%)	
<i>Grade 2</i>	17	(17%)	
<i>Grade 3</i>	15	(15%)	
Serous carcinoma	29	(28%)	
Clear cell carcinoma	7	(7%)	
Uterine carcinosarcoma	10	(10%)	
Mixed carcinoma*	3	(3%)	
Uterine sarcoma	2	(2%)	
FIGO stage			
IA	51	(50%)	
IB	21	(21%)	
II	3	(3%)	
III	18	(18%)	
IV	10	(10%)	
<b>Controls</b>			
Urine	100		61 (55-78)
Self-sample	107		60 (60-60)
Scrape	110		60 (55-60)

\*Patients with endometrial carcinomas of mixed subtypes included one mixed clear cell and endometrioid carcinoma, and two mixed clear cell and serous carcinomas.

Correlation of individual markers between sample types was assessed for paired samples from patients with endometrial cancer. DNA methylation levels of six markers (*ADCYAP1*, *BHLHE22*, *CDO1*, *GHSR*, *SST* and *ZIC1*) were moderately to strongly correlated between the different sample types (Spearman correlation;  $r = .43 - .80$ ). Interestingly, while the remaining three markers (*CDH13*, *GALR1*, and *HAND2*) correlated strongly (Spearman correlation;  $r = .72 - .77$ ) between urine and self-samples, a poor correlation was observed between urine and cervical scrapes (Spearman correlation;  $r = .01 - .14$ ) and self-samples and cervical scrapes (Spearman correlation;  $r = .04 - .09$ ).



**Figure 1:** DNA methylation levels of *ADCYAP1*, *BHLHE22*, *CDH13*, *CDO1*, *GALR1*, *GHSR*, *HAND2*, *SST*, and *ZIC1* in urine, cervicovaginal self-samples and clinician-taken cervical scrapes from healthy female controls (n = 100; n = 107; n = 110, respectively) and endometrial cancer patients (n = 103). DNA methylation levels are shown by the 2log-transformed Ct ratios. Boxplots illustrate medians with lower and upper quartile and range whiskers. Outliers are indicated with black circles. A P-value of < .05 was considered statistically significant. \*\*:  $p \leq 0.01$ ; \*\*\*:  $p \leq 0.001$ ; \*\*\*\*:  $p \leq 0.0001$ . Ct, cycle threshold; EC, endometrial cancer.

Correlation coefficients for the individual markers between the different sample types are presented in Table 2. Correlation coefficients between all markers are illustrated in Supplementary Figure 3, showing that the majority of markers correlated highly between urine and self-samples while less correlation was seen when comparing urine and self-samples with cervical scrapes.

**Table 2:** Correlation of DNA methylation markers between sample types.

	<i>ADCYAP1</i>	<i>BHLHE22</i>	<i>CDH13</i>	<i>CDO1</i>	<i>GALR1</i>	<i>GHSR</i>	<i>HAND2</i>	<i>SST</i>	<i>ZIC1</i>
<b>Urine vs Self-sample</b>	0.72	0.80	0.76	0.72	0.77	0.72	0.72	0.43	0.64
<b>Urine vs Scrape</b>	0.70	0.67	0.09	0.51	0.14	0.63	0.01	0.44	0.58
<b>Scrape vs Self-sample</b>	0.68	0.77	0.09	0.61	0.07	0.62	0.04	0.60	0.61

The Spearman's rank correlation coefficients (*r*) of DNA methylation markers *ADCYAP1*, *BHLHE22*, *CDH13*, *CDO1*, *GALR1*, *GHSR*, *HAND2*, *SST*, and *ZIC1* between paired samples of women diagnosed with endometrial cancer (*n* = 103). The Spearman's rank correlation coefficient was calculated based on 2log-transformed Ct ratios. Ct, cycle threshold.

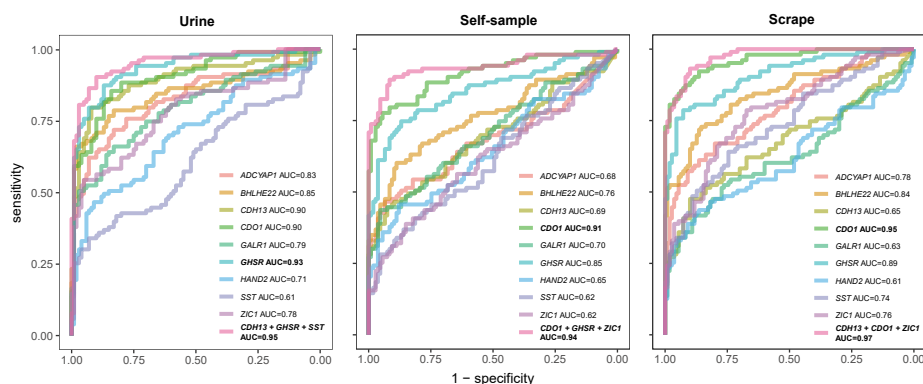
*r* ≤ .19 poor correlation, *r* = .20 - .39 fair correlation, *r* = .40 - .59 moderate correlation, *r* = .60 - .79 strong correlation, *r* ≥ .80 very strong correlation.

### Performance of DNA methylation analysis for endometrial cancer detection

The diagnostic performance of each marker was assessed individually by univariable logistic regression analysis and validated by LOOCV. In urine, the non-cross-validated AUCs of the DNA methylation markers to detect endometrial cancer ranged between 0.61 – 0.93, in cervicovaginal self-samples between 0.62 – 0.91, and in clinician-taken cervical scrapes between 0.61 – 0.95 (Figure 2, Table 3). Most markers, seven out of nine, showed the highest performance in urine: *ADCYAP1* (AUC 0.83), *BHLHE22* (AUC 0.85), *CDH13* (AUC 0.90), *GALR1* (AUC 0.79), *GHSR* (AUC 0.93), *HAND2* (AUC 0.71), and *ZIC1* (AUC 0.78). The remaining markers performed best in clinician-taken cervical scrapes: *CDO1* (AUC 0.95) and *SST* (AUC 0.74). Nonetheless, except for *CDH13* in urine, the 95% confidence interval of AUCs were overlapping between paired sample types.

DNA methylation marker panels, rather than single genes, may increase the diagnostic accuracy for endometrial cancer detection. Multivariable logistic regression with backward selection was applied to identify the most optimal three-marker combinations for each sample type. This selection procedure created marker panels with increased AUC values of 0.95 (95% CI: 0.92-0.98) for urine by combining *CDH13* + *GHSR* + *SST*, 0.94 (95% CI: 0.90-0.97) for cervicovaginal self-samples by combining *CDO1* + *GHSR* + *ZIC1*, and 0.97 (95% CI: 0.96-0.99) for clinician-taken cervical scrapes by combining *CDH13* + *CDO1* + *ZIC1*. Marker panels allowed endometrial cancer detection with increased sensitivity, without a major impact on specificity. This was especially the case in cervicovaginal self-samples and clinician-taken cervical scrapes. The sensitivity and specificity of single genes in urine ranged

from 34% to 87% and 84% to 98%, respectively, and the marker combination yielded a sensitivity and specificity of both 90%. For cervicovaginal self-samples, the sensitivity and specificity of single genes ranged from 28% to 78% and 85% to 95%, respectively, while the marker combination revealed a sensitivity of 89% and specificity of 92%. Similarly, for the clinician-taken cervical scrapes, the sensitivity and specificity of the single genes ranged from 44% to 87% and 67% to 93%, respectively, while the marker combination sensitivity was 93% with a specificity of 90%. Sensitivities and specificities were calculated based on the maximal Youden's Index (*J*) threshold (Supplementary Table 1).



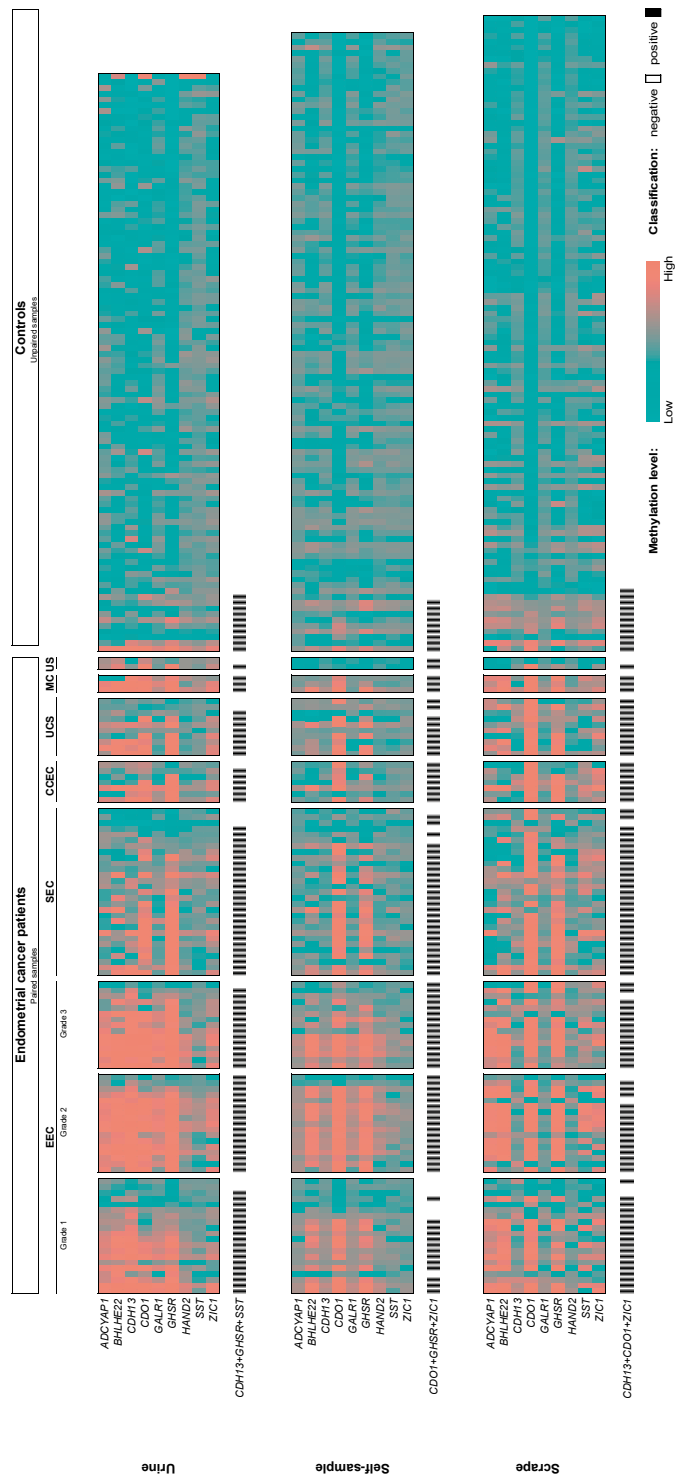
**Figure 2:** Diagnostic performance of individual markers (*ADCVAP1*, *BHLHE22*, *CDH13*, *CDO1*, *GALR1*, *GHSR*, *HAND2*, *SST*, *ZIC1*) and the optimal three-marker panels (based on multivariable logistic regression) for endometrial cancer detection in urine, cervicovaginal self-samples and clinician-taken cervical scrapes. Non-cross-validated ROC curves are shown and quantified by AUC values. Individual genes and marker panels with the highest performance per sample type are depicted in bold. AUC, area under the ROC curve; ROC, receiver operating characteristic.

The diagnostic performance of individual markers and marker panels were validated by LOOCV, which yielded virtually equal AUC values (Table 3), sensitivities, and specificities (Supplementary Table 2) for the single markers and optimal three marker combinations. Additionally, the performance for early-stage endometrial cancer detection was assessed by performing a sub-analysis including only stage I endometrial cancers ( $n = 72$ ). This revealed nearly equal diagnostic performances for both the individual markers and marker panels in urine, cervicovaginal self-samples and clinician-taken cervical scrapes, which were also validated by LOOCV (Supplementary Table 3).

**Table 3:** Diagnostic performance of the nine individual methylation markers (univariable logistic regression) and optimal three-marker panels (multivariable logistic regression) for endometrial cancer detection in the different sample types.

<b>Urine</b>	<b>ADCVAP1</b>	<b>BHLHE22</b>	<b>CDH13</b>	<b>CDO1</b>	<b>GALR1</b>	<b>GHSR</b>	<b>HAND2</b>	<b>SST</b>	<b>ZIC1</b>	<b>CDH13+GHSR+SST</b>
AUC (non-CV; 95%-CI)	0.83 (0.77-0.88)	0.85 (0.79-0.90)	0.90 (0.85-0.94)	0.90 (0.86-0.94)	0.79 (0.73-0.86)	<b>0.93</b> <b>(0.90-0.97)</b>	0.71 (0.64-0.78)	0.61 (0.53-0.69)	0.78 (0.71-0.84)	<b>0.95</b> <b>(0.92-0.98)</b>
Sens (%)	62	66	80	85	63	87	47	34	54	90
Spec (%)	93	98	92	84	86	87	91	93	95	90
AUC (LOOCV)	0.81	0.84	0.89	0.90	0.78	0.93	0.69	0.58	0.76	0.93
<b>Self-sample</b>	<b>ADCVAP1</b>	<b>BHLHE22</b>	<b>CDH13</b>	<b>CDO1</b>	<b>GALR1</b>	<b>GHSR</b>	<b>HAND2</b>	<b>SST</b>	<b>ZIC1</b>	<b>CDO1+GHSR+ZIC1</b>
AUC (non-CV; 95%-CI)	0.68 (0.61-0.76)	0.76 (0.70-0.89)	0.69 (0.62-0.77)	<b>0.91</b> <b>(0.87-0.95)</b>	0.70 (0.63-0.77)	0.85 (0.80-0.91)	0.65 (0.57-0.73)	0.62 (0.54-0.70)	0.62 (0.54-0.69)	<b>0.94</b> <b>(0.90-0.97)</b>
Sens (%)	47	60	45	78	44	75	46	28	39	89
Spec (%)	93	89	93	94	95	87	88	95	85	92
AUC (LOOCV)	0.65	0.76	0.68	0.91	0.69	0.84	0.63	0.60	0.58	0.92
<b>Scrape</b>	<b>ADCVAP1</b>	<b>BHLHE22</b>	<b>CDH13</b>	<b>CDO1</b>	<b>GALR1</b>	<b>GHSR</b>	<b>HAND2</b>	<b>SST</b>	<b>ZIC1</b>	<b>CDH13+CDO1+ZIC1</b>
AUC (non-CV; 95%-CI)	0.79 (0.73-0.85)	0.84 (0.79-0.90)	0.68 (0.60-0.75)	<b>0.96</b> <b>(0.94-0.98)</b>	0.63 (0.55-0.71)	0.91 (0.87-0.95)	0.62 (0.54-0.70)	0.74 (0.67-0.80)	0.78 (0.71-0.84)	<b>0.97</b> <b>(0.96-0.99)</b>
Sens (%)	57	73	48	87	42	79	44	64	79	93
Spec (%)	91	85	90	92	88	93	86	75	67	90
AUC (LOOCV)	0.79	0.84	0.66	0.96	0.61	0.90	0.60	0.73	0.77	0.97

Individual methylation markers and marker panels with the highest AUC value per sample type are depicted in bold. AUCs, including 95% CI, are reported together with sensitivities and specificities based on the maximal Youden's index (J) threshold. LOOCV AUC values are reported without sensitivity and specificity. AUC = area under the receiver operating characteristic curve; CI = confidence interval; LOOCV = leave-one-out cross-validated; non-CV = non-cross-validated; Sens = sensitivity; Spec = specificity.



**Figure 3:** DNA methylation of single markers *ADCYAP1*, *BHLHE22*, *CDH13*, *CD01*, *GALR1*, *GHSR*, *HAND2*, *SST*, and *ZIC1* and optimal marker panels for endometrial cancer detection in urine, cervicovaginal self-samples, and clinician-taken cervical scrapes. Paired samples of cancers are stratified per histological category. In the Mixed carcinoma (MC) category, the first column represents a mixed endometrioid and clear-cell carcinoma and the last two columns are mixed clear-cell and serous carcinomas. Methylation levels (row) are shown per sample (column) and visualized using predicted probabilities on a two-color gradient from green (low methylation level; predicted probability of 0) to red (high methylation level; predicted probability of 1). Black boxes represent methylation positive samples detected using the optimal marker panel (i.e. classified as case by multivariable logistic regression using predicted probability thresholds of  $\geq 0.40$  for urine,  $\geq 0.46$  for self-samples, and  $\geq 0.34$  for scrapes). CCEC, Clear cell endometrial carcinoma; EEC, Endometrioid endometrial carcinoma; MC, Mixed carcinoma; SEC, Serous endometrial carcinoma; UCS, Uterine carcinosarcoma; US, Uterine sarcoma.

Figure 3 visualizes the methylation levels of all individual markers per sample for all patients and controls individually using the predicted probabilities computed during the ROC curve analyses. Samples of controls showed predominantly low methylation levels (green), whereas samples of patients with endometrial cancer showed high methylation levels (red). Paired endometrial cancer cases were stratified by histological subtype. Within the non-endometrioid cancers, increased methylation was predominantly seen in *CDO1* and *GHSR*, followed by *BHLHE22*. The marker panels formed by multivariable logistic regression detected more cancers than the single markers. Samples were classified as positive (black box) based on the Youden's Index (*J*) thresholds of the three-marker panels calculated for urine ( $\geq 0.40$ ), self-samples ( $\geq 0.46$ ) and scrapes ( $\geq 0.34$ ). Using this threshold, 90% (93/103), 89% (92/103), and 93% (96/103) of the cancers were classified as cases and 10% (10/100), 8% (9/107) and 10% (11/110) of the controls were classified as cases in the urine, self-samples and scrapes, respectively.

## DISCUSSION

Our study presents the diagnostic potential of DNA methylation testing in minimally- and non-invasive samples for endometrial cancer detection. The diagnostic performance of nine DNA methylation markers and most optimal three-marker panels for endometrial cancer detection were evaluated and compared between the different sample types. Endometrial cancer detection in samples collected by home-based methods was excellent and comparable to the diagnostic performance of methylation testing in clinician-taken cervical scrapes. Three-marker combinations yielded an AUC value of 0.95 (95% CI: 0.92-0.98), 0.94 (0.90-0.97) and 0.97 (0.96-0.99), for endometrial cancer detection in urine, self-samples and scrapes, respectively. Virtually equal performances were obtained after cross-validation. Excellent diagnostic performances were maintained in stage I endometrial cancers, confirming the ability to detect endometrial cancer at its earliest stage. Our study emphasizes the outstanding potential of DNA methylation analysis using patient-friendly home-based sample collection methods for endometrial cancer detection.

Several discovery screens identified valuable hypermethylated genes as biomarker candidates for endometrial cancer detection in minimally invasive specimens (17-19, 21, 23). The markers *ADCYAP1* (18), *BHLHE22* (17), *CDH13* (18), *CDO1* (17, 24, 32), *GALR1* (21), and *HAND2* (23, 24) tested in our study originate from such discovery screens carried out by different research groups. Except for *CDO1* and *HAND2*, none of these markers have been independently validated before for endometrial cancer detection in minimally- or non-invasive samples. All genes showed a significant difference in

each sample type tested when comparing endometrial cancers with healthy controls. *ADCYAP1* and *CDH13* performed particularly well in urine samples, with AUC values of 0.83 and 0.90, respectively. In line with previous studies (17, 24, 32), *CDO1* allowed endometrial cancer detection with excellent performance in all sample types tested (AUC 0.90-0.96). *HAND2* performed moderately in all sample types (AUC 0.62-0.71), as opposed to previously described performances of 0.91 and 0.96 in vaginal swabs of stage IA and stage IB/II/III patients, respectively (23). On the other hand, the diagnostic performance of *HAND2* found in our study is more similar to the AUC of 0.77 in cervical scrapes as reported by Liew *et al.* (24). *GALR1* performed best in urine (AUC 0.79) but did not reach the AUC value of 0.93 as found in vaginal swabs by Doufekas *et al.* (21). The methylation markers included in our study were originally discovered on tissue material, rather than the presently tested samples types, which could explain differences in their performance.

It has been demonstrated that endometrial cancer is detectable in cervical scrapes, vaginal tampons, vaginal brushes and urine by DNA methylation analysis (20, 23, 25, 33-37). The collection of paired samples in the present study allowed a comprehensive comparison of their diagnostic performance. Most methylation marker levels correlated moderately to strongly between the different sample types, except for *CDH13*, *GALR1* and *HAND2*, which correlated poorly when comparing urine and self-samples with cervical scrapes. Among these markers, a clear difference in performance was observed for *CDH13* in the different sample types, with an AUC value of 0.90 in urine as opposed to AUC values of 0.69 and 0.65 in self-samples and scrapes. These results indicate that methylation markers may not have equal performance in different sample types, which could be explained by the differences in background DNA of each sample type and the source of methylated DNA. While mostly shedded endometrial material is collected by self-samples and scrapes, the full void urine contains both shedded material and transrenally excreted cell-free DNA (25).

The application of previously discovered methylation markers in endometrial carcinomas of non-endometrioid histologies has remained largely unexplored, as previous studies included mostly endometrioid carcinomas. Even though non-endometrioid carcinomas are rare, early detection of this aggressive subtype is critical as they have a higher risk to metastasize and a substantially worse prognosis (38). Our study revealed differences in DNA methylation changes between endometrioid and non-endometrioid cancers. While endometrioid carcinomas showed increased methylation of all methylation markers, the non-endometrioid carcinomas showed particularly increased *CDO1* and *GHSR* methylation, followed by *BHLHE22*. Interestingly, *CDO1* and *GHSR* are also known as pan-cancer markers, as they are described to be highly methylated in many human cancers

(39, 40). Although their performance is excellent, they are probably not specific for endometrial cancer only and combining them with endometrial cancer-specific markers might be valuable for endometrial cancer-specific test development. Methylation of *BHLHE22* and *CDO1* in non-endometrioid cancers has previously been reported by Huang *et al.* (17) and Liew *et al.* (24) who identified and validated the performance of these genes in cervical scrapes. This panel is now commercially available as the MPap® Test (37). Differences in methylation signatures between endometrioid and non-endometrioid tumors should be taken into account during the development of a methylation-based test to allow the detection of all (molecular) subtypes of endometrial cancer.

DNA methylation analysis for endometrial cancer detection offers a sensitive molecular test, applicable to both minimally- and non-invasive sample types. This easy-to-apply approach offers the potential to reduce the number of biopsy procedures, thereby reducing costs and easing pressure on the healthcare system. The cervicovaginal self-sampling device provides a home-based sampling method which is introduced in the Dutch cervical cancer screening program to increase screening participation (41). Logistics around transport and sample processing of this sample type is already in place in diagnostic laboratories in The Netherlands, which eases its implementation for endometrial cancer diagnostics. Urine is another attractive diagnostic sample type for the detection of endometrial cancer (25). This liquid biopsy has gained more interest because it is easy to obtain and preferred by women over other sample types (42). Apart from locally shedded cellular tumor-DNA, urine also contains transrenally excreted tumor-derived cell-free DNA which poses an additional advantage (14, 43, 44).

DNA methylation testing on patient-friendly sample types may contribute to the timely detection of endometrial cancer in patients with symptoms of postmenopausal bleeding. Moreover, this method is promising to screen asymptomatic women at risk for endometrial cancer (*i.e.* women with inherited cancer syndromes, such as Lynch or Cowden) which are currently intensively screened using repeated endometrial biopsies. This approach may reduce the number of invasive procedures within these patient groups and prioritize the use of resources for patients in greater need in times of scarcity. Methylation testing in patient-friendly samples could also be valuable for recurrence detection after curative intent treatment, as recently explored in plasma by Beinse *et al.* (45).

To assess the clinical applicability of this approach for abovementioned purposes, DNA methylation testing needs to be further validated on samples of patients presenting with postmenopausal bleeding with varying final diagnoses (*i.e.* including women without

abnormalities, benign endometrial conditions and cancer) or patient populations with an increased risk of developing endometrial cancer. During finalization of the current article, the clinical utility of simple methylation-based tests in self-collected samples for endometrial cancer detection was also evaluated by Herzog *et al.* (35). Methylation levels of two regions of the *GYPE* gene and the *ZC1112* gene allowed endometrial cancer detection in cervical scrapes, vaginal swabs and self-collected cervicovaginal samples with high accuracy in a cohort of women presenting with postmenopausal blood loss. Of note, the specificity in clinician-collected cervical scrapes was substantially lower (76%) as compared to present findings (92%). Nevertheless, their results are complementary to ours and independently exemplify the potential of epigenomic testing in self-collected samples.

Our study is limited by the fact that the distribution of histopathological subtypes included in our study does not reflect the natural prevalence. The inclusion of patients diagnosed with endometrial cancer mainly occurred in tertiary care cancer centers treating high-grade cancers and rare histopathological subtypes. It is, however, worth noting that early detection of high-grade non-endometrioid cancers is of utmost importance given their worse prognosis (38). Samples were not collected at first clinical presentation but after endometrial cancer diagnosis was made based on a pipelle or hysteroscopic biopsy. This order would be different when home-based sampling would be applied in clinical practice. Sample collection after endometrial biopsy might have facilitated the release of tumor DNA into the urine or vaginal fluid. Yet, the influence of biopsy procedures on the presence of tumor DNA in self-collected specimen is most likely limited as the median time between biopsy and self-sampling was 37 days. In some excluded cases the complete carcinoma was biopsied with no or minimal residual cancer being found during histopathological evaluation of the uterus. It is conceivable that DNA methylation testing will be even more accurate when used at first clinical presentation. Finally, this comparative study had no access to paired samples from controls and did not include controls with postmenopausal bleeding symptoms or benign endometrial conditions. Even though others have shown that most of the markers tested in our study enable discrimination between benign endometrial pathology and cancer (17, 18, 20, 21, 23, 24, 32), this was not validated in the current study.

Strengths of the current study are that nine DNA methylation markers, originated from different discovery screens, were tested on a large series of 626 samples. Over a 100 patients diagnosed with endometrial cancer were included, encompassing the full and heterogeneous range of endometrial cancer histotypes, grades and FIGO (2009) stages. Methylation marker assays were multiplexed to measure the methylation levels of three genes and a reference gene within the same reaction, without loss of PCR efficiency, to

reduce hands-on time, costs and the amount of DNA needed. Self-samples and urines were collected at home, which is an appropriate setting to evaluate the use of home-based sampling for endometrial cancer detection. The collection of paired sample types allowed a comprehensive comparison of their performance.

Our study demonstrates that DNA methylation testing allows endometrial cancer detection with high sensitivity and specificity using a three-marker panel of methylated genes in patient-friendly sample types that can be collected at home. Following validation in additional cohorts, including individuals presenting with postmenopausal bleeding and asymptomatic women at-risk for endometrial cancer, methylation testing could be valuable as a preselection method to inexpensively determine who needs to undergo invasive endometrial tissue sampling and facilitate timely diagnosis.

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### **DATA AVAILABILITY STATEMENT**

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

### **CONFLICT OF INTEREST**

Renske D.M. Steenbergen and Daniëlle A.M. Heideman have a minority share in Self-screen B.V., a spin-off company of Amsterdam UMC, location VUmc. Self-screen B.V. holds patents and products related to the work (methylation markers for cervical screening). The remaining authors declare no potential conflicts of interest.

### **AUTHORS' CONTRIBUTIONS**

BMMW: Resources, data curation, formal analysis, investigation, visualization, methodology, writing-original draft. RvdH: Conceptualization, resources, data curation, formal analysis, investigation, visualization, methodology, writing-original draft. APvS: Investigation, methodology, writing-review and editing. MDJMvG: Resources, writing-review and editing. JCK: Resources, writing-review and editing. JWT: Resources, writing-

review and editing. HRV: Resources, writing-review and editing. WMvB: Resources, writing-review and editing. AH: Methodology, resources, writing-review and editing. LV: Resources, writing-review and editing. DAMH: Resources, writing-review and editing. BILW: Formal analysis, writing-review and editing. NEvT: Conceptualization, supervision, funding acquisition, validation, writing-review and editing, project administration. RDMS: Conceptualization, supervision, funding acquisition, validation, writing-original draft, project administration. MCGb: Conceptualization, supervision, funding acquisition, validation, writing-original draft, project administration.

The work reported in the paper has been performed by the authors, unless clearly specified in the text.

Parts of the PhD thesis of Rianne van den Helder are included in the current manuscript.

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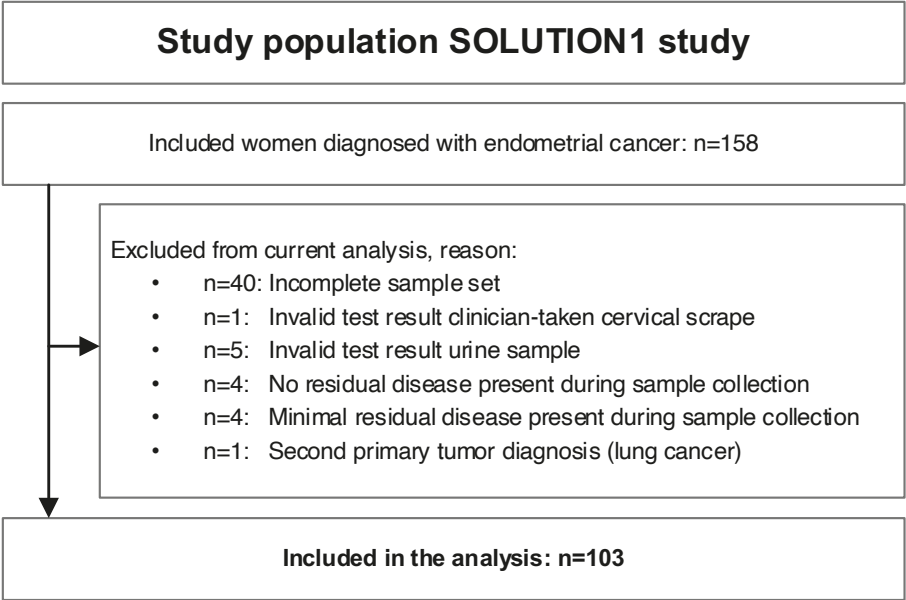
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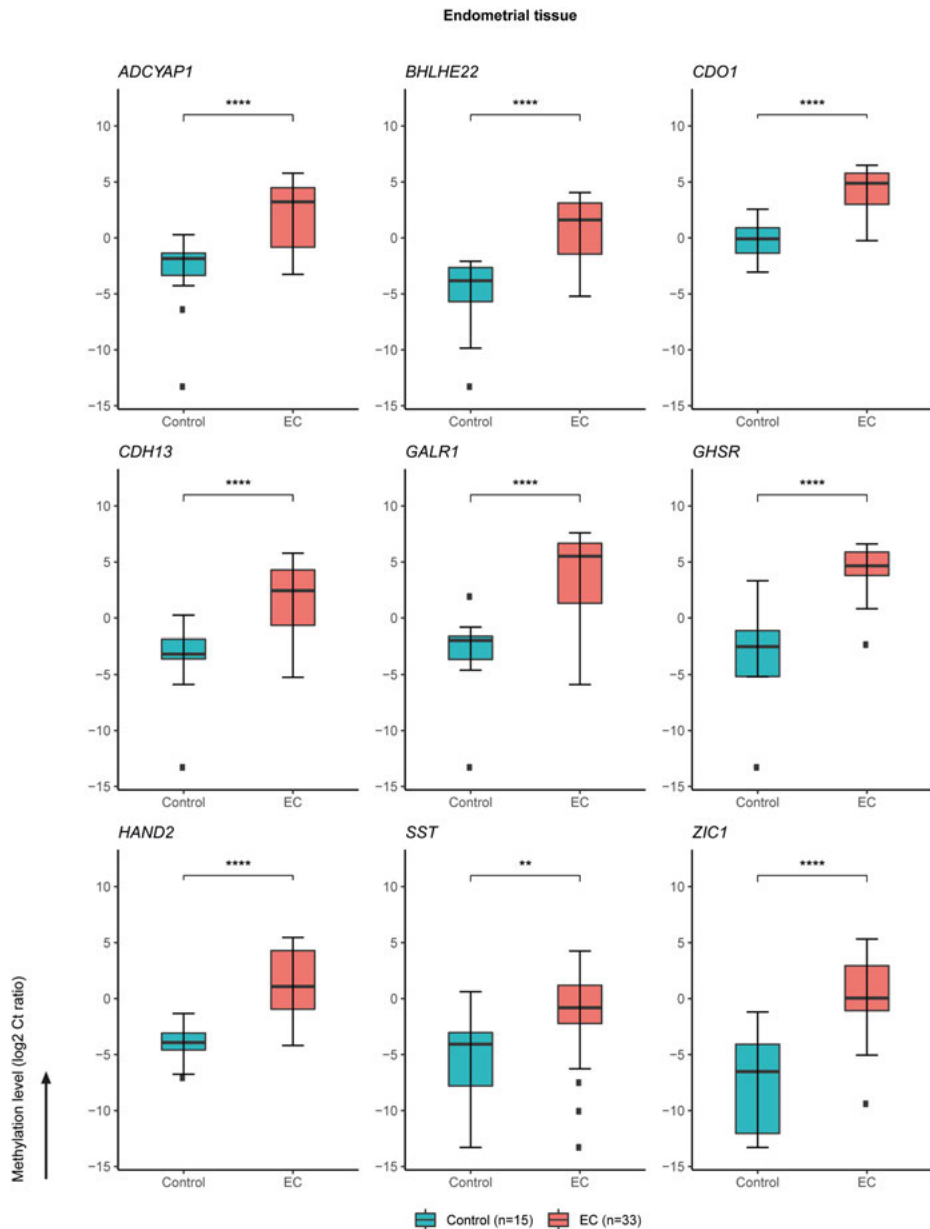
## SUPPLEMENTARY INFORMATION

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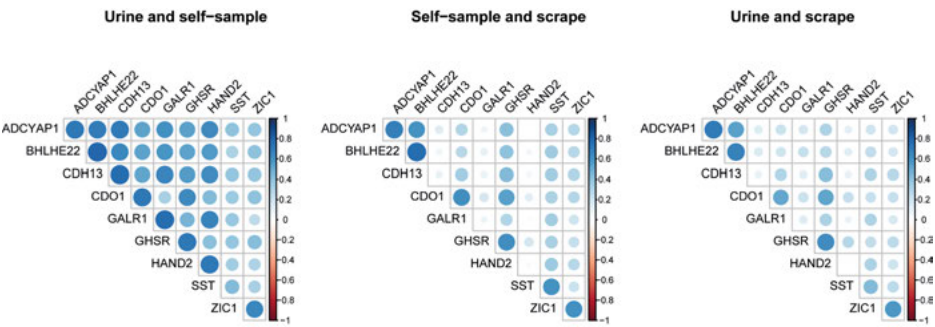
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**Supplementary Figure 1:** Flowchart illustrating excluded endometrial cancer patients (n=55) from the total study population (n=158) due to various reasons: incomplete sample-set (n=40), invalid test-result for DNA methylation analysis (n=6), no residual cancer during sample collection (n=4), minimal residual cancer (*i.e.* < 5 mm) during sample collection, and diagnosed with synchronous lung cancer (n=1).



**Supplementary Figure 2:** DNA methylation levels of *ADCYAP1*, *BHLHE22*, *CDH13*, *CDO1*, *GALR1*, *GHSR*, *HAND2*, *SST*, and *ZIC1* in endometrial cancer (n=33) and normal endometrial tissue (n=15). DNA methylation levels are shown by the 2log-transformed Ct ratios. Boxplots illustrate medians with lower and upper quartile and range whiskers. Outliers are indicated with black squares. A *p*-value of <0.05 was considered statistically significant. \*\*: *p* < 0.01; \*\*\*\*: *p* < 0.0001. EC = endometrial cancer.



**Supplementary Figure 3:** The Spearman's rank correlation coefficients ( $r$ ) of methylation markers *ADCYAP1*, *BHLHE22*, *CDH13*, *CDO1*, *GALR1*, *GHSR*, *HAND2*, *SST*, and *ZIC1* between paired samples of patients diagnosed with endometrial cancer. The Spearman's rank correlation coefficient was calculated based on 2log-transformed Ct ratios. Dot color and size indicate the degree of correlation (*i.e.* the larger and darker the dot, the more correlation).

**Supplementary Table 1:** Youden's Index (*J*) thresholds for maximal sensitivity and specificity calculations of individual markers and marker panels.

	Urine	Self-sample	Scrape
<i>ADCYAP1</i>	0.59	0.57	0.62
<i>BHLHE22</i>	0.59	0.54	0.50
<i>CDH13</i>	0.42	0.55	0.43
<i>CDO1</i>	0.50	0.57	0.55
<i>GALR1</i>	0.53	0.58	0.53
<i>GHSR</i>	0.57	0.55	0.52
<i>HAND2</i>	0.44	0.51	0.62
<i>SST</i>	0.56	0.58	0.51
<i>ZIC1</i>	0.63	0.54	0.46
<i>CDH13+GHSR+SST</i>	0.40	-	-
<i>CDO1+GHSR+ZIC1</i>	-	0.46	-
<i>CDH13+CDO1+ZIC1</i>	-	-	0.34

Thresholds represent predicted probability values.

**Supplementary Table 2:** LOOCV univariable logistic regression analysis on the diagnostic performance of the nine individual markers and LOOCV multivariable logistic regression analysis on the diagnostic performance of the optimal marker combinations for endometrial cancer detection in the different sample types. Individual methylation markers with the highest AUC value per sample type are depicted in bold.

<b>Urine</b>	<b>ADCVAP1</b>	<b>BHLHE22</b>	<b>CDH13</b>	<b>CDO1</b>	<b>GALR1</b>	<b>GHSR</b>	<b>HAND2</b>	<b>SST</b>	<b>ZIC1</b>	<b>CDH13+GHSR+SST</b>
AUC (LOOCV)	0.81	0.84	0.89	0.90	0.78	0.93	0.69	0.58	0.76	0.93
Sens (%)	62	66	79	82	62	80	44	29	54	87
Spec (%)	93	98	92	87	86	93	92	96	94	90
<b>Self-sample</b>	<b>ADCVAP1</b>	<b>BHLHE22</b>	<b>CDH13</b>	<b>CDO1</b>	<b>GALR1</b>	<b>GHSR</b>	<b>HAND2</b>	<b>SST</b>	<b>ZIC1</b>	<b>CDO1+GHSR+ZIC1</b>
AUC (LOOCV)	0.65	0.76	0.68	0.91	0.69	0.84	0.63	0.60	0.58	0.92
Sens (%)	47	60	44	80	44	72	46	28	31	84
Spec (%)	92	88	93	91	93	89	85	93	86	90
<b>Scrape</b>	<b>ADCVAP1</b>	<b>BHLHE22</b>	<b>CDH13</b>	<b>CDO1</b>	<b>GALR1</b>	<b>GHSR</b>	<b>HAND2</b>	<b>SST</b>	<b>ZIC1</b>	<b>CDH13+CDO1+ZIC1</b>
AUC (LOOCV)	0.79	0.84	0.66	0.96	0.61	0.90	0.60	0.73	0.77	0.97
Sens (%)	57	72	48	86	53	75	43	64	67	92
Spec (%)	91	85	87	93	74	95	86	74	78	89

The sensitivity and specificity corresponding to the LOOCV AUCs are computed using the Youden's Index (*J*) threshold. AUC = area under the receiver operating characteristic curve; LOOCV = leave-one-out cross-validated.

**Supplementary Table 3:** Diagnostic performance of the nine individual methylation markers (univariable logistic regression) and optimal three-marker panels (multivariable logistic regression) for early-stage endometrial cancer detection (n=72) in the different sample types.

<b>Urine</b>	<b>ADCYAP1</b>	<b>BHLHE22</b>	<b>CDH13</b>	<b>CDO1</b>	<b>GALR1</b>	<b>GHSR</b>	<b>HAND2</b>	<b>SST</b>	<b>ZIC1</b>	<b>CDH13+GHSR+SST</b>
AUC (non-CV; 95%-CI)	0.86 (0.80-0.92)	0.87 (0.81-0.93)	0.90 (0.85-0.95)	0.89 (0.84-0.94)	0.83 (0.76-0.89)	<b>0.92</b> <b>(0.88-0.96)</b>	0.74 (0.66-0.82)	0.58 (0.49-0.67)	0.78 (0.71-0.85)	<b>0.94</b> <b>(0.90-0.98)</b>
Sens (%)	83	68	82	89	71	89	50	28	56	89
Spec (%)	78	98	92	80	84	82	91	95	95	89
AUC (LOOCV)	0.85	0.87	0.89	0.89	0.82	0.92	0.73	0.55	0.76	0.92
Sens (%)	78	68	82	85	68	88	47	28	56	86
Spec (%)	82	98	90	82	86	83	91	90	94	90
<b>Self-sample</b>	<b>ADCYAP1</b>	<b>BHLHE22</b>	<b>CDH13</b>	<b>CDO1</b>	<b>GALR1</b>	<b>GHSR</b>	<b>HAND2</b>	<b>SST</b>	<b>ZIC1</b>	<b>CDO1+GHSR+ZIC1</b>
AUC (non-CV; 95%-CI)	0.69 (0.60-0.78)	0.78 (0.70-0.85)	0.70 (0.62-0.79)	<b>0.90</b> <b>(0.84-0.95)</b>	0.74 (0.66-0.82)	0.83 (0.77-0.90)	0.69 (0.58-0.75)	0.61 (0.52-0.69)	0.62 (0.53-0.70)	<b>0.92</b> <b>(0.87-0.97)</b>
Sens (%)	53	64	49	75	51	71	50	35	54	86
Spec (%)	93	89	93	94	95	87	88	89	68	92
AUC (LOOCV)	0.68	0.77	0.69	0.89	0.73	0.83	0.65	0.58	0.57	0.91
Sens (%)	53	62	47	78	51	65	47	33	54	83
Spec (%)	92	90	93	91	93	91	88	87	63	92
<b>Scrape</b>	<b>ADCYAP1</b>	<b>BHLHE22</b>	<b>CDH13</b>	<b>CDO1</b>	<b>GALR1</b>	<b>GHSR</b>	<b>HAND2</b>	<b>SST</b>	<b>ZIC1</b>	<b>CDH13+CDO1+ZIC1</b>
AUC (non-CV; 95%-CI)	0.82 (0.76-0.89)	0.86 (0.80-0.92)	0.69 (0.60-0.77)	<b>0.95</b> <b>(0.92-0.98)</b>	0.62 (0.53-0.71)	0.89 (0.84-0.94)	0.60 (0.51-0.70)	0.70 (0.62-0.78)	0.76 (0.68-0.83)	<b>0.97</b> <b>(0.95-0.99)</b>
Sens (%)	71	75	46	82	53	75	40	60	78	93
Spec (%)	80	85	90	94	76	93	86	75	67	89
AUC (LOOCV)	0.82	0.85	0.67	0.95	0.59	0.88	0.57	0.69	0.74	0.96
Sens (%)	60	74	46	89	53	76	39	44	78	89
Spec (%)	91	85	90	86	75	90	84	89	65	90

Individual methylation markers and marker panels with the highest AUC value per sample type are depicted in bold. AUCs, including 95% CI, are reported together with sensitivities and specificities based on the maximal Youden's index (J) threshold. AUC = area under the receiver operating characteristic curve; LOOCV = leave-one-out cross-validated; Sens = sensitivity; Spec = specificity.



# CHAPTER 5

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## MOLECULAR ANALYSIS FOR OVARIAN CANCER DETECTION IN PATIENT-FRIENDLY SAMPLES

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## ABSTRACT

### Background

High ovarian cancer mortality rates motivate the development of effective and patient-friendly diagnostics. Here, we explored the potential of molecular testing in patient-friendly samples for ovarian cancer detection.

### Patients and methods

Home-collected urine, cervicovaginal self-samples, and clinician-taken cervical scrapes were prospectively collected from 54 patients diagnosed with a highly suspicious ovarian mass (benign  $n=25$ , malignant  $n=29$ ). All samples were tested for nine methylation markers, using quantitative methylation-specific PCRs that were verified on ovarian tissue samples, and compared to unpaired patient-friendly samples of 110 healthy controls. Copy number analysis was performed on a subset of urine samples of ovarian cancer patients by shallow whole-genome sequencing.

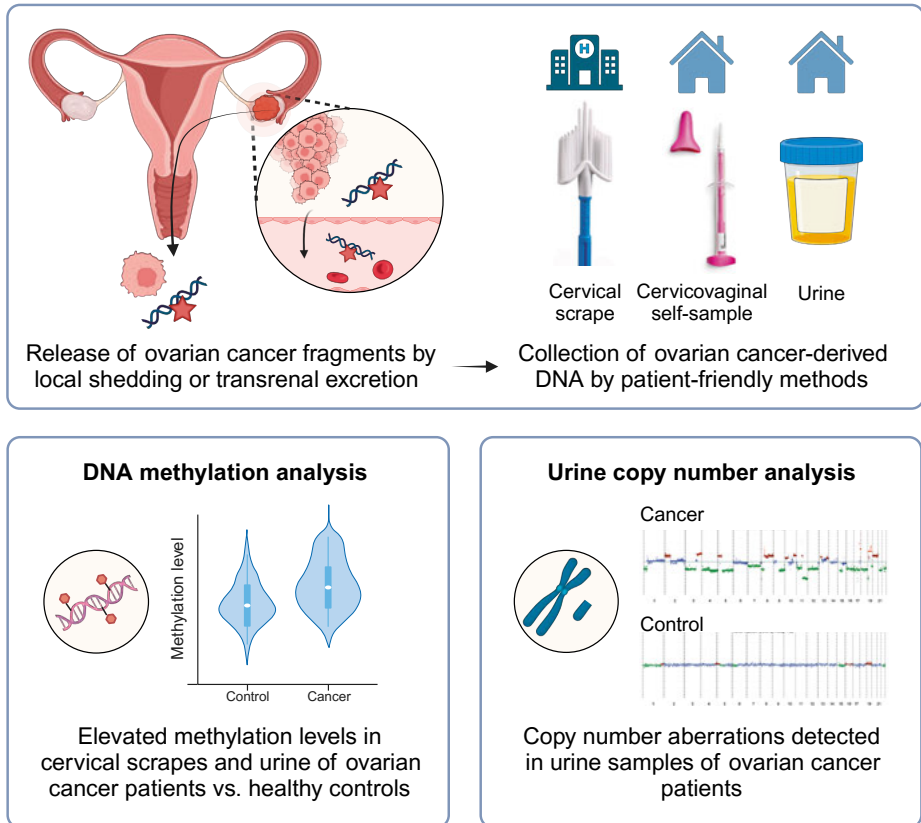
### Results

Three methylation markers were significantly elevated in full void urine of ovarian cancer patients as compared to healthy controls (*C2CD4D*,  $p=0.008$ ; *CDO1*,  $p=0.022$ ; *MAL*,  $p=0.008$ ), of which two were also discriminatory in cervical scrapes (*C2CD4D*,  $p=0.001$ ; *CDO1*,  $p=0.004$ ). When comparing benign and malignant ovarian masses, *GHSR* showed significantly elevated methylation levels in the urine sediment of ovarian cancer patients ( $p=0.024$ ). Other methylation markers demonstrated comparably high methylation levels in benign and malignant ovarian masses. Cervicovaginal self-samples showed no elevated methylation levels in patients with ovarian masses as compared to healthy controls. Copy number changes were identified in 4 out of 23 urine samples of ovarian cancer patients.

### Conclusion

Our study revealed increased methylation levels of ovarian cancer-associated genes and copy number aberrations in the urine of ovarian cancer patients. Our findings support continued research into urine biomarkers for ovarian cancer detection and highlight the importance of including benign ovarian masses in future studies to develop a clinically useful test.

## GRAPHICAL ABSTRACT



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## HIGHLIGHTS

- Ovarian cancer is often diagnosed at an advanced stage with a poor prognosis
- We studied the potential of molecular testing in different types of patient-friendly material for ovarian cancer detection
- Elevated methylation of ovarian cancer-associated genes can be measured in cervical scrapes and urine
- Copy number aberrations are detectable in urine of ovarian cancer patients
- DNA-based testing in cervical scrapes and urine could aid ovarian cancer diagnosis upon further development

## INTRODUCTION

Ovarian cancer is the most lethal gynecological cancer worldwide, accounting for 207,252 deaths in 2020 (1). Due to non-specific or absence of symptoms at an early-stage, patients typically present at a late-stage when prognosis is poor (2). Five-year overall survival rates sharply decrease with higher stage at diagnosis, with 92% survival in early-stage disease compared to only 29% in late-stage disease (3). High mortality rates prioritize the development of novel diagnostic approaches for ovarian cancer. Although more ovarian cancer patients were diagnosed at an earlier stage with screening strategies using conventional imaging and/or serum biomarkers (e.g. CA-125), this did not translate into reduced overall cancer-specific mortality in general and in high-risk populations (4, 5). In fact, the majority of ovarian cancers were not detected during or after the trial. A more accurate and easily accessible test could potentially overcome this problem.

Testing for ovarian cancer using biomarkers related to carcinogenesis could offer such an accurate test. DNA methylation-mediated silencing of tumor suppressor genes occurs early in cancer development and is therefore promising to detect cancer at an early stage (6). Methylation analysis in urine, cervicovaginal self-samples, and clinician-taken cervical scrapes has already been proven to allow reliable detection of cervical (7, 8) and endometrial cancer (9, 10). In urine, even signals of non-urogenital cancers, including colorectal (11) and lung cancer (12, 13), are detectable by methylation testing. The measurement of somatic mutations, aneuploidy, or DNA methylation in clinician-taken cervical scrapes or blood demonstrated the high potential of molecular-based diagnostic tests for ovarian cancer (14-17). However, these molecular changes have not been investigated in home-collected urine and cervicovaginal self-samples of ovarian cancer patients.

In this study, we explored the potential of molecular testing in home-collected urine and cervicovaginal self-samples, and clinician-taken cervical scrapes for ovarian cancer detection. Methylation markers considered suitable for the detection of ovarian cancer included a combination of markers described in studies on cervical and endometrial cancer detection in patient-friendly sample types (*GALR1*, *GHSR*, *MAL*, *PRDM14*, *SST*, and *ZIC1* (10, 18-20)), and ovarian cancer detection in cervical scrapes and plasma (*C2CD4D*, *CDO1*, *NRN1* (17, 21, 22)). In addition, the analysis of somatic copy number aberrations (SCNA) and fragmentation patterns was performed using shallow whole-genome sequencing on a subset of the samples to verify the presence of ovarian cancer-derived DNA in urine.

## MATERIAL AND METHODS

### Study population

This study prospectively included patients with a highly suspicious ovarian mass according to current triage methods (>40% risk of malignancy using the IOTA adnex model) (23, 24). Paired samples (*i.e.* urine, cervicovaginal self-samples, and clinician-taken cervical scrapes) were consecutively collected within the SOLUTION1 study, between July 2018 and September 2022, at the Antoni van Leeuwenhoek hospital, Amsterdam, The Netherlands. Samples were collected from patients who underwent pelvic surgery with post-operatively confirmed ovarian cancer of any stage and histological subtype, and patients with a benign ovarian mass who were referred to a highly specialized tertiary oncology unit for further assessment. Patients scheduled for pelvic surgery, involving exploratory laparotomy to determine the origin of their ovarian mass or cytoreductive surgery, were asked to collect samples prior to surgery. Patients without residual tumor/ovarian mass at time of inclusion or no possibility to collect cytological or urine samples prior to surgery were excluded from participation. Patients diagnosed with a borderline tumor were also excluded to focus on the most distinct tumor types in this feasibility stage (*i.e.* benign and malignant ovarian masses). Patients of which not all three paired sample types (*i.e.* cervical scrape, cervicovaginal self-sample, and urine) were available were not excluded.

Control urine samples were obtained from the URIC biobank, including healthy women without any prior cancer diagnosis within the last five years. Control cervicovaginal self-samples and cervical scrapes were collected from high-risk human papillomavirus (hrHPV)-negative women. Both were retrieved from leftover material of the Dutch national cervical cancer screening program coordinated by the Dutch National Institute for Public Health and the Environment (RIVM).

To verify the discriminatory power of the methylation assays and concordance of copy number profiles, formalin-fixed paraffin-embedded (FFPE) and fresh frozen high-grade serous ovarian cancer (HGSOC) tissue samples were retrieved from the Pathology archives of Amsterdam UMC, locations AMC and VUmc, Amsterdam, The Netherlands. FFPE normal fallopian tube tissues were collected from patients undergoing a hysterectomy for the treatment of benign endometrial conditions.

### Sample collection, processing, DNA extraction, and bisulfite modification

The sample collection, processing, DNA extraction, and bisulfite modification procedures were carried out as described previously for cervical (8, 25) and endometrial cancer (10, 19). A detailed description is provided in the Supplemental Methods. Briefly, urine and

cervicovaginal self-samples were collected at home and clinician-taken cervical scrapes were collected before surgery. Urine was centrifuged and separated into two fractions: the urine supernatant and the urine sediment. Both fractions and the remaining full void urine were stored for further analysis. Following DNA extraction, up to 250 ng of DNA was subjected to bisulfite modification.

### **DNA methylation analysis by quantitative methylation-specific PCR**

Methylation levels of the *C2CD4D* (gene-ID: 100191040), *CDO1* (gene-ID: 1036), *GALR1* (gene-ID: 2587), *GHSR* (gene-ID: 2693), *MAL* (gene-ID: 4118), *NRN1* (gene-ID: 51299), *PRDM14* (gene-ID: 63978), *SST* (gene-ID: 6750), and *ZIC1* (gene-ID: 7545) genes were measured by quantitative methylation-specific polymerase chain reactions (qMSP). Methylation markers were multiplexed to assess the methylation levels of three genes (1: *GHSR/SST/ZIC1*, 2: *CDO1/MAL/PRDM14*, 3: *C2CD4D/GALR1/NRN1*) and a reference gene (*ACTB*, gene-ID: 60) within the same reaction. Methylation analysis of *CDO1*, *GALR1*, *GHSR*, *MAL*, *SST*, *PRDM14*, and *ZIC1* was performed as described previously (10, 18, 19) with a shortened amplicon size of *ACTB*, *MAL* and *ZIC1* to facilitate methylation detection in fragmented urinary DNA. Assays targeting *C2CD4D* and *NRN1* were designed based on gene loci discovered and validated by others (17, 21). Primer and probe information is provided in Supplemental Table 1. Reaction conditions, instrument identifications, and thermocycling parameters are described in the Supplemental Methods. Double-stranded gBlocks™ Gene Fragments (Integrated DNA Technologies) containing the target amplicons and H<sub>2</sub>O were taken along in each run as positive and negative control, respectively. Sample quality and sufficient input was ensured by excluding samples with a *ACTB* quantification cycle (Cq) ≥ 32. Methylation levels were calculated relative to *ACTB* levels by the comparative Cq method:  $2^{-(\text{Cq marker} - \text{Cq ACTB})} \times 100$  (26).

All qMSP assays were designed, multiplexed and optimized according to parameters described earlier (27). Target specificity was validated *in silico* (BLAST). Correct amplicon size was verified by agarose gel electrophoresis. Analytical validation was performed using a dilution series of bisulfite treated methylated DNA from the SiHa cell line (100, 50, 10, 5, 1, 0.5%) within the range of 20 to 0.1 ng (Supplemental Table 2). The discriminatory power of each assay was verified by comparing methylation marker levels in tissue samples of ovarian cancer patients with those measured in normal fallopian tube tissue.

### **Shallow whole-genome sequencing**

Urine cell-free DNA (cfDNA) extracted from urine supernatant samples of ovarian cancer patients was further characterized by shallow whole-genome sequencing (~1x coverage). The cfDNA was quantified and analyzed using a Cell-free DNA ScreenTape

assay of the Agilent 4200 TapeStation System (Agilent) for quality control before sequencing. Sequencing libraries of the first pilot series of urine supernatant DNA were prepared using the ThruPLEX Plasma-seq Kit (Takara Bio, Mountain View, CA, USA) for whole-genome sequencing according to manufacturer's instructions. The remaining samples were prepared using the NEBNext® Enzymatic Methyl-seq (EM-seq) Kit (NEB, Ipswich, MA, USA). EM-seq was performed according to manufacturer's guidelines for standard insert libraries with 14 PCR cycles. Libraries were quantified and quality checked using the D1000 ScreenTape Analysis Assay (Agilent) before pooling. Paired-end 150 base pair (bp) libraries were pooled in equimolar amounts and sequenced on a NovaSeq6000 (Illumina) (GenomeScan, Leiden). The processing of sequencing data and subsequent analysis of SCNA and cfDNA fragmentation patterns are provided in the Supplemental Methods. Shallow whole-genome sequencing of paired FFPE primary tumor tissue was performed to verify copy number profile concordance and is also described in the Supplemental Methods.

### Statistical analysis

Methylation levels were expressed as 2log-transformed Cq ratios and presented in violin plots. Tissue methylation levels were compared between two groups using the non-parametric Mann-Whitney U test. Methylation levels of each gene in the remaining sample types were compared between healthy controls and patients diagnosed with a benign or malignant ovarian mass using the Kruskal-Wallis test. In case of a significant Kruskal-Wallis test ( $p < 0.05$ ), this was followed by post-hoc testing of 1) healthy controls versus malignant ovarian masses, and 2) benign versus malignant ovarian masses using the Mann-Whitney U test with Bonferroni correction.

The correlation between methylation levels of each DNA methylation marker between paired samples of patients diagnosed with ovarian cancer was assessed using Spearman's rank correlation. Correlation coefficient  $r$  was defined as very weak ( $r = 0.00-0.19$ ), weak ( $r = 0.20-0.39$ ), moderate ( $r = 0.40-0.59$ ), strong ( $r = 0.60-0.79$ ), or very strong ( $r = 0.80-1.00$ ) and displayed in correlation matrices.

Fragment size profiles were visualized by density plots and analyzed by comparing cfDNA reads of healthy controls and ovarian cancer patients with low (<5%) and high (≥5%) tumor fractions.

Data was collected using Castor EDC and analyzed using R (version 4.0.3 with packages: cowplot, corrplot, dplyr, ggplot, ggpubr, and rstatix).  $P$ -values are two-sided and considered statistically significant when  $p < 0.05$ .

## RESULTS

### Study population

A total of 428 samples of 164 participants were analyzed within this study. Samples were prospectively collected from 54 patients undergoing pelvic surgery at a tertiary oncology center because of a highly suspicious ovarian mass. Twenty-nine women were diagnosed with ovarian cancer and 25 with a benign ovarian mass. For comparison, 110 unpaired samples of healthy age-matched controls were collected. Sample types included clinician-taken cervical scrapes (control n=40, benign n=22, malignant n=24), cervicovaginal self-samples (control n=40, benign n=24, malignant n=28), full void urine (control n=30, benign n=25, malignant n=28), urine supernatant (control n=29, benign n=25, malignant n=29), and urine sediment (control n=30, benign n=25, malignant n=29). Clinical characteristics of study participants are summarized in Table 1.

**Table 1:** Clinical characteristics of study participants.

	n	%	Age: median (IQR)
<b>Ovarian cancer:</b>	29	(100%)	59 (56 - 67)
<b>Histology</b>			
Serous carcinoma	22	75,9%	
<i>Low-grade</i>	4		
<i>High-grade</i>	18		
Clear cell carcinoma, high-grade*	3	10,3%	
Carcinosarcoma, high-grade	2	6,9%	
Endometrioid carcinoma, low-grade	1	3,4%	
Mucinous carcinoma, low-grade	1	3,4%	
<b>Stage (FIGO 2014)</b>			
IIB	5	17,2%	
IIC	1	3,4%	
IIIA	5	17,2%	
IIIB	4	13,8%	
IIIC	12	41,4%	
IV	2	6,9%	
<b>Benign ovarian mass:</b>	25	(100%)	62 (54 - 69)
<b>Histology</b>			
Serous cystadeno(fibro)ma	8	32,0%	
Mucinous cystadenoma	6	24,0%	
Fibroma	4	16,0%	
Endometriosis cyst	4	16,0%	
Mature teratoma	3	12,0%	

**Table 1:** (Continued)

	n	%	Age: median (IQR)
<b>Healthy controls:</b>	110		
<b>Sample type</b>			
Urine	30		60 (53 - 74)
Cervicovaginal self-sample	40		60 (60 - 60)
Clinician-taken cervical scrape	40		60 (60 - 60)

\*Including one mixed clear cell and low-grade endometrioid carcinoma.

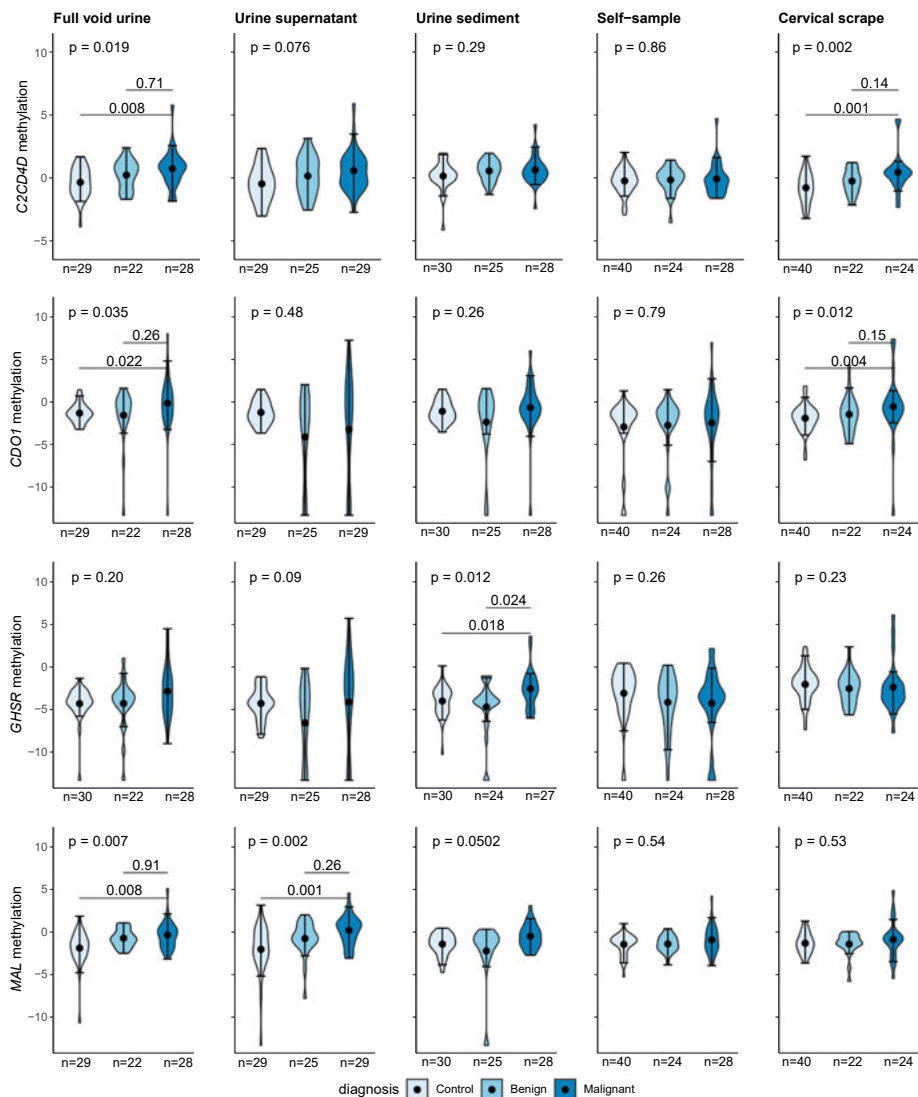
### **DNA methylation levels are elevated in cervical scrapes and urine samples of women with ovarian masses**

The discriminatory power of qMSP assays was verified in tissue, in which all markers showed clear significant differences when comparing methylation levels in normal fallopian tube (n=22) with HGSOC (n=35) tissues ( $p < 0.0001$ ; Supplemental Figure 1, Mann-Whitney U).

The feasibility of ovarian cancer detection in urine by methylation analysis was evaluated by testing nine methylation markers in full void (*i.e.* unfractionated) urine, urine supernatant, and urine sediment of healthy controls and patients diagnosed with a benign or malignant ovarian mass (Figure 1, Supplemental Figure 2-4). When comparing healthy controls with ovarian cancer patients, three markers showed a significant discrimination in full void urine (*C2CD4D*,  $p=0.008$ ; *CDO1*,  $p=0.022$ ; *MAL*,  $p=0.008$ , Mann-Whitney U), one in urine supernatant (*MAL*,  $p=0.001$ ) and one in urine sediment (*GHSR*,  $p=0.018$ , Mann-Whitney U). Benign and malignant masses revealed comparably high methylation levels for most methylation markers, except for *GHSR*. *GHSR* showed significantly elevated methylation levels in the urine sediment of ovarian cancer patients ( $p=0.024$ , Mann-Whitney U; Figure 1, Supplemental Figure 4).

Similarly, the feasibility of ovarian cancer detection in cervicovaginal self-samples and clinician-taken cervical scrapes by methylation analysis was assessed by testing the same methylation markers. While methylation levels of two markers were significantly increased in clinician-taken cervical scrapes of ovarian cancer patients as compared to controls (*C2CD4D*,  $p=0.001$ ; *CDO1*,  $p=0.004$ , Mann-Whitney U), benign and malignant ovarian masses could not be distinguished using these markers (Figure 1, Supplemental Figure 5). None of the markers were significantly elevated in cervicovaginal self-samples when comparing these groups (Figure 1, Supplemental Figure 6).

Numbers were insufficient to compare methylation levels between different histological subtypes and stages.



**Figure 1: Methylation analysis in patient-friendly sample types.** Methylation levels of most discriminating markers *C2CD4D*, *CDO1*, *GHSR*, and *MAL* in full void (unfractionated) urine, urine supernatant, urine sediment, cervicovaginal self-samples, and clinician-taken cervical scrapes of healthy controls and patients diagnosed with a benign or malignant ovarian mass. Methylation levels are expressed by 2log-transformed Cq ratios and bold circles represent medians.

### DNA methylation levels are correlated between paired cervical scrapes and urine samples

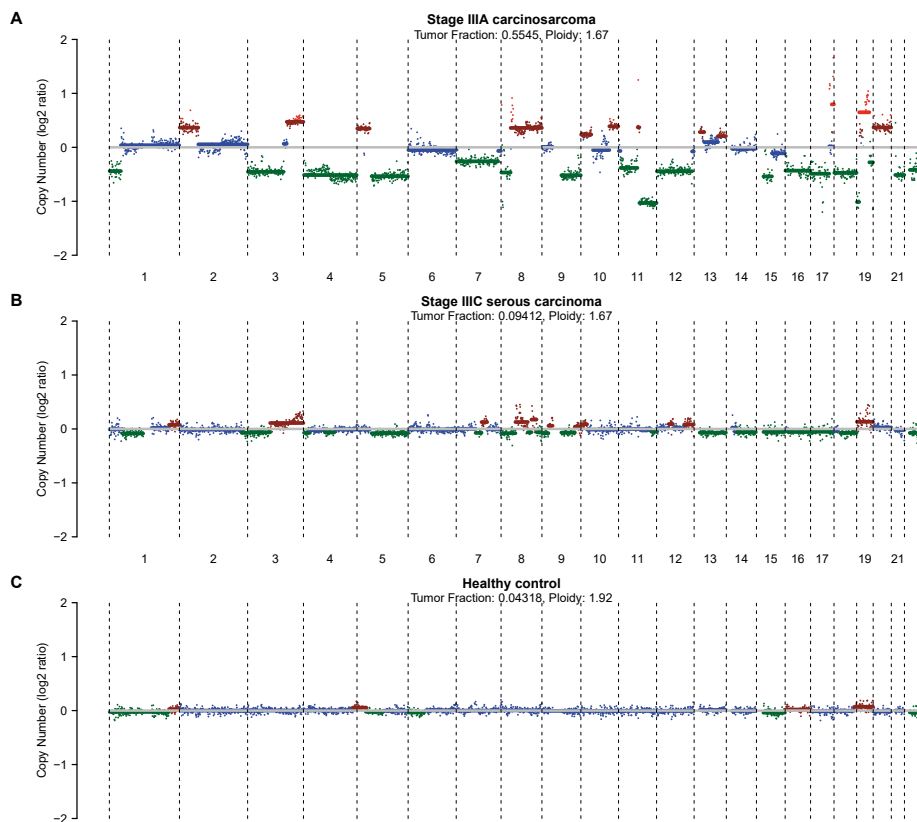
DNA methylation levels of genes significantly discriminating between healthy and malignant in cervical scrapes and urine (*i.e.* *C2CD4D*, *CDO1*, *GHSR*, *MAL*) were compared

between paired samples to assess their correlation (Supplemental Figure 7). Paired cervical scrapes and urine were available for 23 ovarian cancer patients. Individual markers in full void urine correlated moderately to strongly with urine supernatant ( $r = 0.52-0.61$ ) and urine sediment ( $r = 0.67-0.76$ ). The full void urine showed the best correlation with cervical scrapes ( $r = 0.42-0.59$ ), while a weak correlation was observed between the urine supernatant and cervical scrapes ( $r = 0.33-0.45$ ).

### **Copy number aberrations are detectable in urine cell-free DNA**

The presence of ovarian cancer-derived DNA in the urine was verified by analyzing a subset of 25 urine supernatant samples of ovarian cancer patients ( $n=23$ ) and healthy controls ( $n=2$ ) by shallow whole-genome sequencing. Sequencing yielded a sufficient read count for all samples (median mapped paired read count of 55,133,492). Shallow whole-genome sequencing coverage and quality statistics per urine sample are provided in Supplemental Table 3. Aberrant genome-wide copy number profiles were found in 4 out of 23 sequenced urine supernatant samples of ovarian cancer patients (Figure 2, Supplemental Figure 8). Copy number profile concordance between urine and the primary tumor tissue was verified for these cases (Supplementary Figure 8). The patient with the highest tumor fraction also showed the highest methylation levels of MAL in the urine supernatant (Supplemental Figure 9).

Additionally, fragment size distributions were analyzed by comparing cfDNA reads of healthy controls and ovarian cancer patients with low and high tumor fractions. Cancer samples with a high tumor fraction ( $n=4$ ) revealed a shorter modal fragment size of 80 bp as compared to 111 bp in cancer samples with a low tumor fraction ( $n=19$ ) and controls ( $n=2$ ; Supplemental Figure 10).



**Figure 2: Copy number analysis in urine cell-free DNA.** Illustrative examples of genome-wide somatic copy number profiles of urine supernatant samples collected from patients with a stage IIIA carcinosarcoma (**A**), stage IIIC serous carcinoma (**B**), and a healthy control (**C**). Estimated ploidy and tumor fraction are listed at the top of the plot. The y-axis depicts the log2 tumor to normal ratio.

## DISCUSSION

Both elevated methylation levels of a subset of markers and SCNA were detected in home-collected urine samples of ovarian cancer patients by targeted qMSP assays and shallow whole-genome sequencing, respectively. Urine is truly non-invasive and unlocks at home collection of liquid biopsy to reduce in-person visits. Yet, an important finding was that methylation levels in benign cases were similarly high, presenting a challenge for the development of clinically useful tests.

While we tested for methylation markers described and also by us verified to be associated with ovarian cancer, it was found that when tested in our patient-friendly sample types most of these did not distinguish benign from malignant ovarian

masses. Only *GHSR* demonstrated slightly increased methylation levels in the urine sediment. Benign ovarian masses included in this study were highly suspicious for malignancy according to current triage methods (>40% risk of malignancy using the IOTA adnex model) as samples were collected in a tertiary oncology unit. Half of the included patients in our cohort were ultimately diagnosed with a benign ovarian mass, underlining that current triage for referral to tertiary oncology care is suboptimal. The majority of previous studies only included benign controls for methylation marker discovery in tissue but not during marker validation in plasma, as recently reviewed by Terp *et al.* (15), or benign controls were not age-matched to cancers (21). Similarly, studies on ovarian cancer detection in cervical scrapes did not include benign controls (16, 17). The inclusion of age-matched patients diagnosed with benign and malignant ovarian masses is essential to accurately assess the clinical value of DNA methylation testing for ovarian cancer detection.

The presence of ovarian cancer-derived DNA in the urine is currently underexplored. So far, only Valle *et al.* reported on the detection of somatic mutation profiles and *HIST1H2BB/MAGI2* promoter methylation in a small paired series of ascites, blood, tissue, urine, and vaginal swabs of HGSOC patients (28). Their data on two patients revealed that methylation levels in urinary cfDNA correlated stronger with tissue than with blood, indicating the potential of urine-based ovarian cancer detection. Unfortunately, the diagnostic potential of ovarian cancer detection in urine could not be determined in the study of Valle *et al.* as no control samples were included.

In our study, different urine fractions were systematically compared to explore whether a preferred urine sample type for ovarian cancer detection exists. Full void urine most likely contains both genomic and cfDNA, whereas the urine sediment is enriched for genomic DNA and the urine supernatant for transrenally excreted cfDNA (29). This assumption is confirmed by the strong correlation for *CDO1* between cervical scrapes and urine sediment, while cervical scrapes and urine supernatant correlated weakly to moderately. Most methylation markers significantly differentiated between healthy controls and ovarian cancer patients in the full void urine (3/12), followed by urine supernatant (1/12), and the urine sediment (1/12). These outcomes suggest that tumor-derived methylation signals can originate from genomic DNA as well as transrenally excreted cfDNA. Yet, larger samples sizes are needed to determine whether a preferred urine sample type for methylation analysis exists.

In the present study, genes with elevated methylation levels in HGSOC tissue, were not always measurable in urine. Our qMSP assays were designed to facilitate the detection of methylation in small DNA fragments present in the urine as shown in our previous

studies (8, 10, 12). Yet, the current assays may not reach the limit of detection needed for the low tumor-derived methylation signals. Nucleic acids that are released from the bladder epithelium may further dilute the ovarian cancer signal in urine. Another explanation for the absence of tumor-derived methylation signals of some genes in the urine could be linked to the origin of urinary cfDNA. Urine cfDNA is described to be even shorter as compared to plasma cfDNA (modal size of 82 vs. 167 basepairs) (30). Differences in fragmentation patterns between plasma and urine are likely caused by Dnase1 cleavage activity in the urine and high concentrations of urea and salt that affect histone-DNA binding (31). Histone-bound DNA is more protected against degradation as compared to DNA that is not histone-bound (32). Hypothetically, hypermethylated regions of interest that are not histone-bound could be further degraded and become unmeasurable. We partly accounted for this by including methylation markers with proven diagnostic value in plasma in our selection (*i.e.* *C2CD4D*(21, 22), *CDO1*(22)), which both appeared suitable for ovarian cancer detection in urine.

Clear SCNA profiles harboring common chromosomal gains (*e.g.* 1q, 3q, 7q, 8q) and losses (*e.g.* 17p, 19q, 22q) could be obtained from four urine supernatant samples of ovarian cancer patients, verifying the presence of tumor-derived DNA in the urine (33). Furthermore, a focal amplification at chromosome 19 was identified in the urine of one patient with stage IIIA serous carcinoma, which is a clinically relevant alteration that has previously been described in a subgroup of serous ovarian cancers (34). Aneuploidy was detected previously in cervical scrape samples of ovarian cancer patients using the PapSEEK test (16). We also observed shorter fragment sizes in urine supernatant samples with a high tumor fraction, which is another indication for the presence of tumor-derived DNA in the urine, as shown previously in urine samples of glioma patients (30).

Given the feasibility of ovarian cancer detection in cervical scrapes by DNA methylation analysis (14, 17), similar findings were expected for self-collected cervicovaginal samples. While *C2CD4D* and *CDO1* distinguished healthy versus malignant in cervical scrapes, none of the markers showed elevated methylation levels in cervicovaginal self-samples. Our findings are in line with those of van Bommel *et al.* who reported that mutation analysis in cervicovaginal self-samples of ovarian cancer patients was not feasible (35). None of the pathogenic mutations found in surgical specimens could be detected in cervicovaginal self-samples. Ovarian cancer signals might be more diluted in cytological specimens collected from areas further away from the ovaries. This was also observed for the PapSEEK test, which detected 45% of ovarian cancers when using intrauterine sampling (Tao brush) as compared to 17% when using endocervical sampling (Pap brush) (16).

Nevertheless, considering our relatively small sample size, we do not exclude the use of cervicovaginal self-samples for ovarian cancer detection yet. The optimization of pre-analytical factors, such as increased input of original sample or improved DNA isolation methods, could enhance the ovarian cancer signal in vaginal samples. Alternatively, a non-tumor DNA driven approach could be useful for ovarian cancer detection in cervicovaginal self-samples, as recently described by Barrett *et al* (36). Their signature consisted of epigenetic differences in cervical cells and allowed ovarian cancer detection in cervical scrapes with an area under the receiver operating characteristic curve value of 0.76. Larger cohort studies, such as the Screenwide study (37), will provide further insight into the use of cervicovaginal self-samples for ovarian cancer detection.

Strengths of this study include the collection of a unique paired sample series of both patients diagnosed with a benign ovarian mass and with a malignant ovarian tumor, covering most histological subtypes. Moreover, urine and cervicovaginal self-samples were collected from home to assess the feasibility and potential of home-based sampling for ovarian cancer. The successful sequencing of urine cfDNA of ovarian cancer patients provides opportunities for future (epi)genome profiling using short- or long-read sequencing technologies. Although we have demonstrated the potential diagnostic value of urine for ovarian cancer, this study is limited by still relatively low sample numbers and the lack of early-stage cancers ( $\leq$  FIGO stage 2A). Given the heterogeneous nature of benign and malignant ovarian masses, larger sample series are needed to conclude on the clinical applicability of home-collected cervicovaginal self-samples and urine for ovarian cancer detection. Furthermore, direct comparisons with paired plasma samples using DNA-based and other molecular biomarkers (e.g. HE4) would be informative for future studies.

This study supports limited existing data on ovarian cancer detection in cervical scrapes by DNA methylation analysis. Moreover, it provides first proof of concept that urine yields increased methylation levels of ovarian cancer-associated genes and contains ovarian cancer-derived DNA as demonstrated by SCNA analysis. Our findings support continued research into urine biomarkers for ovarian cancer detection and highlight the importance of including benign ovarian masses in future studies. Molecular biomarker testing in patient-friendly samples could facilitate earlier ovarian cancer detection and triage women presenting with an ovarian mass to manage specialist referral. Yet, further studies investigating alternative urine (methylation) biomarkers are warranted to develop a clinically useful test.

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### **Ethics statement**

All patients participating in the SOLUTION1 study were 18 years or older and signed informed consent before sample collection. Ethical approval was obtained by the Medical Ethical Committee of the VU University Medical Center for the use of samples collected within the SOLUTION1 study (METc: 2016.213, Trial registration ID: NL56664.029.16), samples stored in the URIC biobank (TcB 2018.657), and samples archived in the biobank containing leftover material of the Dutch national cervical cancer screening program (TcB 2020.245). The Code of Conduct for Responsible Use of Left-over Material of the Dutch Federation of Biomedical Scientific Societies was adhered for the use of tissue specimen.

### **Data and code availability**

The sequencing dataset generated and analyzed during the current study is available in the European Genome-Phenome Archive repository, under accession number EGAD00001010848. The DNA methylation dataset generated and analyzed during this study is available from the corresponding author on reasonable request.

### **Conflict of interest statement**

RS has a minority share in Self-screen B.V., a spin-off company of Amsterdam UMC, location VUmc. Self-screen B.V. holds patents and products related to the work. RS and FM are co-inventors on multiple patents related to methylation markers and cfDNA analysis, respectively. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

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## SUPPLEMENTARY MATERIALS

### Supplemental Methods

#### Sample collection and processing

Urine and cervicovaginal self-samples were collected at home for which all participants received a package including materials needed for collection and transport. Participants were instructed to collect urine before the cervicovaginal self-sample. Cervicovaginal self-samples were collected according to the provided user manual using the Evalyn® brush (Rovers Medical Devices, Oss, The Netherlands), which is a clinically validated self-sampling method (1). Urine was collected in 3x30 mL tubes containing the storage buffer Ethylenediaminetetraacetic acid (EDTA; final concentration 40 mM) to preserve nucleic acids during transport. Clinician-taken cervical scrapes were collected prior to surgery using a Cervex-Brush (Rovers Medical Devices) and directly placed in 10 mL Thinprep PreservCyt medium (Hologic, Marlborough, MA, US). Samples were sent to the Pathology department of Amsterdam UMC, location VUmc, within 72 hours by regular mail and processed directly after arrival.

Urine was processed as described in our previously validated processing and storage protocol (2). Briefly, a total of 15 mL of urine was centrifuged at 3000g for 10 min to separate the urine into two fractions: the urine supernatant and urine sediment. Both fractions and the remaining full void (*i.e.* unfractionated) urine were stored at -20°C. Cytological samples were processed as described previously for cervical (3) and endometrial cancer (4). Cervicovaginal self-samples were stored in 1.5 mL ThinPrep PreservCyt medium upon arrival. Cervicovaginal self-samples and cervical scrapes were stored at 4°C.

Formalin-fixed paraffin-embedded (FFPE) and fresh frozen tissue specimens were consecutively sectioned of which the first and last sections were Hematoxylin and Eosin (H&E) stained for histopathological review by a pathologist to confirm the presence of ovarian cancer or normal fallopian tube tissue.

#### DNA extraction and bisulfite modification

DNA from full void urine (30 mL patients diagnosed with ovarian mass; 40 mL controls), urine sediment (15 mL original volume), and urine supernatant (15 mL) was extracted as described previously (5, 6). In short, both full void urine and urine supernatant were isolated with the Quick DNA urine kit (Zymo Research, Irvine, CA, US) and urine sediment using the DNA mini and blood mini kit (Qiagen, Hilden, Germany). DNA from cervicovaginal self-samples and clinician-taken cervical scrapes was isolated as

described before (3), using the NucleoMag 96 Tissue kit (Machery-Nagel) and a Microlab Star robotic system (Hamilton, Germany). DNA of FFPE tissue samples was isolated using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany). DNA of fresh frozen tissue samples was isolated using the DNeasy Blood & Tissue kit (Qiagen). DNA yield was quantified using a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, US). Up to 250 ng of extracted DNA was subjected to bisulfite modification using the EZ DNA Methylation Kit (Zymo Research) to convert unmethylated cytosines. All procedures were performed according to manufacturer's guidelines.

### **Reaction conditions and instrument identifications of quantitative methylation-specific PCR**

Up to 50 ng of modified DNA was mixed with Epitect Multiplex PCR Mastermix (Qiagen, Venlo, Netherlands), 2.5-5.0  $\mu$ M of each primer, and 5.0-10.0  $\mu$ M of each hydrolysis probe in a total volume of 12.5  $\mu$ l. Thermocycling conditions were: 95°C for 5 minutes, 45 cycles at 95°C for 15 seconds, 59/60/63°C for 1 minute, and 72°C for 1 minute. Quantitative methylation-specific PCR (qMSP) assays were performed using a ViiA7 real-time PCR-system (Applied Biosystems, Foster City, CA, USA) or an ABI-7500 real-time PCR-system (Applied Biosystems, Waltham, MA, US) for *GHSR/SST/ZIC1*. The qMSP data was analyzed with manual thresholds and automatic baseline settings using QuantStudio™ Real-Time PCR Software (v. 1.6.1) and 7500 Software (v. 2.3).

### **Analysis of somatic copy number aberrations and cell-free DNA fragmentation patterns**

Processing of the sequencing data was performed by a pipeline controlled by Snakemake (v. 7.14.0). In brief, sequencing adapters and indexes were trimmed by the bbdut.sh (v. 38.79) [<https://sourceforge.net/projects/bbmap/>] in paired mode with parameters 'ktrim=r k=23 mink=11 hdist=1' and the adapter reference dataset provided with the software. Trimmed non-converted samples were mapped to the GRCh38 human genome assembly (GeneBank accession: GCA\_000001405.28) using bwa mem (v. 0.7.17) [<https://github.com/lh3/bwa>]. Enzymatically converted reads were mapped to the same assembly using biscuit (v. 1.0.2.20220113) [<https://huishenlab.github.io/biscuit/>]. For both non-converted and converted samples, reads with a mapping quality lower than 5, unmapped reads, secondary mappings, chimeric and PCR duplicates were filtered using samtools (v. 1.12) [<https://github.com/samtools/samtools>] and sambamba markdup (v. 0.8.1) [<https://lomereiter.github.io/sambamba/>]. Reads passing the filtering step were submitted for somatic copy number aberrations (SCNA) analysis and tumor fraction estimation using the ichorCNA software (v. 0.3.2.0) (7) using default settings, except the use of an in-house panel-of-normals from shallow whole-genome sequencing, setting the non-tumor fraction parameter restart values to c(0.95,0.99,0.995,0.999). The tumor

fraction with the highest log likelihood was reported. Fragmentation patterns of urine cfDNA for both non-converted and converted samples were analyzed by retrieving the fragment sizes of the trimmed and filtered reads using picard CollectInsertSizeMetrics (v. 2.22.2) with HISTOGRAM\_WIDTH=1000 [<https://gatk.broadinstitute.org/hc/en-us>].

Shallow whole-genome sequencing for the analysis of SCNA in paired FFPE primary tumor tissue was performed as described previously with a few adaptations (8). Sequencing libraries were prepared using the KAPA HyperPlus Kit (Roche, Basel, Switzerland), following manufacturer's protocol. Libraries were sequenced using a NextSeq2000 (Illumina). Sequence reads were aligned to the GRCh38 human genome assembly using bwa mem (v. 0.7.17). PCR duplicates (marked by Picard v. 2.20.8), as well as low-quality reads (MAPQ < 37), were filtered out using samtools (v. 0.1.1830). Reads passing the filtering step were submitted for SCNA analysis using ichorCNA software as described for urine samples.

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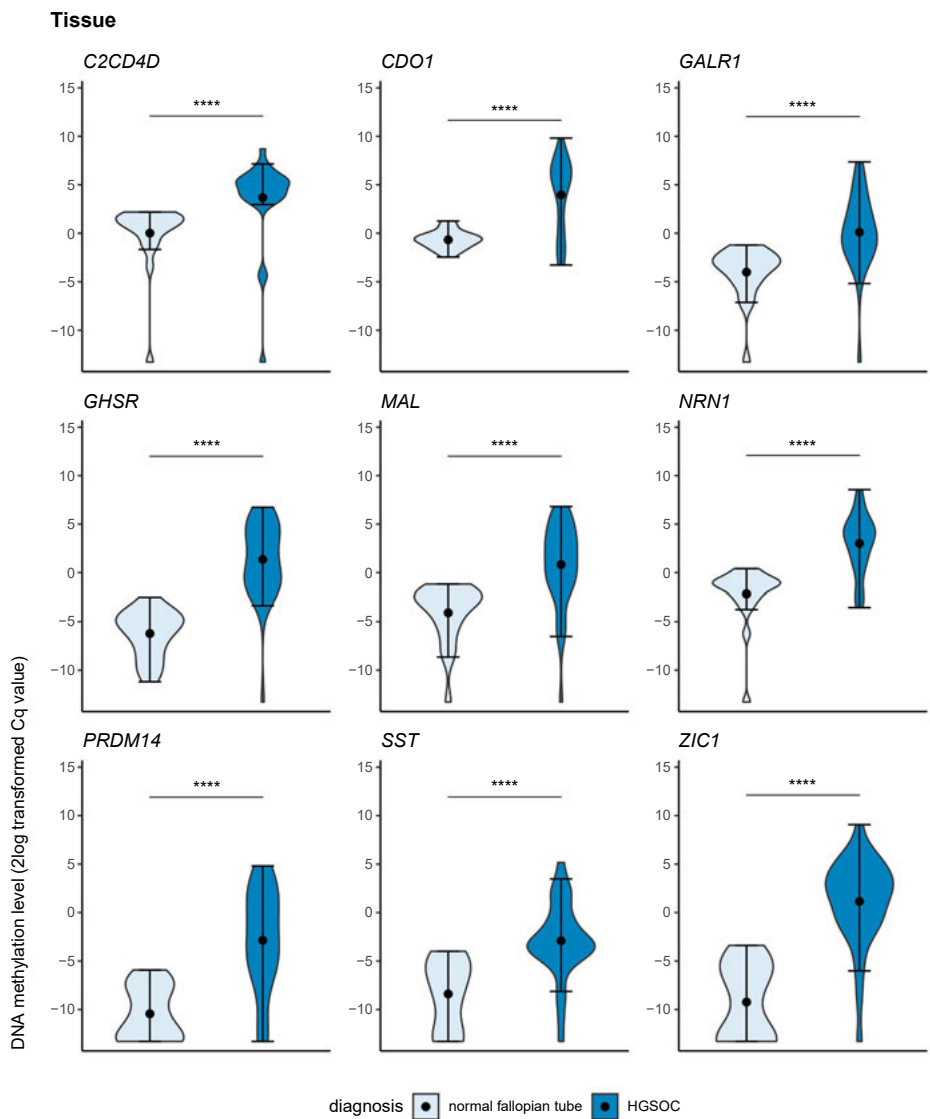
## SUPPLEMENTAL FIGURES

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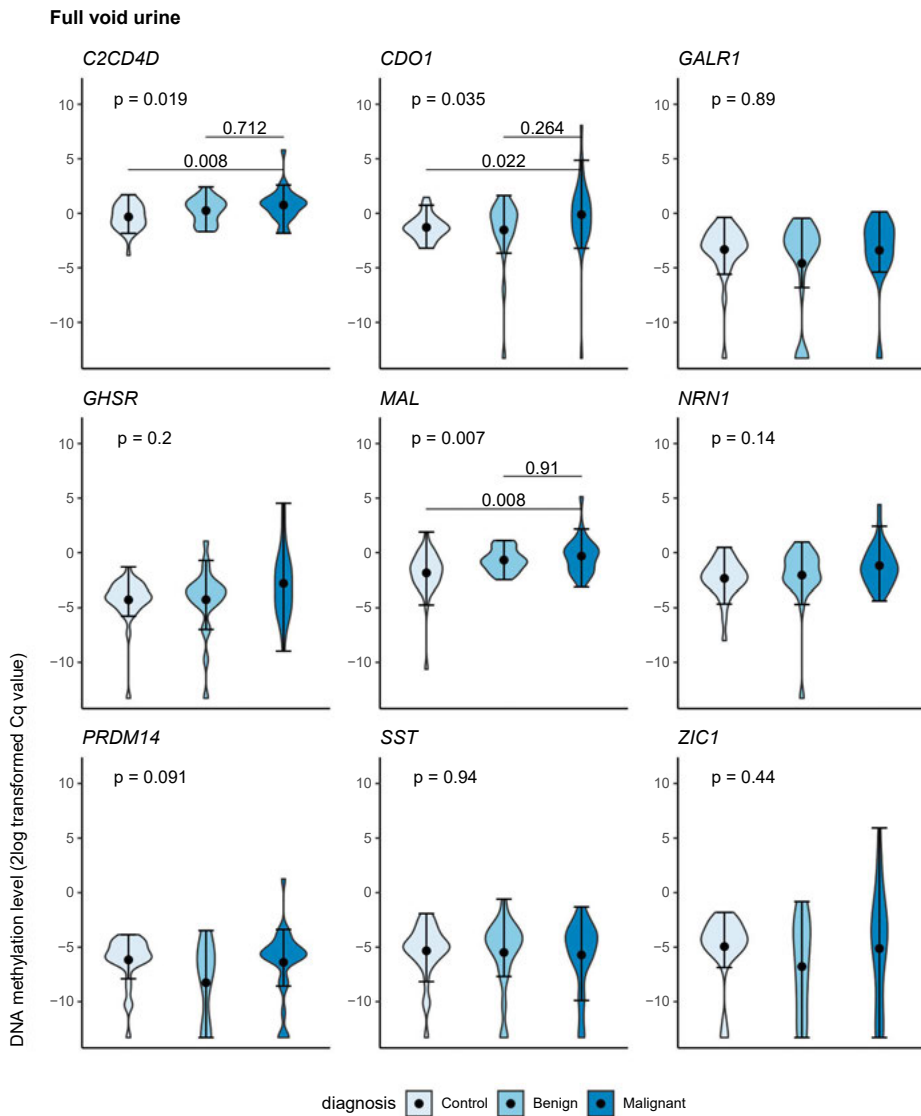
<b>Supplemental Figure 1</b>	DNA methylation levels of <i>C2CD4D</i> , <i>CDO1</i> , <i>GALR1</i> , <i>GHSR</i> , <i>MAL</i> , <i>NRN1</i> , <i>PRDM14</i> , <i>SST</i> , and <i>ZIC1</i> in high-grade serous ovarian cancer (n=35) and normal fallopian tube tissue (n=22). DNA methylation levels are shown by 2log-transformed Cq ratios. Violin plots represent medians with lower and upper quartile and range whiskers. A <i>p</i> -value of <0.05 was considered statistically significant. ****: <i>p</i> < 0.0001. Cq = quantification cycle; HGSOC = high-grade serous ovarian cancer.
<b>Supplemental Figure 2</b>	DNA methylation levels of <i>C2CD4D</i> , <i>CDO1</i> , <i>GALR1</i> , <i>GHSR</i> , <i>MAL</i> , <i>NRN1</i> , <i>PRDM14</i> , <i>SST</i> , and <i>ZIC1</i> in full void ( <i>i.e.</i> unfractionated) urine of healthy controls (n=30), and women diagnosed with a benign (n=27) or high-stage malignant ovarian mass (n=28). DNA methylation levels are shown by 2log-transformed Cq ratios. Violin plots represent medians with lower and upper quartile and range whiskers. A <i>p</i> -value of <0.05 was considered statistically significant. Cq = quantification cycle.
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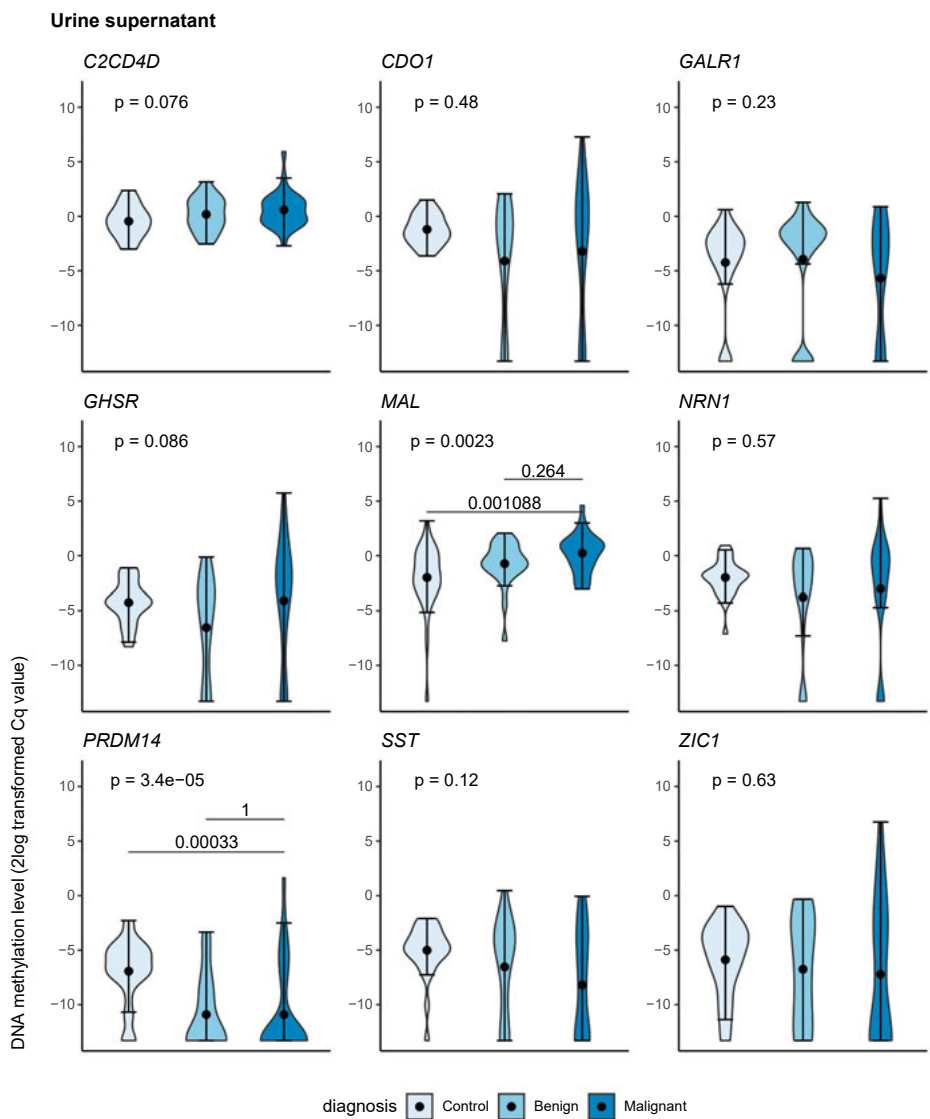
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<b>Supplemental Figure 9</b>	Scatter plot indicating the relation between <i>MAL</i> methylation levels and the tumor fraction as estimated by ichorCNA in urine supernatant samples. <i>MAL</i> methylation levels are shown by 2log-transformed Cq ratios. <i>MAL</i> was the most discriminating marker between urine supernatant samples of healthy controls and ovarian cancer patient and therefore plotted against the tumor fraction. The patient with the highest tumor fraction in urinary cfDNA also showed the highest <i>MAL</i> methylation, as seen in the upper right part of the plot. Cq = quantification cycle.
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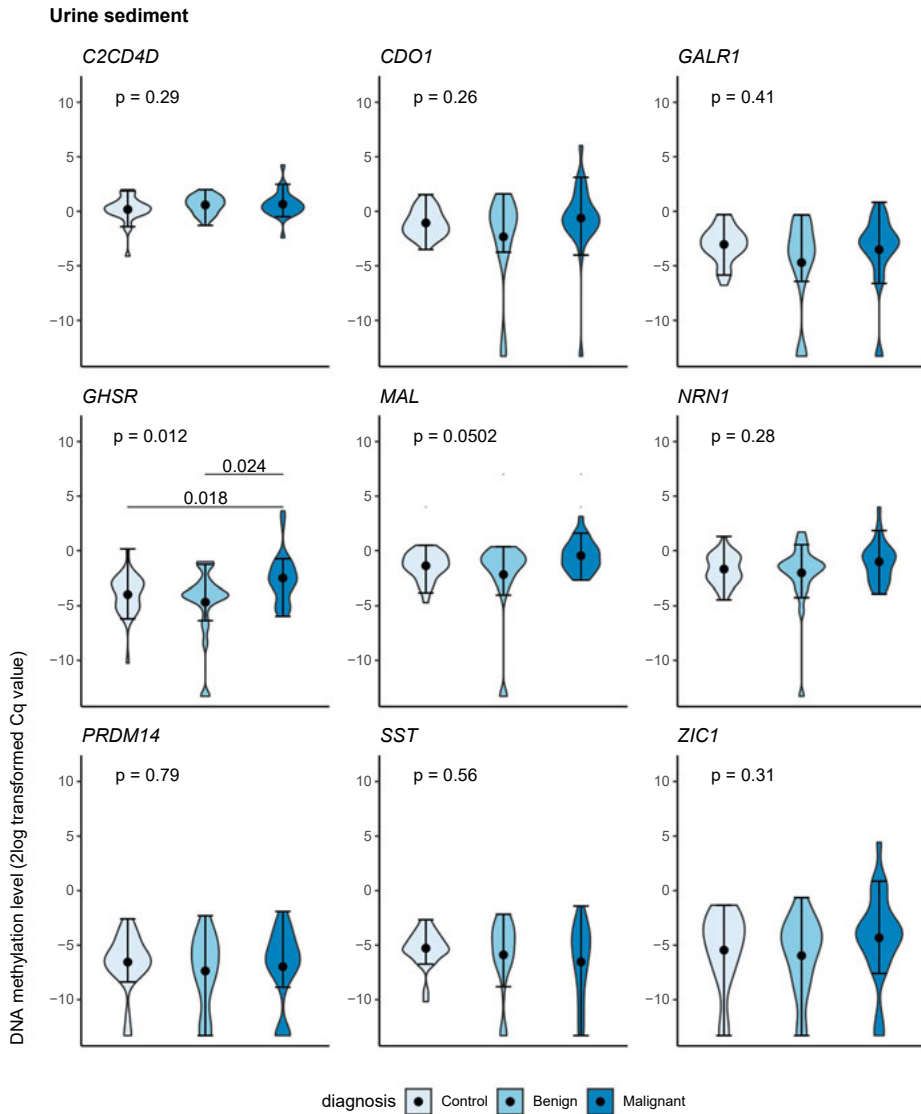
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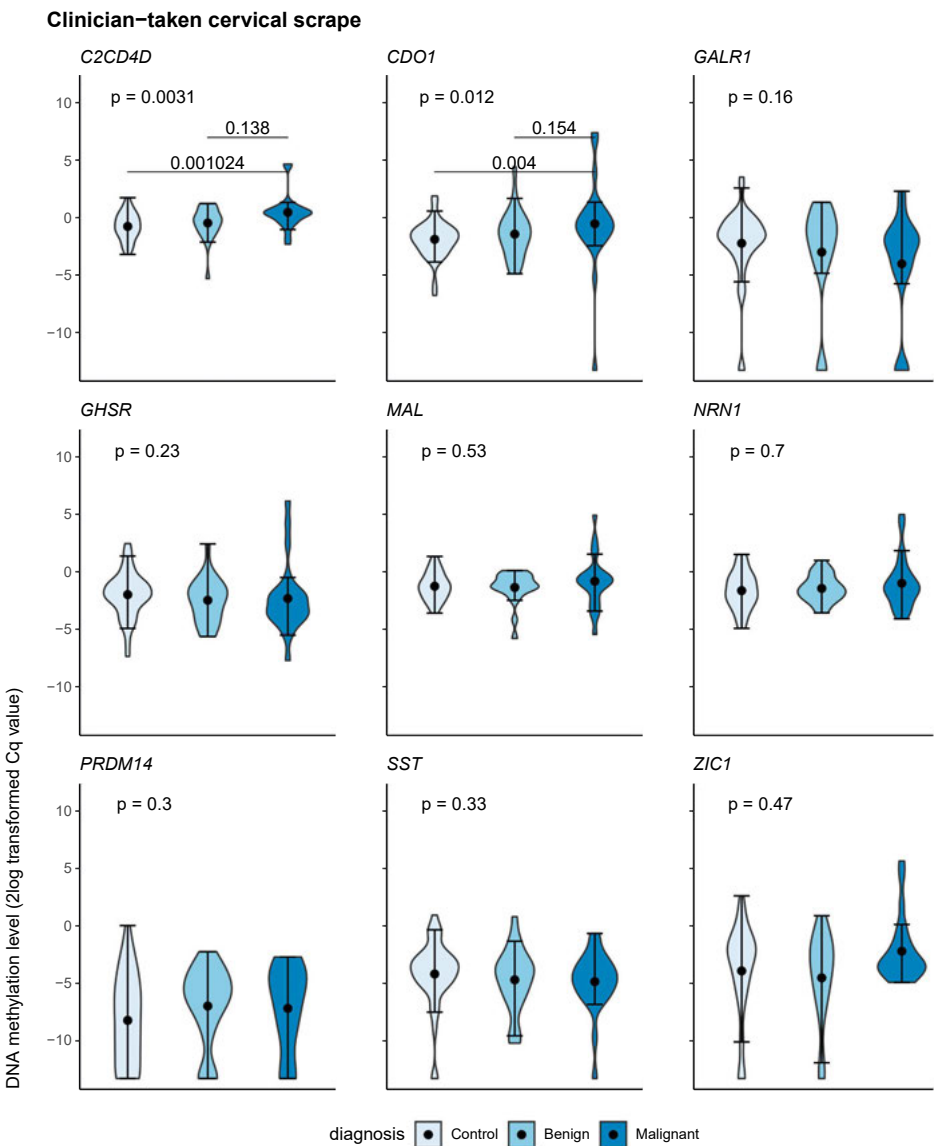
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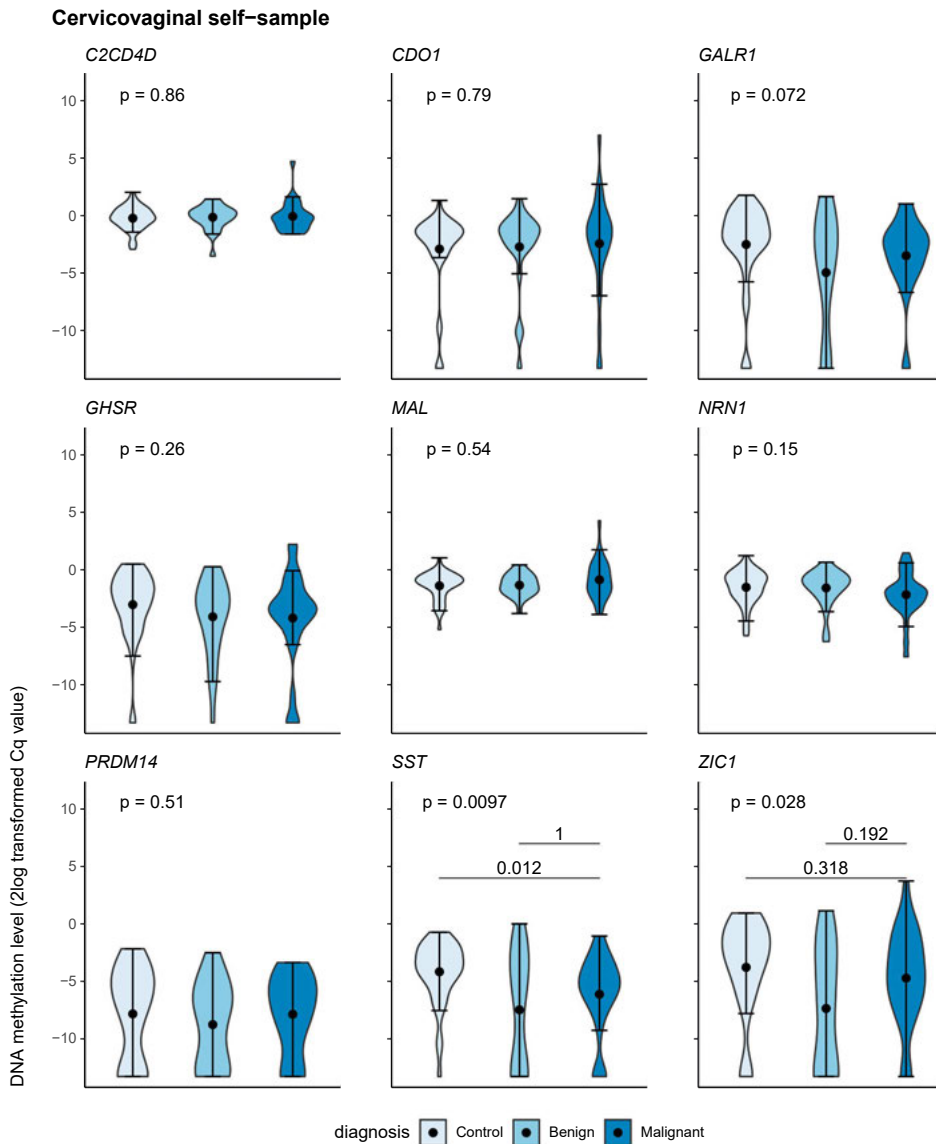
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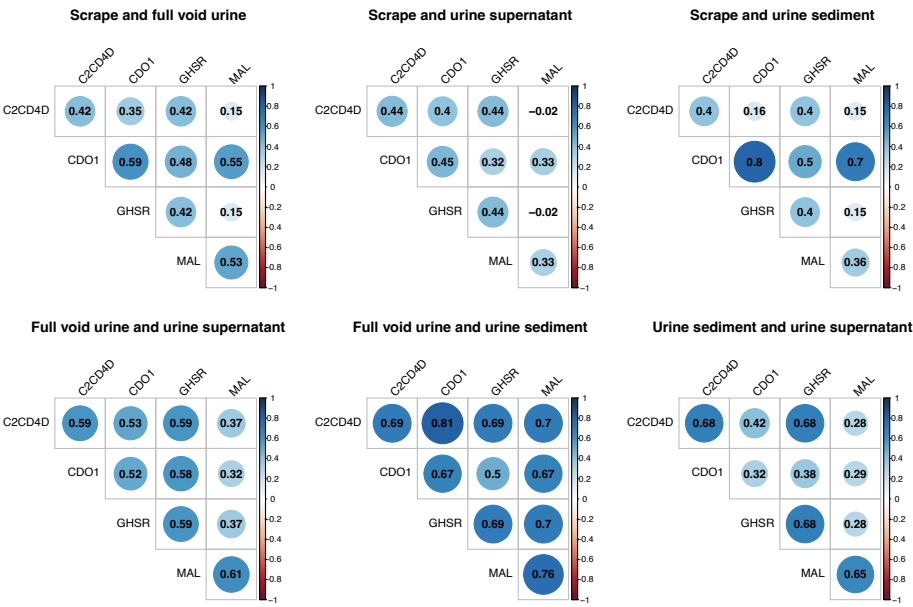
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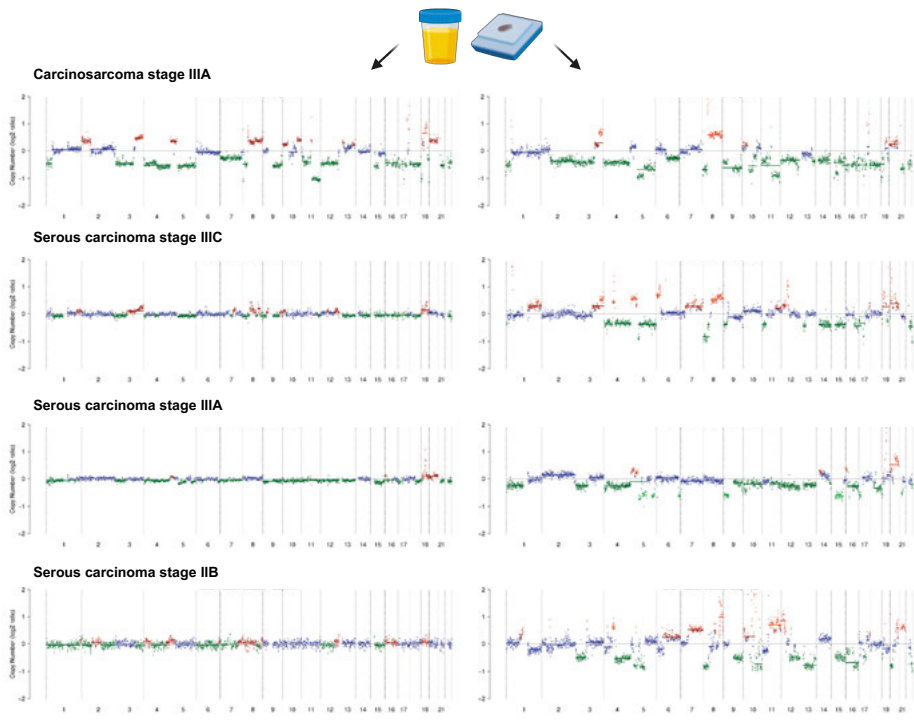
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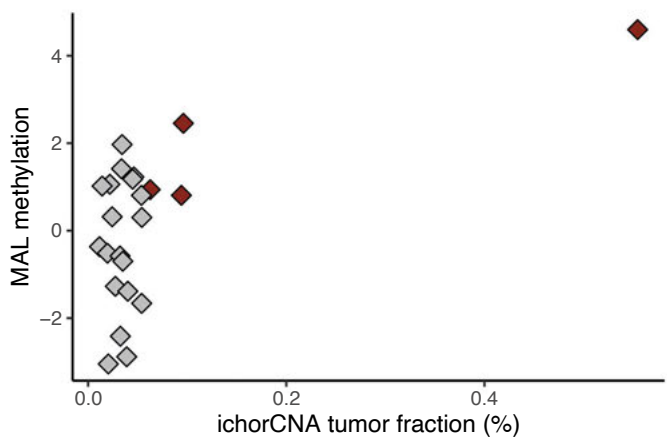
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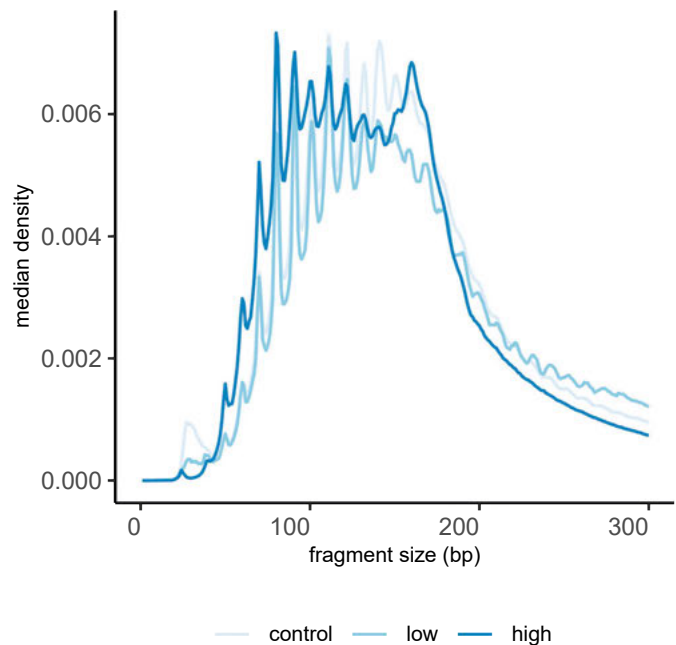
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**Supplemental Figure 9:** Scatter plot indicating the relation between *MAL* methylation levels and the tumor fraction as estimated by ichorCNA in urine supernatant samples. *MAL* methylation levels are shown by 2log-transformed Cq ratios. *MAL* was the most discriminating marker between urine supernatant samples of healthy controls and ovarian cancer patient and therefore plotted against the tumor fraction. The patient with the highest tumor fraction in urinary cfDNA also showed the highest *MAL* methylation, as seen in the upper right part of the plot. Cq = quantification cycle.



**Supplemental Figure 10:** Fragment size distributions for cfDNA reads of urine supernatant samples from healthy controls (n=2) and ovarian cancer patients with a low (<5%, n=19) and high ( $\geq$ 5%, n=4) tumor fraction determined from shallow whole-genome sequencing. The cfDNA with a high tumor fraction revealed a shorter modal fragment size (80 bp) than cfDNA with a low tumor fraction and controls (111 bp).

**Supplemental Table 1:** Primer and probe information of multiplex quantitative methylation-specific PCR assays.

Multi-plex	Target	Forward primer	Reverse primer	Probe	5'Dye- 3'Quencher	Amplicon size	Amplicon location	Tm (°C)
1	<i>GHSR</i>	available upon request	available upon request	available upon request	FAM - BHQ1	88	available upon request	63
1	<i>SST</i>	GGTTGGTTG CGTTGTTATC	CTACAAAACTAAC- GAAATCTAAATCCG	CCGATAACACAAC- CCAAA	VIC - MGB-Eclipse®	84	Chr3: 187670184- 187670267	63
1	<i>ZIC1</i>	available upon request	available upon request	available upon request	DFO - BHQ2	84	available upon request	63
2	<i>CDO1</i>	CGTTTTTTTC- GTTTTATTTCGTCG	CCTCCGAC- CCTTTTATCTACG	TGTGGTTCGCGAC- GTTGGGACGT	FAM - BHQ1	69	Chr5: 115816927- 115816995	60
2	<i>MAL</i>	CGCGTAGTATTAAGTA- GAGAGGTTTCG	ACCGCCGACCCCTTCC	CCACTAAACCGAC- GCTAATTCGACGCT	DFO - BHQ2	85	Chr2: 95025113- 95025197	60
2	<i>PRDM14</i>	TTACGTGTTATTGTGG- GGATTC	ATATCTATTCCTA- ATACCTAAAAAC- GAAACG	AAACGCCTTAAAC- GCTAAAAAACTTCG- CCTC	JOE - BHQ2	88	Chr8: 70071718- 70071805	60
3	<i>C2CD4D</i>	CGTGGGTCGTAGTTGG- TAGTATAG	AACCCGCGACTCGCGG	CGCCGAACCGCCC	DFO - MGB-Eclipse®	84	Chr1: 151838369- 151838452	59
3	<i>GALP1</i>	available upon request	available upon request	available upon request	FAM - BHQ1	65	available upon request	59
3	<i>NRN1</i>	available upon request	available upon request	available upon request	JOE - BHQ1	65	available upon request	59
1,2	<i>ACTB</i>	available upon request	available upon request	available upon request	CY5 - MGB-Eclipse®	68	available upon request	63, 60
3	<i>ACTB</i>	available upon request	available upon request	available upon request	CY5 - MGB-Eclipse®	108	available upon request	59

A {base} indicates a locked nuclear acid (LNA). Amplicon locations are based on GRCh38.109. Sequences not provided are available upon reasonable request from Self-screen B.V.

**Supplemental Table 2:** Analytical validation of multiplex quantitative methylation-specific PCR assays.

<b>Multiplex</b>	<b>Target</b>	<b>Slope</b>	<b>R2</b>	<b>Efficiency (%)</b>
1	<i>GHSR</i>	-3,38	1,00	97,52
1	<i>SST</i>	-3,24	0,99	103,69
1	<i>ZIC1</i>	-3,23	0,99	104,00
1	<i>ACTB</i>	-3,39	0,99	97,26
2	<i>CDO1</i>	-3,21	0,99	104,78
2	<i>MAL</i>	-3,28	0,98	101,89
2	<i>PRDM14</i>	-3,37	0,99	98,15
2	<i>ACTB</i>	-3,38	0,99	102,39
3	<i>C2CD4D</i>	-3,46	0,98	94,56
3	<i>GALR1</i>	-3,27	0,99	102,03
3	<i>NRN1</i>	-3,36	0,99	98,64
3	<i>ACTB</i>	-3,38	0,99	97,76

Data is based on serial dilution series of bisulfite treated methylated DNA from the SiHa cell line (100, 50, 10, 5, 1, 0.5%) within the range of 20 to 0.1 ng.

**Supplemental Table 3:** Shallow whole-genome sequencing coverage and quality statistics.

Sample ID	Library preparation	Total number of sequenced reads	Total number of uniquely mapped non-duplicate reads	Total number of covered bases	Median coverage per base	Percentage of targeted bases with coverage $\geq 10\times$
OC_feas_1	ThruPLEX Plasma-seq Kit (Takara Bio)	34443206	33229566	4600768397	1.0X	0.3%
OC_feas_2	ThruPLEX Plasma-seq Kit (Takara Bio)	4841322	4534802	438559552	0.0X	0.1%
OC_feas_3	ThruPLEX Plasma-seq Kit (Takara Bio)	8670936	8489020	551021318	1.0X	0.2%
OC_feas_4	ThruPLEX Plasma-seq Kit (Takara Bio)	6622584	4903274	313628741	9.0X	48.7%
OC_feas_5	ThruPLEX Plasma-seq Kit (Takara Bio)	3983596	3719015	212249544	0.0X	0.0%
OC_feas_6	ThruPLEX Plasma-seq Kit (Takara Bio)	38765800	37853097	2052308677	0.0X	0.1%
OC_feas_7	ThruPLEX Plasma-seq Kit (Takara Bio)	117445252	116737292	6597660157	0.0X	0.1%
OC_feas_8	ThruPLEX Plasma-seq Kit (Takara Bio)	39243440	33682508	1942114454	0.0X	0.1%
OC_feas_9	NEBNext® Enzymatic Methyl-seq (EM-seq) Kit	55213392	55133492	4817069501	2.0X	1.5%
OC_feas_10	NEBNext® Enzymatic Methyl-seq (EM-seq) Kit	36361574	36295918	3146361198	1.0X	0.3%
OC_feas_11	NEBNext® Enzymatic Methyl-seq (EM-seq) Kit	214080118	214025057	17885220140	7.0X	33.5%
OC_feas_12	NEBNext® Enzymatic Methyl-seq (EM-seq) Kit	153789314	153494635	12073110695	5.0X	16.5%
OC_feas_13	NEBNext® Enzymatic Methyl-seq (EM-seq) Kit	61933416	61703055	5195041255	2.0X	1.8%
OC_feas_14	NEBNext® Enzymatic Methyl-seq (EM-seq) Kit	41047082	40545733	3625729946	1.0X	0.6%
OC_feas_15	NEBNext® Enzymatic Methyl-seq (EM-seq) Kit	61948248	61749958	5148741517	2.0X	1.6%
OC_feas_16	NEBNext® Enzymatic Methyl-seq (EM-seq) Kit	102101482	101856451	8564737514	3.0X	7.3%
OC_feas_17	NEBNext® Enzymatic Methyl-seq (EM-seq) Kit	201244050	201125135	14932769622	6.0X	26.6%
OC_feas_18	NEBNext® Enzymatic Methyl-seq (EM-seq) Kit	182260894	182096275	13778672708	6.0X	22.2%
OC_feas_19	NEBNext® Enzymatic Methyl-seq (EM-seq) Kit	41249044	41097514	3636279625	1.7X	0.6%
OC_feas_20	NEBNext® Enzymatic Methyl-seq (EM-seq) Kit	152163250	152091965	12414842765	5.9X	17.0%
OC_feas_21	NEBNext® Enzymatic Methyl-seq (EM-seq) Kit	57461346	57378412	4707118763	2.2X	1.1%
OC_feas_22	NEBNext® Enzymatic Methyl-seq (EM-seq) Kit	50560614	50490796	4565501330	2.2X	1.3%
OC_feas_23	NEBNext® Enzymatic Methyl-seq (EM-seq) Kit	38128212	37980864	3460774777	1.7X	0.5%
OC_feas_24	NEBNext® Enzymatic Methyl-seq (EM-seq) Kit	106041952	105808160	9403139563	4.5X	8.9%
OC_feas_25	NEBNext® Enzymatic Methyl-seq (EM-seq) Kit	59473824	59277401	4954810846	2.4X	1.5%

Trimmed non-converted and enzymatically converted reads were mapped to the GRCh38 human genome assembly (GeneBank accession: GCA\_0000001405.28). Targeted bases are defined as whole-genome.





## PART 2

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### NON-SMALL CELL LUNG CANCER DETECTION IN URINE



# CHAPTER 6

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## DETECTION OF NON-METASTATIC NON-SMALL CELL LUNG CANCER IN URINE BY METHYLATION-SPECIFIC PCR ANALYSIS: A FEASIBILITY STUDY

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\*Equal contribution.

## ABSTRACT

### Background

Lung cancer has the highest cancer-related mortality worldwide and earlier detection could improve outcomes. Urine circulating tumor DNA (ctDNA) represents a true non-invasive means for ambulant sample collection. In this prospective study, the potential of urine for perioperative detection of non-metastatic non-small cell lung cancer (NSCLC) using ctDNA methylation analysis is evaluated.

### Methods

Preoperative urine samples of 46 surgical NSCLC patients and 50 sex and age-matched controls were analyzed for DNA methylation of NSCLC-associated methylation markers *CDO1*, *SOX17*, and *TAC1*, using quantitative methylation-specific PCR (qMSP). The accuracy for NSCLC detection was determined by univariable and multivariable logistic regression analysis, followed by leave-one-out cross-validation. Fourteen additional urine samples were collected postoperatively to evaluate whether DNA methylation levels alter after surgery with curative intent.

### Results

Methylation levels of *CDO1* and *SOX17* were significantly elevated in patients compared to controls ( $P = .016$  and  $P < .001$ , respectively). This marker combination yielded an area under the receiver operating curve (AUC) value of 0.71 upon leave-one-out cross-validation for non-metastatic NSCLC detection in urine. Stage I patients tended to have higher methylation levels of *SOX17* as compared to stage III patients. Similar methylation levels were found across the different histological subtypes of NSCLC. In some patients with preoperative elevated methylation levels, reduced methylation levels were found in post-operative urine samples.

### Conclusions

Urine *CDO1* and *SOX17* showed increased methylation levels in NSCLC patients as compared to sex- and age-matched controls. This demonstrates that urine ctDNA methylation analysis may provide an interesting non-invasive means to detect non-metastatic NSCLC. Further studies are needed to validate the clinical usefulness of this approach and to assess the potential of post-operative monitoring.

### HIGHLIGHTS

- Non-small cell lung cancer is often diagnosed at an advanced stage.
- Urine is a valuable and patient-friendly source of tumor DNA for early diagnosis.
- Increased methylation levels of *CDO1* and *SOX17* in urine are diagnostically relevant.
- Urine methylation tests could support primary and recurrent lung cancer diagnoses.

## INTRODUCTION

Lung cancer is one of the most commonly diagnosed cancer types worldwide, and accounts for the highest cancer-related mortality in many countries (1). In early-stage patients, local ablative modalities such as radiotherapy and surgery can be curative (2, 3). This is illustrated by a favorable prognosis of patients with stage I and II NSCLC, with a 5-year survival varying from 52 to 93% (4). Despite curative intent treatments, these patients eventually develop recurrences in approximately 30%, mostly attributable to hematogenous metastases (5). These numbers underline the importance of early detection of NSCLC, and motivate the initiation of large-scale screening trials, such as the NLST and NELSON trials, that investigate the value of low-dose computed tomography (LDCT) in detecting lung cancer in at-risk populations (6-9). A critical issue raised by the NLST is the high rate of false positives (96%) found with LDCT screening. Similar concerns regarding potential over-diagnosis were raised following the NELSON trial (9). Although an algorithm accounting for the tumor volume doubling time reduced the number of false positives, still, high numbers of false positives were found, resulting in unnecessary diagnostic procedures. This hampers the implementation of LDCT screening in Europe (10) and emphasizes the urgent need for additional strategies to discriminate between patients with lung cancer and nonmalignant lesions.

Plasma-based liquid biopsies are playing an ever-increasing role in the clinical practice of mainly actionable genomic alteration positive advanced-stage NSCLC (11, 12). Tumor-shed cell-free DNA (cfDNA) in the blood, often referred to as circulating tumor DNA (ctDNA), can enable non-invasive NSCLC detection through DNA sequencing (13, 14). However, a less known but promising modality for identifying ctDNA is the use of DNA methylation, *i.e.*, the covalent attachment of methyl ( $\text{CH}_3$ ) to cytosine bases located in cytosine-guanine (CpG) dinucleotides, involved in the regulation of gene transcription. In many cancer types, epigenetic dysregulation appears at the early stages of oncogenesis through the hypermethylation of promoter regions of tumor suppressor genes (15). Methylation-based ctDNA analysis could thus be of interest to incorporate in a multi-dimensional lung cancer screening algorithm with LDCT (16-19).

Besides plasma, urine offers an alternative viable source of ctDNA (20-23). Plasma ctDNA can translocate to urine if sufficient fragmentation occurs, enabling renal passage. Urine ctDNA allows for the same diagnostics as ctDNA derived from plasma or sputum, including the detection of NSCLC-specific driver mutations (24) and changes in DNA methylation (19, 25). Moreover, urine has several advantages over plasma, as it is truly non-invasive and does not require healthcare professionals to collect and provides a stable environment for DNA when handled correctly (26).

We considered the markers *CDO1*, *SOX17*, and *TAC1* as most interesting methylation marker candidates to evaluate the detection of non-metastatic NSCLC in the urine. This is based on their diagnostic potential for detecting NSCLC in sputum (17, 27), plasma (17, 19), and urine (19). Previously, high diagnostic efficacy of these methylation markers was shown in genome-wide discovery studies using both tissues from The Cancer Genome Atlas (TCGA) database (17, 19, 27) and other discovery cohorts (27). *CDO1* was also specifically identified and validated as a biomarker for stage I NSCLC detection in minimally invasive samples (27).

In this study, the diagnostic potential of DNA methylation analysis for non-metastatic NSCLC detection in urine was evaluated by assessing the previously described NSCLC methylation markers *CDO1*, *SOX17* and *TAC1* (17, 19, 27, 28) in preoperative urine samples. Furthermore, we explored the methylation levels of these genes in postoperative urine samples to evaluate whether methylation levels altered after surgery.

## MATERIAL AND METHODS

### Study design and population

This was a single-institution prospective study from a non-screening population in The Netherlands. Eligible patients, planned for anatomical pulmonary resection for (suspected) NSCLC, were consecutively enrolled and urine was collected between March 2018 and September 2020 at the outpatient clinic of the Department of Pulmonary diseases of the Amsterdam UMC, a tertiary referral center in Amsterdam, the Netherlands. Patients were older than 18 years, diagnosed with NSCLC of any histological subtype, did not undergo any anti-cancer (induction) therapy for at least one year prior to sampling and had no diagnosis of any type of other cancer in the last 5 years preceding lung cancer diagnosis. The cancer stage was determined using the 8<sup>th</sup> edition of the TNM classification system of the International Association for the Study of Lung Cancer (IASLC) (29).

For exploration purposes, also postoperative urine samples were collected during the course of the study to evaluate whether methylation levels alter after resection of the tumor. Hence, postoperative samples were only collected in a subset of patients at various time points after surgery with curative intent.

Control samples were obtained from healthy volunteers through the Urine Controls (URIC) Biobank. URIC participants were selected for eligibility through a questionnaire to exclude controls with a cancer history in the previous 5 years. Furthermore, age, sex,

and smoking history were documented. URIC participants were selected on having the same age range as the NSCLC cases.

The Medical Ethical Committee board of the Amsterdam UMC approved the study design including the collection of urine from NSCLC patients (no. 2017.333 and no. 2017.545) and healthy volunteers (no. 2017.112). Written informed consent was obtained from all participants of this study.

### **Urine collection and processing**

Preoperative urine samples were collected autonomously by participants at home at least two weeks before planned pulmonary surgery. Ambulant urine collection was realized by providing participants a collection kit which included a large collection container (300 ml) and three 30 ml collection tubes. The collection tubes contained 2 ml of 0.6 M Ethylenediaminetetraacetic acid (EDTA) as a preservative agent (final concentration of 40 mM). Study participants sent their urine samples to the Department of Pathology of Amsterdam UMC, location VUmc by mail, where samples were processed within 24-72 h, following collection. This collection and storage protocol was previously validated (26). Postoperative urine samples were usually collected at outpatient clinic visits, or by autonomous collection at home as described above. To acquire the urine supernatant fraction, samples were centrifuged at 3000g for 15 min. All urine samples were stored at -20 °C until DNA isolation.

### **DNA isolation and bisulfite modification**

DNA was isolated from 20 ml urine supernatant using the Quick DNA urine kit (Zymo Research, Irvine, CA, US). Extracted DNA was eluted in 50 µl elution buffer, after which DNA concentrations were measured using the Qubit™ dsDNA HS Assay (Invitrogen, Carlsbad, CA, US). To allow for DNA methylation analysis, up to 250 ng of isolated DNA was subjected to sodium bisulfite treatment using the EZ DNA Methylation kit (Zymo Research, Irvine, CA, US). All procedures were performed according to the manufacturer's guidelines.

### **DNA methylation analysis**

DNA methylation analysis was performed using quantitative methylation-specific PCR (qMSP), as described previously (25). Briefly, a multiplex qMSP targeting the hypermethylated promoter regions of 3 genes (*CDO1*, *SOX17*, and *TAC1*) and a reference gene (*ACTB*) was developed based on gene loci discovered in Hulbert *et al.* (17) and adjusted for NSCLC detection in urine in Liu *et al.* (19). Amplicon sizes did not exceed 70 base pairs, facilitating the detection of methylation in small DNA fragments present in urine. The qMSP analysis was performed on a ViiA7 real-time PCR-system (Applied

Biosystems, Foster City, CA, USA), using Epiect Multiplex PCR Mastermix (Qiagen, Venlo, Netherlands) and 2.5–5.0  $\mu\text{M}$  of each primer and 5.0–10.0  $\mu\text{M}$  of each probe in a total volume of 12.5  $\mu\text{l}$ . As a positive control, double-stranded gBlocks™ Gene Fragments (Integrated DNA Technologies) containing the target regions were taken along. H<sub>2</sub>O was taken along as a negative control during each run. Samples with a *ACTB* Cycle threshold (Ct) value exceeding 32 were excluded from methylation analysis to ensure sample quality and sufficient input. Methylation marker abundance was calculated relative to *ACTB* levels (Ct-ratio), using the following formula:  $2^{-(\text{Ct}_{\text{MARKER}} - \text{Ct}_{\text{ACTB}})} \times 100$ .

### **Data analysis**

For comparison of categorical data between groups, the  $\chi^2$  test was used. All calculations of methylation levels were performed using square root transformed Ct-ratios. Differences in DNA methylation levels between cases and controls, smokers and non-smokers, stages, histological subtypes, and tumors with and without nodal involvement were compared using the Mann Whitney U test. *P*-values <.05 (two-sided) were considered statistically significant.

The performance of individual methylation markers was assessed by univariate logistic regression analysis. To determine whether a combination of markers improved discrimination between cases and controls, multivariate logistic regression using backward selection was applied. The predicted probabilities obtained from the logistic regressions, representing the probability for the presence of NSCLC, were visualized using receiver operating characteristic (ROC) curves, including the area under the curve (AUC) value for each sample and individually per sample. Model performance was evaluated by AUC values with confidence intervals, and sensitivity and specificity at the Youden's Index (*J*) threshold (30). This threshold was used to define marker cut-offs based on the predicted probabilities that maximizes the sum of sensitivity and specificity. The predictive performance of the individual markers and marker combination were assessed outside the set by leave-one-out cross-validation (LOOCV). Samples were considered positive if any of the individual markers was classified as positive ('believe-the-positive') (31). Statistical testing was performed using SPSS (SPSS 22.0, IBM Corp., NY, USA). Logistic regression analyses and LOOCV were executed using R version 4.0.3 (Vienna, Austria. UR).

## **RESULTS**

### **Study population**

A total of 46 patients who underwent pulmonary surgery with curative intent for NSCLC and 50 controls were included. NSCLC patients and controls showed no statistically significant

differences between sex ( $P = .43$ ) and age ( $P = .64$ ). The proportions of never, former, and current smokers significantly differed between NSCLC patients and controls ( $P < 0.001$ ).

Two urine samples of NSCLC patients had insufficient quality ( $ACTB\ Ct \geq 32$ ) and were therefore excluded from further analysis, resulting in a study population of 44 patients for present methylation analysis. All control samples met the DNA quality criteria. Clinical characteristics of NSCLC patients and controls with valid qMSP results are depicted in Table 1.

**Table 1:** Baseline characteristics of NSCLC patients (n=44) and controls (n=50).

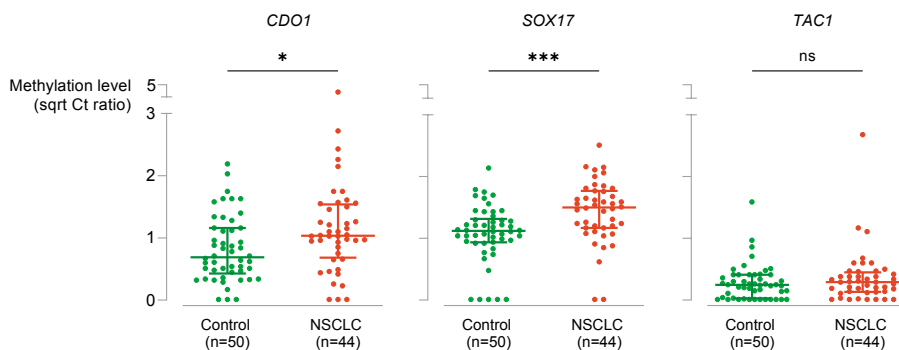
	NSCLC		Controls		P
Age					
Median (IQR)	66 (62-72)		66 (59-80)		.64
Sex					
	n	%	n	%	
Male	21	47.7	28	56.0	.43
Female	23	52.3	22	44.0	
Stage*					
	n	%			
1	28	63.6	NA		
2	4	9.1			
3	12	27.3			
4	0	0.0			
Histology					
	n	%			
LUAD	27	61.4	NA		
LUSC	16	36.4			
NOS	1	2.3			
Smoking					
	n	%	n	%	
Never smokers	3	6.8	30	60.0	<0.001
Former smokers (stopped >1yr)	12	27.3	16	32.0	
Current smokers (active or stopped <1yr)	28	63.6	4	8.0	
Unknown	1	2.3	0	0.0	

\*Staging was according to the 8th edition of the TNM criteria. IQR = interquartile range, LUAD = lung adenocarcinoma, LUSC = lung squamous cell carcinoma, NOS = carcinoma not otherwise specified, NSCLC = non-small cell lung cancer.

### DNA methylation levels in pre-operative urine samples

DNA methylation levels of markers *CDO1*, *SOX17*, and *TAC1* relative to *ACTB* were determined in the urine supernatant of 44 surgical NSCLC patients and 50 controls. Methylation levels of *CDO1* and *SOX17* were significantly higher in NSCLC patients as compared to controls ( $P = .016$  and  $P < .001$ , respectively), while *TAC1* did not show significant differences between groups ( $P = .347$ , Figure 1). As the proportion of smokers differs between the NSCLC

patients and controls, methylation levels of smokers and non-smokers were compared within both groups. The methylation levels did not significantly differ between smokers and non-smokers diagnosed with NSCLC. Likewise, methylation levels of smoking controls were comparable to non-smoking controls (Supplementary Figure 1).



**Figure 1: Methylation levels in the urine supernatant samples.** Methylation levels of markers *CDO1*, *SOX17*, and *TAC1* in the urine supernatant of surgical NSCLC patients and controls. Data is depicted as the median with an interquartile range of square root transformed Ct ratios. The green and red circles represent the DNA methylation levels of individual controls and cases, respectively. A *P*-value of .05 was considered statistically significant. \* =  $P < .05$ , \*\*\* =  $P < .001$ , ns = not significant. NSCLC = non-small cell lung cancer.

### DNA methylation levels in relation to disease severity and histology

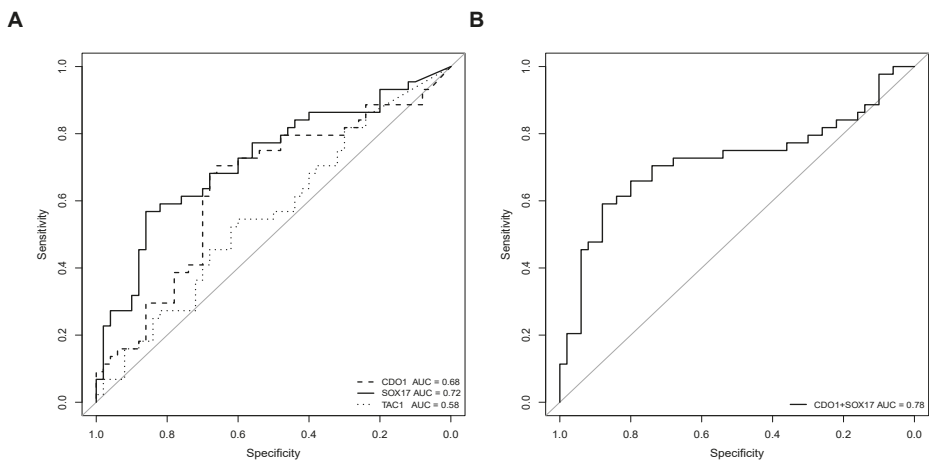
The methylation levels of each marker were compared between the different cancer stages and histological subtypes of NSCLC (Supplementary Figure 2). Due to low patient numbers in the stage II group ( $n=4$ ), only stage I ( $n=28$ ) could be compared with stage III ( $n=12$ ). While *CDO1* and *TAC1* showed no significant difference between these stages, a trend toward higher methylation levels in stage I cases was seen for *SOX17*. In line with these findings, *SOX17* levels were also found to be lower in tumors with lymph node involvement, which are overrepresented in the stage III group (Supplementary Figure 3). None of the markers showed a significant difference between the histological subtypes lung adenocarcinoma (LUAC,  $n=27$ ) and lung squamous cell carcinoma (LUSC,  $n=16$ ). The histological subtype not otherwise specified (NOS) could not be taken along in this comparison, due to the low number of patients in this group ( $n=1$ ).

### Diagnostic performance of DNA methylation analysis for pre-operative NSCLC detection

Univariate logistic regression analysis was performed and individual AUCs were calculated for each marker (Figure 2A). The AUCs obtained for *CDO1*, *SOX17*, and *TAC1* were 0.68 (95% CI: 0.54-0.76), 0.72 (95% CI: 0.61-0.83), and 0.58 (95% CI: 0.46-0.69), respectively.

Validation by LOOCV yielded similar AUCs of 0.64, 0.72, and 0.56 for *CDO1*, *SOX17*, and *TAC1*, respectively. Sensitivities and specificities based on the maximal Youden's Index (*J*) threshold, varied from 0.48 to 0.68 and 0.66 to 0.86, respectively (Table 2).

To evaluate potential complementarity between markers, multivariate logistic regression with backward selection was used (Figure 2B). The backward selection rejected *TAC1* from the final model, yielding an AUC of 0.78 (95% CI: 0.68-0.87) for *CDO1* and *SOX17* combined. Upon validation by LOOCV, an AUC of 0.71 was achieved, with a sensitivity of 0.55 and specificity of 0.86 based on a 'believe-the-positive' algorithm (Table 2).



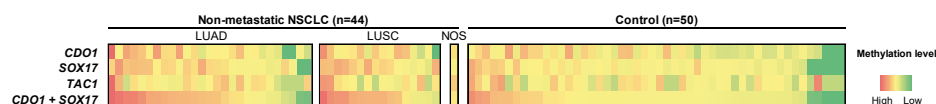
**Figure 2: Diagnostic potential of individual methylation markers and marker combination.** Non-CV receiver operator characteristic (ROC) curves of *CDO1*, *SOX17*, *TAC1* (A) and *CDO1*/*SOX17* combined (B). Results of individual markers and the marker combination are quantified by the area under the curve (AUC) value. Non-CV = non-cross-validated.

**Table 2:** Univariable logistic regression analysis on diagnostic performance of the three individual markers (*CDO1*, *SOX17*, *TAC1*) and multivariable logistic regression analysis on diagnostic performance of the optimal marker combination (*CDO1*+*SOX17*) for NSCLC detection.

Methylation marker(s)	<i>CDO1</i>	<i>SOX17</i>	<i>TAC1</i>	<i>CDO1</i> + <i>SOX17</i>
AUC (non-CV; 95% CI)	0.68 (0.54-0.76)	0.72 (0.61-0.83)	0.58 (0.46-0.69)	0.78 (0.68-0.87)
Sensitivity	0.68	0.57	0.48	0.57
Specificity	0.66	0.86	0.68	0.86
AUC (LOOCV)	0.64	0.72	0.56	0.71
Sensitivity	0.68	0.55	0.46	0.55
Specificity	0.64	0.82	0.58	0.86

Non-CV AUC values of individual markers and marker combination *CDO1* + *SOX17*, including 95% CI, are reported together with sensitivity and specificity based on the Youden's Index (*J*) threshold. LOOCV AUC values are reported together with sensitivity and specificity based on a 'believe-the-positive' algorithm. AUC = area under the receiver operating characteristic curve, CI = confidence interval, LOOCV = leave-one-out cross-validated, non-CV = non-cross-validated.

The DNA methylation levels of *CDO1*, *SOX17*, *TAC1*, and *CDO1 + SOX17* combined were visualized for each individual sample using predicted probabilities (Figure 3). Urine samples of surgical NSCLC patients were stratified per histological category, including LUAD, LUSC and NOS. Sorting the samples on predicted probabilities found for *CDO1 + SOX17* combined illustrates the added value of using a marker panel, instead of individual markers. Methylation levels were highly variable among both the surgical NSCLC patients and controls. Predicted probabilities ranged from 0.13 (green) to 0.56 (red). The majority of cases showed high methylation levels (red/orange) and most controls showed low methylation levels (yellow/green) for the combined marker panel.



**Figure 3: DNA methylation of *CDO1*, *SOX17*, *TAC1*, and *CDO1+SOX17* combined in urine samples of surgical NSCLC patients per histological category and healthy controls.** The methylation levels are shown per sample (column) and visualized using predicted probabilities in a three-color gradient from green (lowest predicted probability – 0.13, indicating low methylation levels) to red (highest predicted probability – 0.56, indicating high methylation levels). Samples are stratified per histological category, including LUAD, LUSC, and NOS, and sorted based on the predicted probabilities of the optimal marker panel *CDO1 + SOX17*. LUAD = lung adenocarcinoma, LUSC = lung squamous cell carcinoma, NOS = carcinoma not otherwise specified.

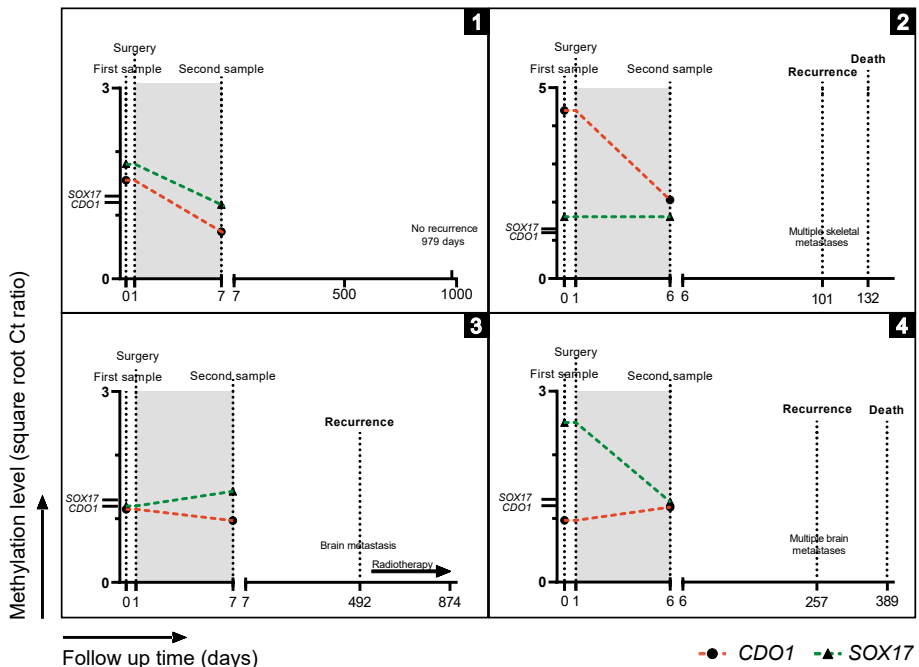
### DNA methylation in post-operative urine samples

The primary objective of this study was to evaluate the diagnostic potential of DNA methylation analysis in urine of NSCLC patients undergoing surgery with curative intent. Upon acquisition of preliminary results thereof, postoperative urine samples were also collected from a subset of the enrolled NSCLC patients to assess the methylation levels after resection of the tumor. Postoperative urine samples were collected from 14 NSCLC patients, 6 or 7 days (n=4) or 63 to 974 days (n=10) after surgery with curative intent (Supplementary Table 1). The upper quartile value (*i.e.* 75% percentile) of methylation levels in control samples was used to arbitrarily define a threshold (1.2 for *CDO1* and 1.3 for *SOX17*) for preoperatively elevated methylation levels. Since *TAC1* showed no differences in urine methylation between cases and controls, defining such a threshold was not possible and therefore only methylation levels of *CDO1* and *SOX17* were evaluated in postoperative samples.

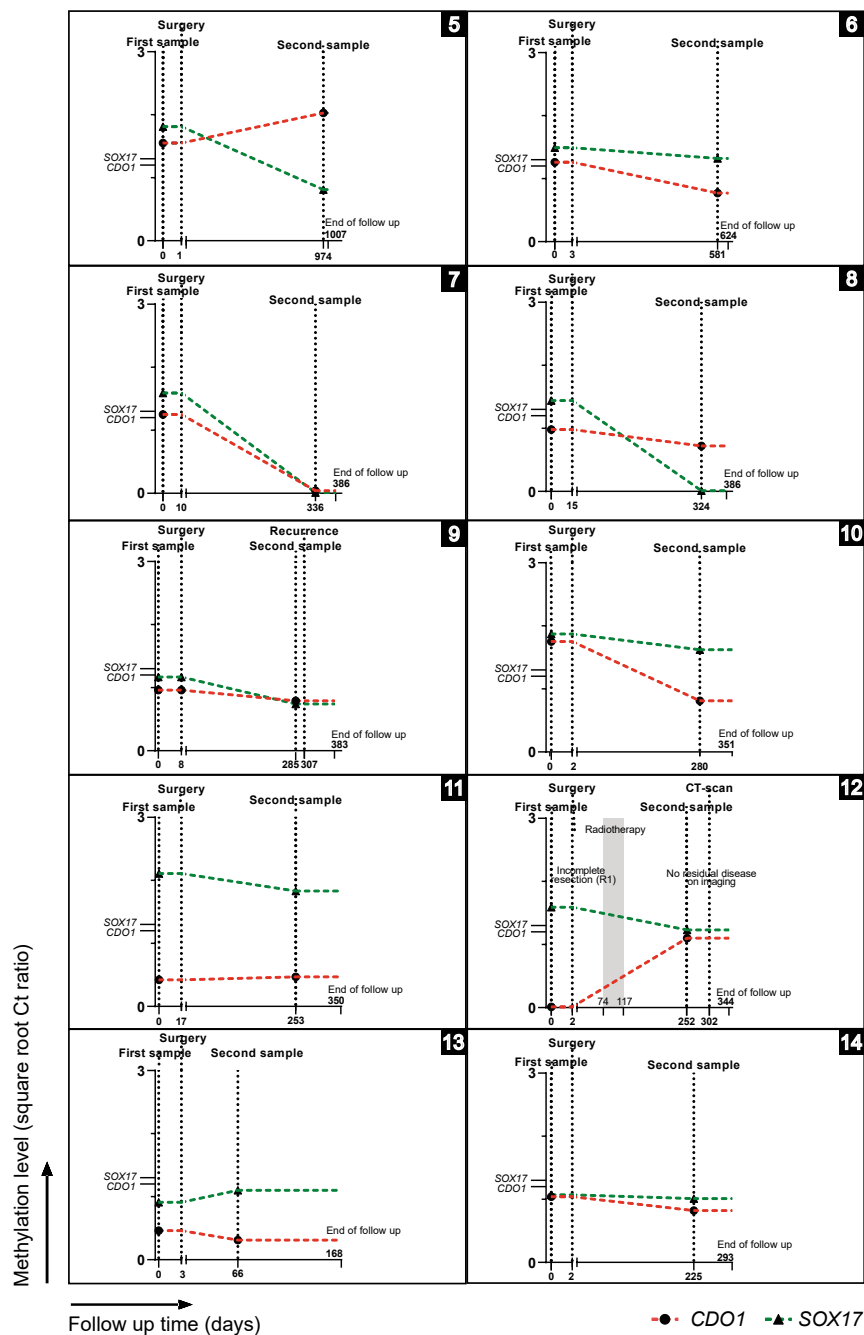
To explore if methylation levels were altered shortly after curative intent surgery, 4 patients provided urine samples before and 6 or 7 days after surgery (Figure 4). In 3 patients with preoperatively elevated methylation levels for both markers (patient 1) or a single marker (patient 2 and 4), methylation levels were reduced in the post-operative

samples. Patient 2, 3, and 4 were diagnosed with recurrent disease after 101-497 days of follow-up, and patient 1 showed no recurrence up to 979 days of follow-up. Despite the fact that no clear pattern of methylation can be seen shortly after curative intent surgery in these few individual cases, it is remarkable that the only patient in which both markers were clearly elevated preoperatively, a decrease was seen postoperatively (patient 1).

To obtain an impression on whether an increase in urine DNA methylation could be indicative of the presence of disease recurrence, another subset of patients (n=10) provided a second urine sample between 63 and 974 days after surgery (Figure 5). Seven of these patients (patients 5-8 and 10-12) showed elevated methylation levels of at least one marker pre-operatively, which were all reduced in the post-operative samples. None of these patients were diagnosed with recurrent disease during follow-up. Since only a single patient had recurrent disease after 307 days of follow-up (patient 9) and showed low methylation levels in both pre- and post-operative urine samples no correlation between recurrence and methylation could be assessed.



**Figure 4: Pre- and postoperative methylation levels of *CDO1* and *SOX17* for the detection of residual disease or early distant metastasis.** Methylation levels of *CDO1* (circles) and *SOX17* (triangles) measured before (first sample) and shortly after (second sample) surgery. Moments of sampling, presence of recurrence, treatment, and patient survival are also represented for each case. Methylation levels are depicted as square root transformed Ct ratios. Note that the methylation levels of patient 2 are represented on a different y-axis to show the high *CDO1* methylation level measured in the first sample. The upper quartile value of methylation levels in control samples is visualized on the y-axes to illustrate an arbitrarily defined threshold (1.2 for *CDO1* and 1.3 for *SOX17*) for preoperatively elevated methylation levels.



**Figure 5: Pre- and postoperative methylation levels of *CDO1* and *SOX17* for the detection of disease recurrence.** Methylation levels of *CDO1* (circles) and *SOX17* (triangles) measured before (first sample) and 63-974 days after (second sample) surgery. Moments of sampling, presence of ----->

-----> recurrence, treatment, and patient survival are also represented for each case. Methylation levels are depicted as square root transformed Ct ratios. The upper quartile value of methylation levels in control samples is visualized on the y-axes to illustrate an arbitrarily defined threshold (1.2 for *CDO1* and 1.3 for *SOX17*) for preoperatively elevated methylation levels. Of note, in patient 12, the primary tumor was not completely resected, indicated by an R1 classification of the surgical specimen. For this reason, this patient underwent radiotherapy after which no residual disease was determined during follow-up.

## DISCUSSION

This prospective biomarker study demonstrated that urine of non-metastatic NSCLC patients contains elevated levels of the DNA methylation markers *CDO1* and *SOX17*, as compared with urines of sex- and age-matched controls. When combined, the two methylation markers yielded a cross-validated AUC of 0.71 for the detection of non-metastatic NSCLC. The results from the present study are amongst the first to demonstrate that detection of NSCLC-specific ctDNA in urine is feasible through DNA methylation analysis.

In a 2020 pioneer study, Liu and Hulbert *et al.* demonstrated that urinary DNA methylation analysis in cancer-specific loci, including *CDO1*, *SOX17*, *TAC1*, and *HOXA9*, was significantly associated with the diagnosis of NSCLC (19). By combining with plasma DNA methylation analysis, high accuracy could be achieved. In the present study, *SOX17* was the most discriminating marker, as was the case in the pioneering study (AUC 0.72 and AUC 0.78, respectively). Methylation marker *TAC1* was not increased in NSCLC patients in this study, while it had an equal performance as *CDO1* in the study by Liu *et al.* This finding could be explained by technical differences and differences in the source populations of cases and controls. In this respect, also stage of disease may be of importance as in our previous study on metastatic NSCLC patients, opposed to non-metastatic NSCLC in present study, *TAC1* was found to be increased in urine as compared to healthy controls. Yet, *TAC1* showed a lower reproducibility as compared to the markers *CDO1* and *SOX17* (25).

Surprisingly, methylation levels of *SOX17* were highest in urine from stage I NSCLC patients. This could be partly due to the differences in number of cases per stage of which the majority (64%) were stage I tumors. Yet, in the abovementioned pioneer study of Liu *et al.* (19), also no differences were found when comparing both plasma and urine of low (stage I and II) versus high stage (stage III and IV) NSCLC tumors. Although counterintuitive, the absence of ctDNA in advanced cancer patients with a high tumor burden and presence of metastasis has been remarked previously (32). A study on colorectal cancer detection in urine showed that the presence of the primary tumor may influence the detection of methylated DNA, with higher methylation levels in

patients in which the primary tumor was still present (33). In line with recent literature showing that *CDO1*, *SOX17*, and *TAC1* are highly methylated in both LUAD and LUSC (17), similar methylation levels were found in both histological subtypes of NSCLC.

Other studies investigating the use of urine for NSCLC detection focused mainly on oncogenic driver mutations or mutations that develop resistance to targeted therapies. Several studies have described the detection of clinically actionable mutations in urine, including *EGFR* (19, 24, 34-36) and *KRAS* (19), indicating the presence of NSCLC-derived DNA in this body fluid. Interestingly, Yu *et al.* (36) found that the detection of *EGFR* mutations in urine was more accurate in predicting the outcome of NSCLC patients as compared to plasma. Furthermore, it has been demonstrated that urine allows detecting mutations that were sometimes not found in concordant tissue or plasma (37-39). Monitoring response to systemic therapy using urine ctDNA has also been examined for NSCLC (35, 40, 41). Hu *et al.* (35) examined the use of urine for the detection of early NSCLC relapses in *EGFR*-positive patients and found that elevated urine DNA concentrations after first-line therapy may already indicate the presence of minimal residual disease. These data underline the potential value of urine as a liquid biopsy for tumor response monitoring and the clinical management of NSCLC.

To our knowledge, there is no data available about perioperative dynamics of methylated DNA in urine for lung cancer patients. In a small pilot of 14 patients, we investigated whether methylation levels alter after surgery with curative intent. Although we found a reduction in methylation levels of some markers post-operatively and in patients without recurrence, no conclusive results were obtained. Whether methylation analysis could be useful for therapy monitoring and the detection of disease recurrence warrants further investigation in larger cohorts using a broader panel of methylation markers. Currently, the MEDAL trial is ongoing, which is a prospective observational trial in which both plasma ctDNA mutations and methylation are utilized as prognostic biomarkers for surgical NSCLC patients (42).

The current feasibility study has several limitations. Our control group consisted of healthy volunteers. Since age and sex could influence background methylation levels (43), the selection of controls was based on these characteristics. However, while almost all NSCLC patients had a smoking history (91%), only less than half of the control group reported a smoking history (40%). It is therefore possible that the methylation results obtained in the NSCLC group can at least partially be attributed to the general changes in DNA methylation patterns associated with tobacco use (44). Yet, in line with our findings, for methylation levels of *CDO1*, *SOX17*, and *TAC1* in particular, independent studies have reported that methylation of these genes allow the prediction of NSCLC

independent of smoking status (17, 19), although smoking status was defined differently. To further rule out bias due to smoking status, more smoking controls should be included in future studies. Furthermore, to determine whether the urine assay is able to differentiate between lung cancer and other pulmonary diseases, future studies should also include patients with non-malignant pulmonary pathology. Nevertheless, from a screening perspective, subjects without any (pulmonary) medical history will represent a large proportion of the screening population. A second potential limitation of the present study is the use of only one urine sample per patient as previous work found that DNA methylation levels might vary greatly over time, both between- and within advanced-stage NSCLC patients (25).

A key strength of the current study is that study participants collected urine from home and sent it to the laboratory by regular mail. This is an appropriate setting to evaluate the use of a self-collected specimen to make screening more accessible. Another strength is the measurement of three methylation markers and a reference gene within a single PCR reaction to reduce costs, time, and the amount of input DNA. Although numbers were small, the collection of a second urine sample after surgery of a subset of patients allowed exploring whether methylation levels altered after surgery with curative intent.

Several technical improvements can be suggested for future studies. In this study, a commercial kit for column-based DNA extraction was used. However, other protocols that enable isolation of short fragmented urine cfDNA, such as methylation on beads (17, 19), hybridization capture, and Q Sepharose DNA isolation (45), might prove superior. Furthermore, bisulfite-free procedures using modified sequencing techniques or nanotechnology-based electrochemical biosensors might facilitate a more sensitive and robust detection of DNA methylation (46-48). Due to the dynamic nature of cfDNA in the urine of NSCLC patients, collecting multiple urine samples per patient could also increase the accuracy of NSCLC detection in urine (25). A genome-wide screen across gene promoter regions using urinary cfDNA of non-metastatic NSCLC patients may yield more accurate biomarkers applicable to urine samples. The combination of DNA methylation with other ctDNA aberrations, such as mutations, copy number alterations or differences in fragment lengths (49), but also non-DNA tumor derivatives in urine such as proteomics or metabolomics, might further improve the performance of urine-based cancer tests (50, 51).

In conclusion, the present study demonstrates technical feasibility of detecting non-metastatic NSCLC in urine using ctDNA methylation analysis. Further research including more patients is needed to validate this approach.

### **Acknowledgments**

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### **Disclosure of interest**

RS has a minority stake in Self-screen B.V., a spin-off company of VU University Medical Center Amsterdam. SB, RS, IB, and GK are named inventors on patent applications related to the detection of methylated DNA in urine. All other authors have no conflict of interest to declare.

### **Author Contributions**

SB, IB, and RS designed the study. SB recruited patients, collected clinical samples, and clinical data. IB and CD guided and provided the collection of clinical samples. BW, DW, and AH were involved in the design and optimization of the multiplex methylation assay. BW, SB, MT, TB, and DW performed laboratory work. RS supervised laboratory experiments. BW, SB, and BL-W carried out statistical analyses. BW, SB and RS drafted the first version of the manuscript. All authors contributed to the interpretation of the data and revised the manuscript critically. Funding was acquisitioned by GK, IB, and RS. All authors approved the final version of the manuscript.

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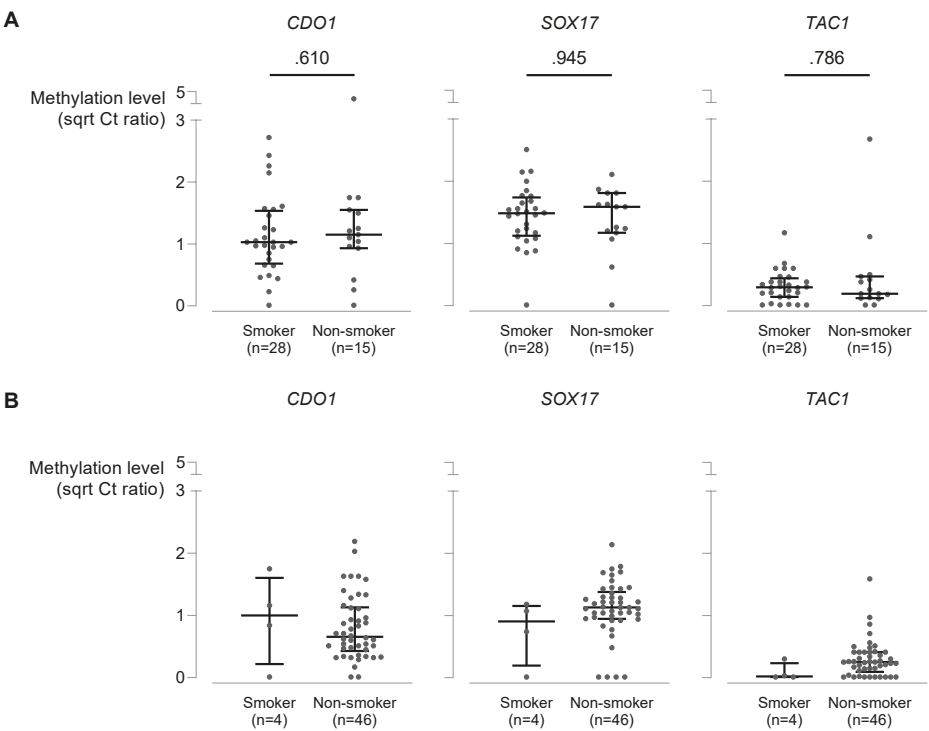
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SUPPLEMENTAL MATERIALS

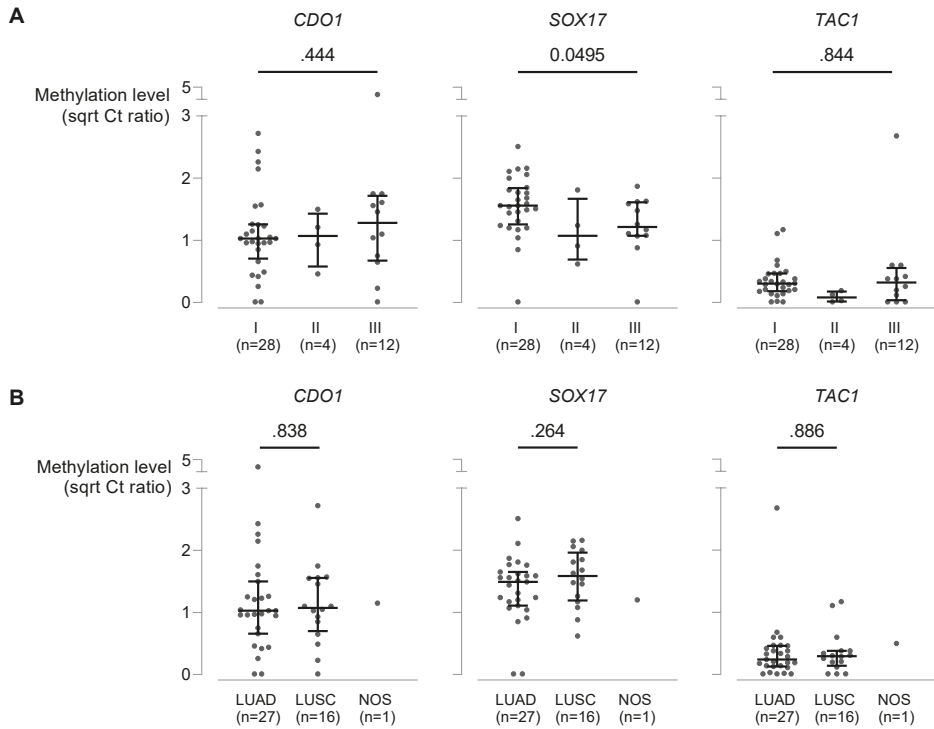
**Supplementary Table 1:** Additional clinical data of surgical NSCLC patients of which pre- and postoperative methylation levels were evaluated for the detection of residual disease (patient 1 to 4) or disease recurrence (patient 5 to 14).

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Time first sample to surgery (days)	1	1	1	1	1	3	10	15	8	2	17	2	2	3
Time surgery to second sample (days)	6	5	6	5	973	578	326	309	277	278	236	252	223	63
Cancer stage*	1	3	1	1	1	1	1	1	1	3	1	3	3	2
Residual tumor classification (R)	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Histology	LUSC	LUAD	NOS	LUAD	LUSC	LUAD	LUAD	LUAD	LUAD	LUAD	LUAD	LUAD	LUAD	LUAD
Disease free survival (days)	-	101	492	257	1007	624	386	386	307	351	350	0	293	168
Total follow-up time (days)	979	132	874	389	1007	624	386	386	383	351	350	344	293	168
Alive	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

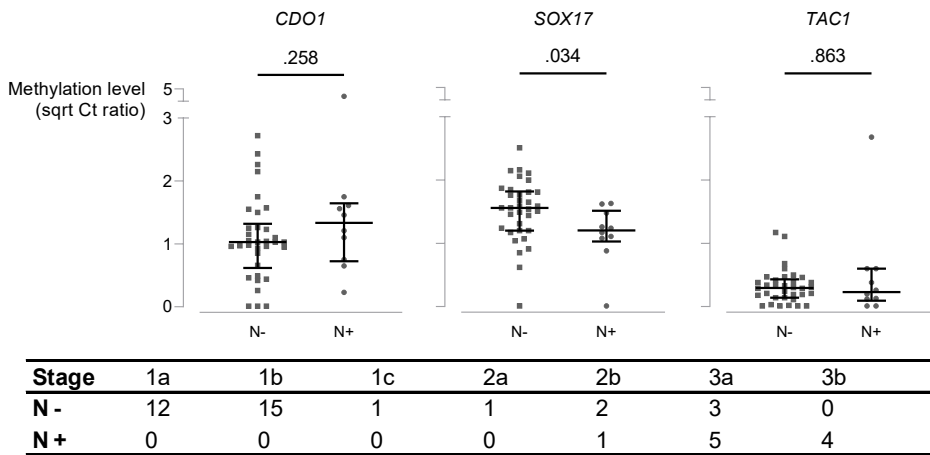
\*Staging according the 8th edition of TNM criteria. LUAD = lung adenocarcinoma, LUSC = lung squamous cell carcinoma, NOS = carcinoma not otherwise specified, NSCLC = non-small cell lung cancer.



**Supplementary Figure 1: Methylation levels in smokers and non-smokers.** Methylation levels of markers *CDO1*, *SOX17*, and *TAC1* in the urine supernatant of surgical NSCLC patients (**A**) and controls (**B**), stratified by smoking status. Data is depicted as the median with an interquartile range of square root transformed Ct ratios. Each symbol represents a single case. A *P*-value of <.05 was considered statistically significant.



**Supplementary Figure 2: Methylation levels of NSCLC patients per cancer stage and histological subtype.** Methylation levels of markers *CDO1*, *SOX17*, and *TAC1* in the urine supernatant of surgical NSCLC patients, stratified by cancer stage (**A**) and histological subtype (**B**). Data is depicted as the median with an interquartile range of square root transformed Ct ratios. Each symbol represents a single case. A *P*-value of  $<.05$  was considered statistically significant. Statistical testing was not performed for NOS due to the low number of patients in this group ( $n=1$ ). LUAD = lung adenocarcinoma, LUSC = lung squamous cell carcinoma, NOS = carcinoma not otherwise specified.



**Supplementary Figure 3: Methylation levels of NSCLC patients without (N-) and with (N+) lymph node involvement.** Methylation levels of markers *CDO1*, *SOX17*, and *TAC1* in the urine supernatant of surgical NSCLC patients, stratified by nodal involvement. Data is depicted as the median with an interquartile range of square root transformed Ct ratios. Each symbol represents a single case. The distribution of nodal involvement per stage is depicted underneath. A *P*-value of <.05 was considered statistically significant. LUAD = lung adenocarcinoma, LUSC = lung squamous cell carcinoma, NOS = carcinoma not otherwise specified.





# CHAPTER 7

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## DYNAMICS OF METHYLATED CELL-FREE DNA IN THE URINE OF NON-SMALL CELL LUNG CANCER PATIENTS

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DNA in the urine of non-small cell lung cancer patients. *Epigenetics*. Oct 4:1-13.

\*Equal contribution.

## ABSTRACT

High levels of methylated DNA in urine represent an emerging biomarker for non-small cell lung cancer (NSCLC) detection and are the subject of ongoing research. This study aimed to investigate the circadian variation of urinary cell-free DNA (cfDNA) abundance and methylation levels of cancer-associated genes in NSCLC patients. In this prospective study of 23 metastatic NSCLC patients with active disease, patients were asked to collect six urine samples during the morning, afternoon, and evening of two subsequent days. Urinary cfDNA concentrations and methylation levels of *CDO1*, *SOX17*, and *TAC1* were measured at each time point. Circadian variation and between- and within-subject variability were assessed using linear mixed models. Variability was estimated using the Intraclass Correlation Coefficient (ICC), representing reproducibility. No clear circadian patterns could be recognized for cfDNA concentrations or methylation levels across the different sampling time points. Significantly lower cfDNA concentrations were found in males ( $p=0.034$ ). For cfDNA levels, the between- and within-subject variability were comparable, rendering an ICC of 0.49. For the methylation markers, ICCs varied considerably, ranging from 0.14 to 0.74. Test reproducibility could be improved by collecting multiple samples per patient. In conclusion, there is no preferred collection time for NSCLC detection in urine using methylation markers, but single measurements should be interpreted carefully, and serial sampling may increase test performance. This study contributes to the limited understanding of cfDNA dynamics in urine and the continued interest in urine-based liquid biopsies for cancer diagnostics.

## INTRODUCTION

Lung cancer remains the leading cause of cancer-related deaths worldwide (1). Screening programs have shown that cancer-related mortality could be reduced by using low-dose computed tomography (LDCT) screening in selected high-risk patients (2-4). Combining this approach with molecular marker testing in liquid biopsies could further improve the screening selection and management of positive LDCT screening tests.

The analysis of methylated cell-free DNA (cfDNA) in liquid biopsies is a promising, safe, and easily applicable tool that is now being investigated for the detection of lung cancer. Methylation, an epigenetic DNA modification that regulates gene expression, is known as a critical process, involved in early lung cancer development and progression (5). Amongst liquid biopsies, blood and sputum are the most commonly reported sources of cfDNA for methylation analyses (6-14). On the other hand, urine is an upcoming means for liquid biopsy analyses in lung cancer diagnostics (10, 15, 16). Urine-based liquid biopsies are of particular interest, as the collection is completely non-invasive and can be performed at home. Moreover, large volumes can be collected regularly, which allows for repetitive sampling at frequent intervals.

Despite encouraging developments of urine-based liquid biopsies for lung cancer detection, this technique is not yet ready for implementation into clinical practice. Over the past years, considerable improvements have been achieved by optimization and standardization of pre-analytical conditions (17-20). However, one of the major remaining questions regarding the yield of cfDNA is the uncertainty on whether the circadian rhythm leads to variations in the amounts of methylated DNA in urine. There is also a limited understanding of the range of biological variation of methylated cfDNA in the urine of lung cancer patients. Biological variability refers to the random fluctuation of analyte concentrations around a homeostatic set point (within-subject variability), which varies per individual (between-subject variability) (21). Previous studies have focused exclusively on the abundance of cfDNA in plasma of healthy controls and lung cancer patients, which appeared to vary greatly within (22) and between individuals (23), and during the day (24).

The aim of this study, therefore, was to investigate the dynamics of methylated cfDNA in the urine of lung cancer patients to estimate both between- and within-subject variability, and to evaluate whether a preferred urine collection time and sampling frequency exist.

## METHODS

### Study population

In this prospective cohort study, patients with histologically confirmed NSCLC were consecutively recruited between November 2019 and January 2020 at the outpatient clinic of the Department of Pulmonology of the Amsterdam University Medical Center, location VUmc, Amsterdam. Inclusion criteria of NSCLC patients involved being diagnosed with active disease (*i.e.* before anti-cancer therapy or at disease progression after therapy) without the presence or history of any other primary malignancies. The revised eighth edition of the American Joint Committee on Cancer/Union for International Cancer Control Tumor-Node-Metastasis (TNM) Staging was used to determine tumor stage (25). Other relevant patient characteristics that were documented included sex, age, weight, tobacco use, therapy during study, survival, and histological subtype.

As controls, urine samples from healthy volunteers were collected through the Urine Controls (URIC) Biobank. Inclusion criteria of controls involved not having any cancer diagnosis in the past 15 years. Sex and age were registered from each participant.

Informed consent was acquired from each participating individual before urine collection. Ethical approval was obtained by the Medical Ethical Committee of the VU University Medical Center for both the DAYTIME study (No. 2017.333 and 2017.545) and the use of the URIC biobank (No. 2017.112).

### Urine sample collection and processing

Each patient was carefully instructed to collect 30 mL of urine at three different time points for two subsequent days, adding up to a total of six samples per patient. To this end, special collection kits were designed, containing clear illustrated instructions, collection tubes, and postal envelopes. The three time points comprised morning (6:00 AM – 11:00 AM), afternoon (12:00 noon– 5:00 PM), and evening (6:00 PM – 12:00 midnight). Patients registered the time of urine collection and shipped their urine samples to the Pathology department of Amsterdam UMC, location VUmc, by regular mail. To ensure the preservation of genetic material in the urine, collection tubes contained 2 mL 0.6 M ethylenediaminetetraacetic acid (EDTA) as a preservative agent (final concentration 40 mM), and sample processing was performed within 72 h after collection. Urine samples of healthy volunteers were retrieved from the URIC biobank, which were collected once at a random time point of the day, according to the same collection protocol.

Urine samples of patients and controls were processed similarly. Up to 30 mL (patients) or 40 mL (controls) full void urine was centrifuged at  $3000 \times g$  for 15 min to obtain the urine supernatant fraction, which was stored at  $-20^{\circ}\text{C}$ . This collection and storage procedure has been validated for reliable DNA methylation detection in urine (18).

### Cell-free DNA extraction and bisulfite conversion

The urinary cfDNA was extracted from 20 mL (patients) or 40 mL (controls) urine supernatant using the Quick DNA urine kit (Zymo Research, Irvine, CA, US). Previous research showed that differences in urine collection volume in a similar range (4-20 mL) have limited effects on DNA yield, eliminating this potential bias (26). DNA concentration was measured using the Qubit™ dsDNA HS Assay (Invitrogen, Carlsbad, CA, US). Depending on the yield, up to 250 ng purified DNA was subjected to bisulfite conversion using the EZ DNA Methylation Kit (Zymo Research). All procedures were carried out according to the manufacturer's instructions.

### DNA methylation analysis by quantitative methylation-specific PCR (qMSP)

Promoter hypermethylation detection of the *CDO1*, *SOX17*, and *TAC1* genes was carried out by qMSP using a ViiA7 real-time PCR system (Applied Biosystems, Foster City, CA, USA). For each reaction, up to 50 ng modified DNA was mixed with the EpiTect MethyLight Master Mix (Qiagen), and 2.5-5.0  $\mu\text{M}$  of each primer and 5.0-10.0  $\mu\text{M}$  of each probe in a total volume of 12.5  $\mu\text{L}$ . Primer and probe sequences used for *CDO1* and *TAC1* were kindly provided by Dr. A. Hulbert (University of Illinois at Chicago, Chicago, IL, US) and listed in (10). Primer and probe sequences of *SOX17* were redesigned within the same genomic region as reported before (10), using a locked nucleic acid probe to enhance specificity (Supplementary Table 1). The qMSP reactions were multiplexed as described previously (27) to assess the methylation levels of all genes within the same reaction. *ACTB* was also included in the multiplex and used as a reference gene for normalization and quality assessment. Sample series from each patient were processed in the same run.

Double-stranded gBlocks™ Gene Fragments (Integrated DNA Technologies) containing the amplicon sequences of all targets and *ACTB* were used as technical quality control and H<sub>2</sub>O was taken along as negative control during each qMSP run. Cycle threshold (Ct) values were measured at a fixed threshold. Sample quality and sufficient input were ensured by excluding samples with a *ACTB* Ct value exceeding 32 from methylation analysis. The discriminatory power of the qMSP was verified by testing 11 pairs of tumors and adjacent normal tissues from NSCLC patients of a previously published cohort (28, 29).

### Statistical analyses

The cfDNA concentration was expressed in ng/mL urine and transformed using an inverse hyperbolic sine function to enhance normality of the data. The methylation levels of the target genes were normalized according to the Ct value of the reference gene *ACTB*  $2^{-(Ct_{\text{marker}} - Ct_{\text{ACTB}})} \times 100$  to obtain Ct ratios, and square root transformed. Linear mixed-effects models were fitted separately for the repeated measurements of cfDNA concentration and methylation levels of each marker. Linear mixed-effects models contain both fixed (*i.e.* constant across the population) and random (*i.e.* varying per individual) effects, enabling estimation of both between- and within-subject variation (30). Models incorporated a random intercept for each patient to account for within-patient correlation and included explanatory variables day (*i.e.* day 1 and day 2) and part of the day (*i.e.* morning, afternoon, and evening) as fixed effects.

Models were estimated using restricted maximum likelihood (REML). Additional patient characteristics (*i.e.* sex, age, weight, therapy during study, survival, and histological subtype) were considered for inclusion as fixed effects by backward stepwise selection ( $p \geq 0.05$  for removal). Tobacco use could not be included as fixed effect due to missing data. Final models are available in the Supplementary material. The assumptions of linearity, normality of the residuals and random effects, and homoscedasticity (*i.e.* constant variance of the residuals) were checked visually using diagnostic plots (31).

Differences in cfDNA concentration and methylation levels during the day and between the two days were evaluated by Type II Wald Chi-square tests. Model estimates and corresponding 95%-confidence intervals (CI), between-subject variances ( $\sigma^2$ ), within-subject variances ( $\tau_{00\text{subject}}$ ), and intraclass correlation coefficients (ICC) were tabulated for both the cfDNA concentration and methylation levels of *CDO1*, *SOX17*, and *TAC1*. The ICC indicates the resemblance of repeated measurements and describes the proportion of between-subject variability with respect to the total variability (between plus within). The ICC can range from zero to one, with zero indicating a poor reproducibility and one indicating a perfect reproducibility (32).

Differences in time were displayed in boxplots, demonstrating the cfDNA concentrations and methylation marker levels measured between the different days and time points at a group level. Between- and within-subject differences were visualized by conditional scatterplots, showing the cfDNA concentrations and methylation marker levels measured at each time point for each patient individually, stratified for sex.

The added value of collecting multiple urine samples was determined by 1) comparing the methylation levels measured in the urine of patients (n=23) and controls (n=60),

by including all available patient samples ( $n=138$ ), and 2) random sampling to compute the statistical difference between methylation levels of cases and controls when only one urine sample would have been collected. Linear mixed-effects models were fitted as described above with subject as a random effect to account for repeated measures in the patient group. Likewise, differences in methylation levels between patients and controls were tested as described above. Final models are available in the Supplementary material. Random sampling was conducted according to (33). Briefly, only one urine sample of each patient was randomly selected from the six available samples to compare the methylation levels of each marker between patients and controls, which was repeated 100 times in total. A median  $p$ -value was computed to summarize the outcome of 100 rounds of random sampling.

Statistical analyses were performed in R (v.3.6.1) and Rstudio (v.1.1.463). For statistical tests,  $p$ -values  $<0.05$  were considered statistically significant.

### ***R packages***

Linear mixed models were computed using the 'lme4' package (30), combined with the Companion to Applied Regression 'car' package (34) for statistical testing. Normalization of the cfDNA concentration data was performed according to the 'bestNormalize' package (35). The 'sjPlot' package (36) was used to test model assumptions and extract model summaries. Boxplots and conditional scatterplots were computed using the 'ggplot2' package (37) and 'lattice' package (38), respectively.

## **RESULTS**

### **Patient characteristics**

A total of 23 patients with NSCLC were included, of which relevant clinical and pathological features are presented in Table 1. The median age at diagnosis was 69 (range 65-75) and nine patients were female. The patient cohort covered the major histological subtypes of NSCLC, with TNM stages ranging from IIb to IVb. The majority of patients were current or former smokers.

### **Variation in cfDNA concentration**

#### ***Variation during the day and between days***

Total cfDNA concentrations of all urine samples ( $n=138$ ) were quantified by Qubit and compared within and between days by a linear mixed model approach. The cfDNA concentrations measured across the six different time points are shown in Figure 1. No significant differences were found between the morning, afternoon, and evening,

or between the two days. Parameter estimates and corresponding 95% confidence intervals and variance components are displayed in Table 2. The cfDNA concentration found in males was significantly lower as compared to females ( $p=0.034$ ; Wald test). Age, weight, therapy during urine collection, survival, tumor stage, or tumor histology were not associated with the cfDNA concentration.

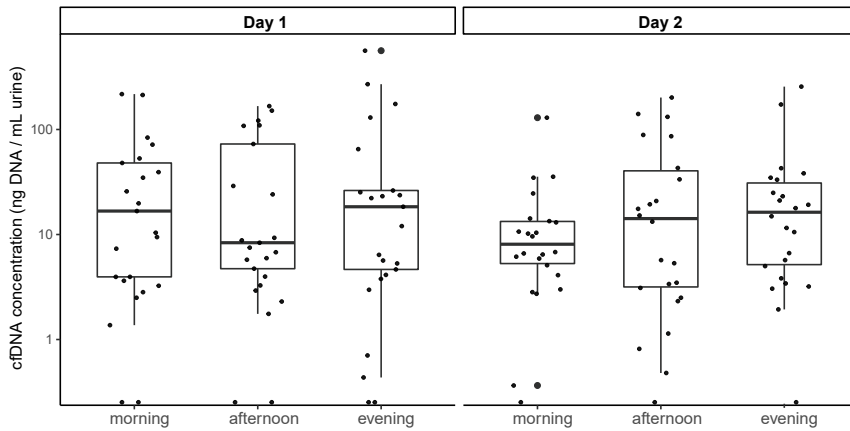
**Table 1:** Baseline characteristics of the 23 NSCLC patients.

<b>Age</b>		
Median (IQR)	69 (65-75)	
<b>Sex</b>	<b>n</b>	<b>%</b>
Female	9	39.1
Male	14	60.9
<b>Histology</b>	<b>n</b>	<b>%</b>
Adenocarcinoma	15	65.2
Squamous cell carcinoma	5	21.7
Carcinoma NOS	3	13.0
<b>TNM Stage*</b>	<b>n</b>	<b>%</b>
IIb	1	4.3
IIIa	3	13.0
IIIb	3	13.0
IVa	10	43.5
IVb	6	26.1
<b>Smoking status</b>	<b>n</b>	<b>%</b>
Current	4	17.4
Former	13	56.5
Never	1	4.3
Unknown	5	21.7

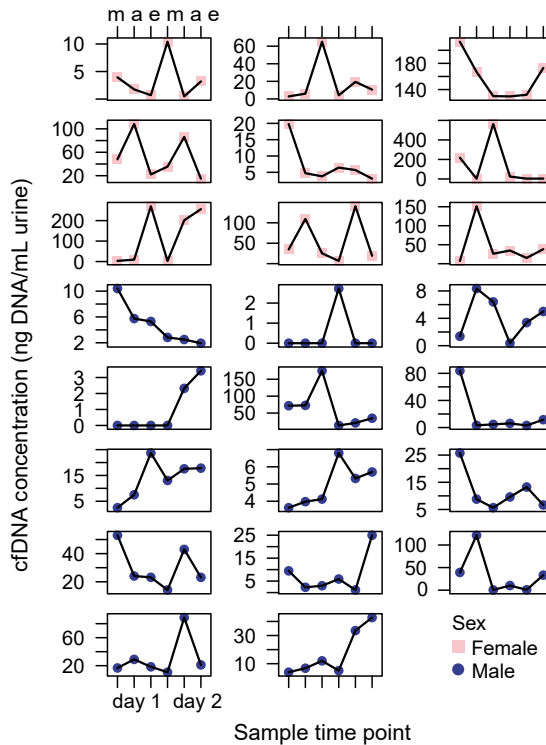
\*Staging was conform the revised 8th edition of tumor-node-metastasis (TNM) criteria. IQR = interquartile range, NSCLC = non-small cell lung cancer, NOS = not otherwise specified.

### *Between- and within-subject variation*

Similarly, the variation in cfDNA concentration was assessed at the individual patient level, as illustrated in Figure 2. The ICC value was 0.49, meaning that 49% of the variance is due to variability between patients, and 51% of the variance can be explained by variability within patients. Parameter estimates of cfDNA concentrations measured over time are summarized in Table 2.



**Figure 1:** Logarithmic representation of cfDNA concentrations (ng/mL urine) measured at different collection time points, illustrating the median and IQR of each collection time point. Outliers are indicated by bold circles located outside the whiskers of the boxplot. No significant differences were found within or between the days. cfDNA = cell-free DNA; IQR = interquartile range.



**Figure 2:** Conditional scatterplots displaying the between- and within-subject variability of the urinary cfDNA concentration of each NSCLC patient across the six sampled time points (m = morning, a = afternoon, e = evening), stratified by sex (pink square = female, blue circle = male). cfDNA = cell-free DNA; NSCLC = non-small cell lung cancer.

**Table 2:** Parameter estimates of cfDNA concentration in the urine of NSCLC patients measured across the different sampling time points according to the fitted linear mixed model corrected for sex.

<i>cfDNA concentration</i>			
<b>Fixed effects</b>	<b>Estimates</b>	<b>95%-CI</b>	<b><i>p</i></b>
(Intercept)	19.51	(8.31, 45.72)	
day [2]	-0.13	(-0.52, 0.25)	0.512
time [afternoon]	0.11	(-0.36, 0.60)	0.638
time [evening]	0.25	(-0.22, 0.76)	0.298
sex [male]	-1.32	(-4.00, -0.08)	<b>0.034</b>
<b>Random Effects</b>			
$\sigma^2$	1.27		
$\tau_{00}$ subject	1.24		
ICC	0.49		
N subject	23		
Observations	138		

cfDNA concentration estimates are presented in ng DNA/mL urine.  $\sigma^2$  = within-subject variability;  $\tau_{00}$  = between-subject variability; cfDNA = cell-free DNA; ICC = Intraclass Correlation Coefficient. NSCLC = non-small cell lung cancer.

## Variation in methylation levels

### *Variation during the day and between days*

DNA methylation levels of *CDO1*, *SOX17*, and *TAC1* were measured in all urine samples ( $n=138$ ) by qMSP (Figure 3). Five urine samples were excluded from the analysis based on an *ACTB* Ct value of  $\geq 32$ . The discriminatory power of the qMSP was verified by comparing methylation levels in 11 pairs of NSCLC and adjacent normal tissues (Supplementary Figure 1). Differences in time were assessed by a linear mixed model framework. None of the studied markers showed systematic differences in methylation levels during the day or between the two days (Table 3). Methylation levels found were independent of sex, age, weight, therapy during urine collection, survival, tumor stage, and tumor histology. For each marker, a significant association between methylation level and the cfDNA concentration was observed ( $p < 0.05$ ; Wald test).

### *Between- and within-subject variation*

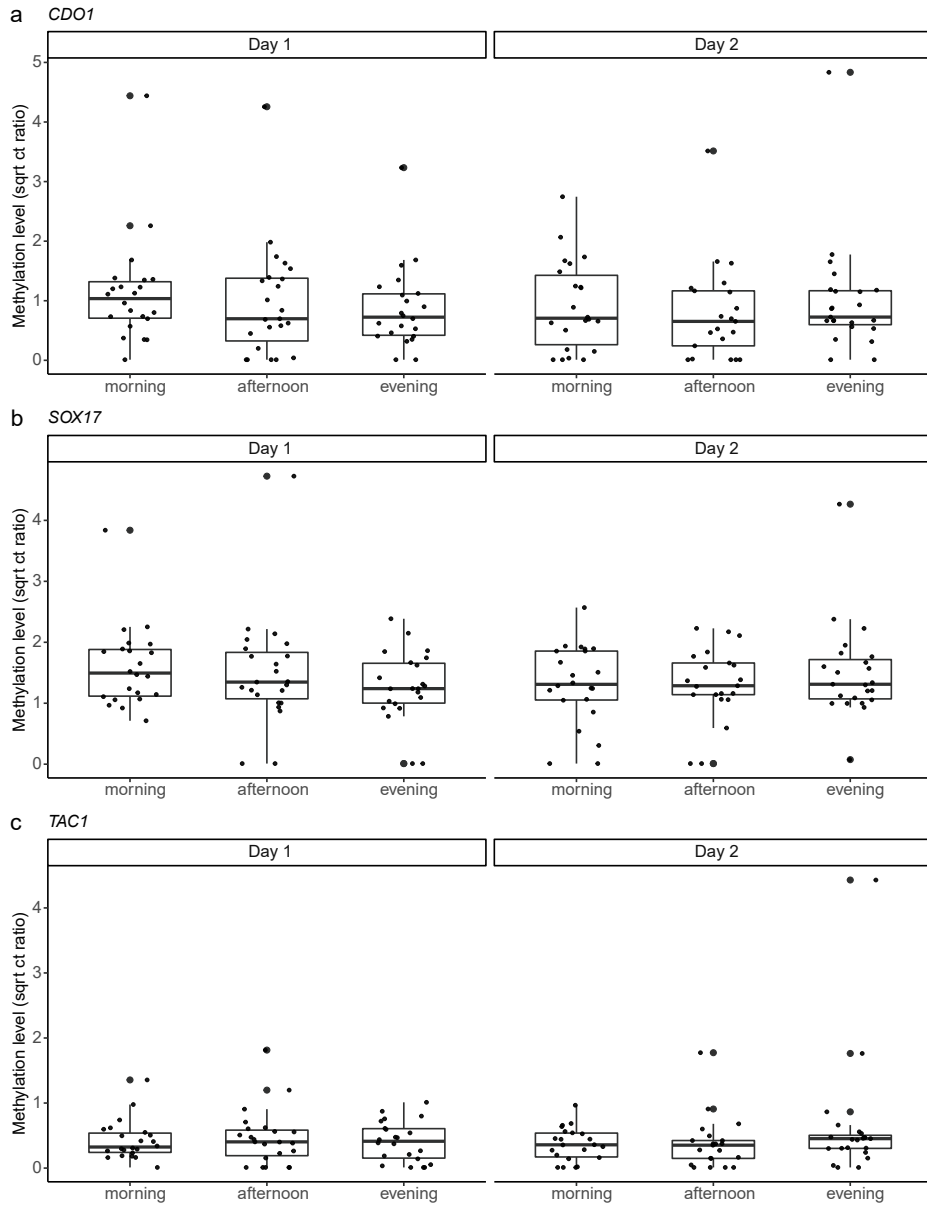
The variation of DNA methylation levels between and within individual patients is displayed in Figure 4 and was examined within the same linear mixed model with subject as a random effect. The ICC values of the markers *CDO1*, *SOX17*, and *TAC1* were 0.74, 0.57, and 0.14, respectively (Table 3). This indicated that 26% of the variation observed in *CDO1* methylation levels is due to variability within patients, as opposed to 43% for *SOX17* and 86% for *TAC1*. Model assumptions were not violated as indicated by diagnostic tests (Supplementary Material).

**Table 3:** Parameter estimates of DNA methylation levels of *CDO1*, *SOX17* and *TAC1* measured over time in urine samples of NSCLC patients according to the fitted linear mixed model.

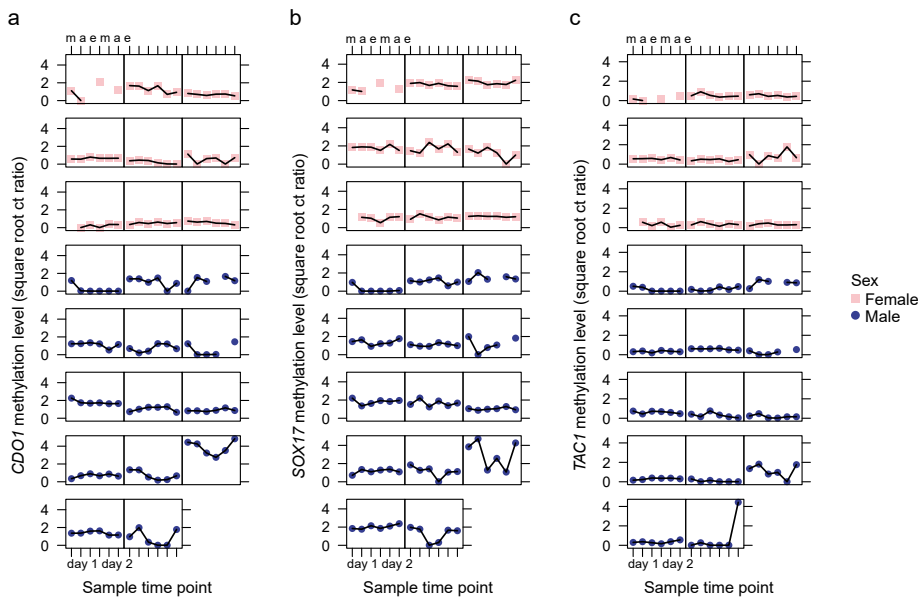
<i>CDO1</i>				<i>SOX17</i>			<i>TAC1</i>		
<b>Fixed effects</b>	<b>Estimates</b>	<b>95%-CI</b>	<b>p</b>	<b>Estimates</b>	<b>95%-CI</b>	<b>p</b>	<b>Estimates</b>	<b>95%-CI</b>	<b>p</b>
(Intercept)	0.80	(0.39, 1.20)		0.83	(0.48, 1.17)		0.14	(-0.11, 0.38)	
day [2]	-0.14	(-0.32, 0.04)	0.136	-0.06	(-0.25, 0.13)	0.528	0.01	(-0.18, 0.20)	0.915
time [afternoon]	-0.09	(-0.27, 0.09)	0.343	-0.10	(-0.29, 0.09)	0.295	0.10	(-0.09, 0.28)	0.313
time [evening]	-0.07	(-0.22, 0.08)	0.379	-0.05	(-0.21, 0.10)	0.511	0.03	(-0.12, 0.18)	0.674
DNA concentration	0.08	(0.01, 0.15)	<b>0.026</b>	0.21	(0.14, 0.28)	<b>&lt;0.001</b>	0.08	(0.02, 0.14)	<b>0.005</b>
<b>Random Effects</b>									
$\sigma^2$	0.19			0.20			0.20		
$\tau_{00}$ subject	0.55			0.27			0.03		
ICC	0.74			0.57			0.14		
N subject	23			23			23		
Observations*	133			133			133		

DNA methylation level estimates are presented as square root transformed ct ratios. Methylation levels of all markers were independent of sex, age, weight, therapy during urine collection, survival, tumor stage, and tumor histology.  $\sigma^2$  = within-subject variability;  $\tau_{00}$  = between-subject variability; ICC = Intraclass Correlation Coefficient; NSCLC = non-small cell lung cancer.

\*Five urine samples were excluded from the analysis based on an *ACTB* Ct value of  $\geq 32$ .



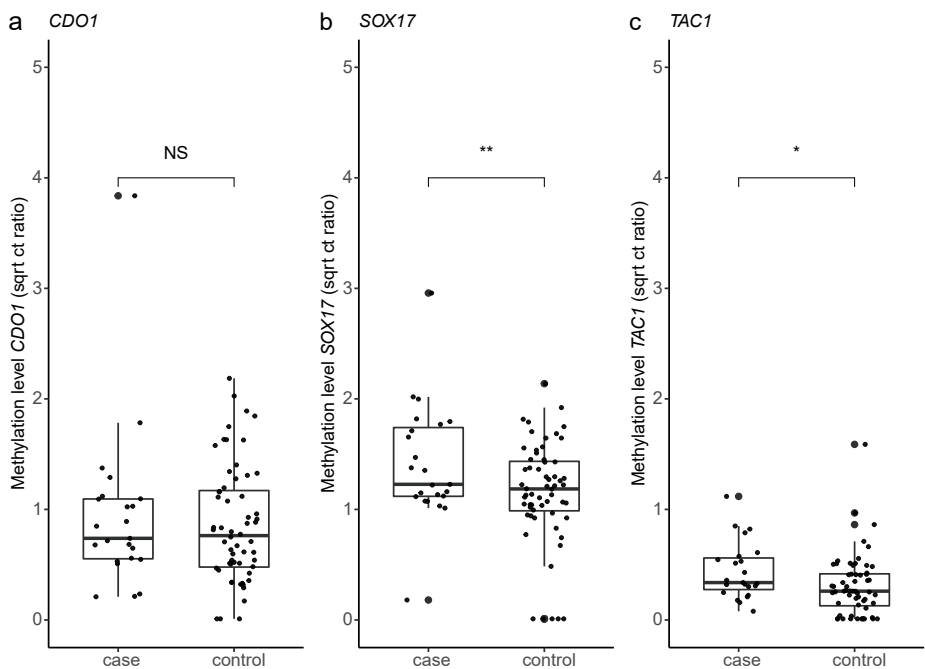
**Figure 3:** Methylation levels of *CDO1* (a), *SOX17* (b), and *TAC1* (c) measured in the urine of NSCLC patients at different collection time points illustrating the median and IQR of each collection moment. Methylation levels are normalized according to the reference gene *ACTB* and presented as square root Ct ratios. No significant differences were found within or between the days. IQR = interquartile range; NSCLC = non-small cell lung cancer.



**Figure 4:** Conditional scatterplots displaying the between- and within-subject variability of *CDO1* (a), *SOX17* (b), and *TAC1* (c) methylation levels of each patient across the six sampled time points (m = morning, a = afternoon, e = evening), stratified by sex (pink square = female, blue circle = male). Missing data points indicate excluded urine samples with an *ACTB* value of  $\geq 32$ .

### Prolonged urine sampling

To explore whether collecting multiple urine samples provides a more accurate test outcome, methylation levels of *CDO1*, *SOX17*, and *TAC1* were also measured in urine samples of healthy controls ( $n=60$ ). The control cohort had a median age of 69 (range 58-79) and 30 controls were female. The qMSP Ct values are provided in the Supplementary Data file (online). The discriminatory power of each marker was first evaluated when including six samples per patient and compared to the levels found in controls. Linear mixed models were used to correct for repeated measurements in the patient group. Significant differences in methylation levels of cases and controls were found for *SOX17* (Wald test,  $p=0.030$ ), and also *TAC1* showed a trend toward significance (Wald test,  $p=0.059$ ), both independent of age and sex (Figure 5 and Supplementary Table 2).



**Figure 5:** Methylation levels of *CDO1* (a), *SOX17* (b), and *TAC1* (c) measured in the urine of NSCLC patients (n=23) and healthy controls (n=60). Methylation levels are normalized to the reference gene *ACTB* and presented as square root Ct ratios. Case values represent the mean methylation level measured in the six collected urine samples. Outliers are indicated by bold circles located outside the whiskers of the boxplot. \* $p < 0.10$  (suggestive evidence), \*\* $p < 0.05$  (moderate evidence).

Next, only one urine sample of each patient was compared against the control group by random sampling. The results of 100 sampling rounds are summarized as median  $p$ -value. Table 4 shows the discriminatory power ( $p$ -value) of each methylation marker between patients and controls when collecting one or six urine samples per patient. This comparative analysis indicated that the discriminatory power of *TAC1* and *SOX17* decreases when only one urine sample was taken into account, instead of six.

**Table 4:** Statistical differences of *CDO1*, *SOX17* and *TAC1* methylation levels between NSCLC patients (n=23) and healthy controls (n=60) when collecting one or six urine samples per patient.

Sample(s)	<i>CDO1</i>	<i>SOX17</i>	<i>TAC1</i>
one (random sampling*)	0.662	0.059	0.133
six	0.711	<b>0.030</b>	0.059

Numbers represent  $p$ -values found when comparing methylation levels found in patients and controls using the Wald-test.

\* $P$ -values of one urine sample represent the median  $p$ -value of 100 rounds of random sampling. NSCLC = non-small cell lung cancer.

## DISCUSSION

Insight into the dynamics of urinary cfDNA is essential to determine whether a preferred collection time and sampling frequency exist, and to correctly interpret molecular analyses. Analysis of the circadian variation of the cfDNA concentration in the urine of NSCLC patients revealed substantial variation between and within subjects, but no clear circadian pattern. Similarly, also for methylation levels of lung cancer markers no clear circadian pattern was found, whereas the biological variation was high.

Data of the current study suggests that the moment of urine collection does not significantly affect the urinary cfDNA concentration in NSCLC patients with active disease. Similarly, no day-to-day variation in urinary cfDNA concentration was found. So far, only the dynamics of cfDNA in plasma have been explored. Madsen *et al.* (24) reported similar results with stable cfDNA amounts in the plasma of lung cancer patients during the day and between days. Contradictory findings have been described for cfDNA concentrations in the plasma of healthy subjects. While constant cfDNA concentrations were observed by Wagner *et al.* (23), other studies demonstrated a significant decrease during the day in healthy subjects (24, 39). Previous studies also did not find a day-to-day variation of cfDNA in plasma (22, 24, 40), in line with the current findings. The only patient characteristic that influenced urinary cfDNA concentration levels in this study was sex, with a significantly higher concentration found in females, following previous studies (41-43).

The proportion of between-subjects variation was expressed using the ICC value, where an ICC value of one indicates a perfectly reproducible test. Although the interpretation of the ICC value differs amongst studies, it has been suggested that ICC values below 0.50 reflect poor reproducibility, values between 0.50 and 0.75 moderate reproducibility, values between 0.75 and 0.90 good reproducibility, and values above 0.90 equal excellent reproducibility (44). The cfDNA concentrations measured in the six urine samples per patient showed between- and within-subject variability of comparable size, approaching a moderate reproducibility (0.49). In other words, both the baseline cfDNA concentration of each patient and the random fluctuation around this baseline contribute equally to the observed variation in cfDNA concentrations. Substantial between- and within-subjects variability has also been reported for serial measurements of cfDNA in plasma of healthy subjects (23, 24).

Methylation levels of the *CDO1*, *SOX17*, and *TAC1* genes were also not affected by the time of urine collection. This is in accordance with the stable detection of *EGFR* mutations in the plasma of lung cancer patients collected during three time points within one day

(45). However, alternative results have been described for methylation of the *SEPT9* gene in plasma samples of a small group of 11 colorectal cancer patients. The highest *SEPT9* methylation levels were found at midnight, detecting all (pre)cancers included in the study, as compared to 77.7% of the cases during the other time points.

Methylation levels of *CDO1* and *SOX17* reached moderate to good reproducibility (0.74 and 0.57), while *TAC1* showed poor reproducibility (0.14). The reproducibility of the markers seems to reflect the level of DNA methylation detected. *TAC1* with the lowest ICC showed the lowest methylation levels of the three markers studied. Comparable variation within subjects has been observed for the mutant allele concentration of tumor-specific mutations in *KRAS* and *P53* in the plasma of non-progressive lung cancer patients (22). From a patient monitoring perspective, the between-subject variation observed in this study implies that evaluating DNA methylation levels within individual patients, using longitudinal testing, may be more useful than using a dichotomous population-based threshold. Also, contrary to the moment of sampling, additional value was observed with collecting multiple urine samples for markers with the highest within-subject variability. This suggests that detecting lung cancer in urine will become more likely when multiple urine samples are being collected. Collecting urine at multiple time points has also been proposed by Liu *et al.* (10) who published the proof-of-concept study for lung cancer detection in urine by the analysis of methylated DNA. A previous study by Hubers *et al.* (46) indicated that prolonged sampling increased the sensitivity of lung cancer detection by methylation analysis in sputum, with a slight decrease in specificity. Other options to improve test accuracy would be to increase urine volume, as shown for bladder cancer detection in urine (47), or to pool several urine samples before DNA isolation, as suggested for gene polymorphism analysis (43). The significant association found between the methylation levels of each marker and the cfDNA concentration of the urine sample indicates that adjusting the threshold of the reference gene, used for normalization and for excluding samples with insufficient DNA quality or quantity, could also increase the test reproducibility and accuracy.

The current study has several limitations. Due to the substantial biological variance observed, our sample size may have been too small to accurately address systematic changes of cfDNA concentration and methylation levels in time. Nonetheless, the total of 138 urine samples included is similar or even higher as compared to previous studies assessing the biological variation of cfDNA in plasma (22-24). Moreover, because this study only included patients with active disease, further studies are warranted to examine the biological variation of cfDNA in patients with early-stage or non-progressive disease. Apart from that, the variability of the current marker selection in the urine of healthy controls and individuals at risk for lung cancer (e.g. heavy smokers or patients

diagnosed with chronic obstructive pulmonary disease) remains to be determined as no longitudinal sample sets of such subjects were available. This will provide essential information since biomarkers with small biological variability or even negative values in controls are clinically most useful for diagnostic and prognostic purposes. Detectable changes in such biomarkers will most likely reflect disease processes and not merely natural occurring variation (48).

The strengths of the study include its relatively large sample size and the measurement of both cfDNA concentrations and DNA methylation levels of three genes at each collection time point. Moreover, the use of a standardized and reproducible urine processing protocol limited pre-analytical variance (18). Together with a sophisticated linear mixed modelling approach, this allowed an accurate estimation of the within- and between-subject variation of all analytes assessed in the current study. Furthermore, although not collected longitudinally, the inclusion of a representative control group enabled evaluation of the potential benefit of prolonged sampling.

In conclusion, no clear circadian pattern of methylated cfDNA in the urine of NSCLC patients was observed, implying that no preferred time of urine collection exists. Nevertheless, the observed between- and within-patient variation indicates that single methylation marker measurements should be interpreted carefully, and that collecting multiple urine samples may increase the chance of detecting lung cancer in urine. Improved understanding of the dynamics of urinary cfDNA provides a fundamental step toward the development of urine-based biopsies and their translation into clinical practice.

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### **Disclosure of interest**

RS has a minority stake in Self-screen B.V., a spin-off company of VU University Medical Center Amsterdam. SB, RS, IB, and GK are named inventors on patent applications

related to the detection of methylated DNA in urine. All other authors have no conflict of interest to declare.

### **Contributors**

SB, IB, and RS designed the study. SB and BW recruited patients, collected clinical samples and performed laboratory work. SB collected clinical data. JV, SH, and IB guided and provided the collection of clinical samples. RS supervised laboratory experiments. BW and MvdW carried out statistical analyses. BW and RS drafted the first version of the manuscript. All authors contributed to the interpretation of the data and revised the manuscript critically. SB, BW, and RS can verify the accuracy of all raw data in this study. Funding was acquisitioned by GK, IB, and RS. All authors approved the final version of the manuscript.

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## SUPPLEMENTARY MATERIAL

### Supplementary Methods

Further detailed description of following methods:

1. Verification of methylation assay efficiency
2. Linear mixed-effects models

#### 1. Verification of methylation assay efficacy

Promoter hypermethylation detection of the *CDO1*, *SOX17*, and *TAC1* genes was carried out by quantitative methylation-specific PCR. The efficiency of this assay was verified using 11 pairs of tumours and adjacent normal tissues from NSCLC patients of a previously published cohort. Differences in DNA methylation levels between cancerous and non-cancerous tissue was evaluated by comparing the square root cycle threshold (ct) ratios. Methylation levels were displayed in boxplots and tested for statistical significance using the nonparametric paired samples Wilcoxon test.

#### 2. Linear mixed-effect models

*Linear mixed-effects modelling for the effect of the circadian rhythm on cfDNA concentration and methylation levels of CDO1, SOX17, and TAC1*

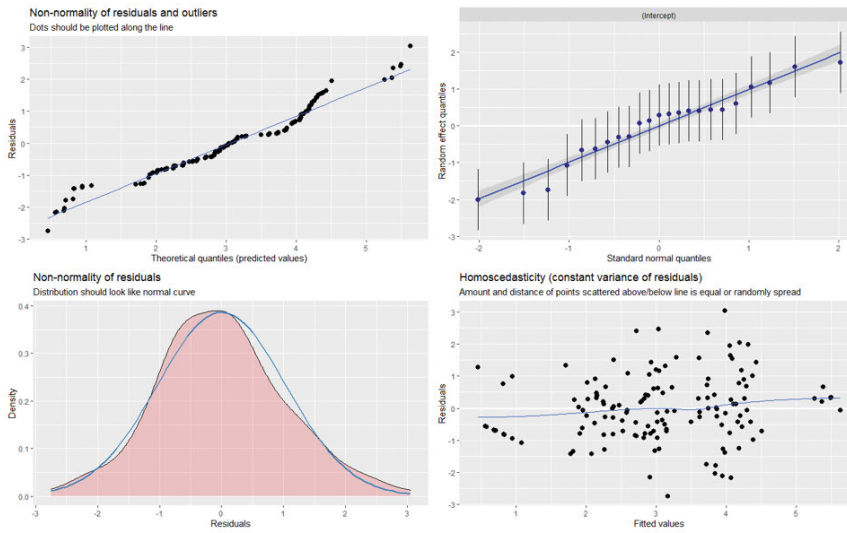
Models were fitted by backward stepwise elimination ( $p \geq 0.05$  for removal) to select fixed and random parts of the linear mixed model using the 'lmerTest' package in R.

Final models are displayed below:

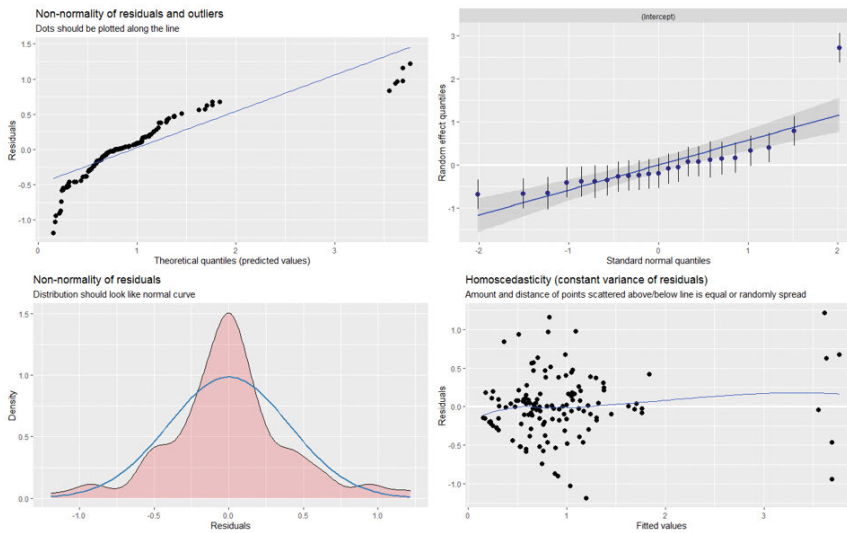
#	Response variable	Final model
1	cfDNA concentration	<code>lmer(DNAconc ~ time + day + gender + (1 Sample  subject), data = DAYTIME_R)</code>
2	<i>CDO1</i> methylation level	<code>lmer(CDO1sqrt ~ time + day + (1 Sample subject), data = DAYTIME_R)</code>
3	<i>SOX17</i> methylation level	<code>lmer(SOX17sqrt ~ time + day + (1 Sample subject), data = DAYTIME_R)</code>
4	<i>TAC1</i> methylation level	<code>lmer(TAC1sqrt ~ time + day + (1 Sample subject), data = DAYTIME_R)</code>

#### Testing model assumptions

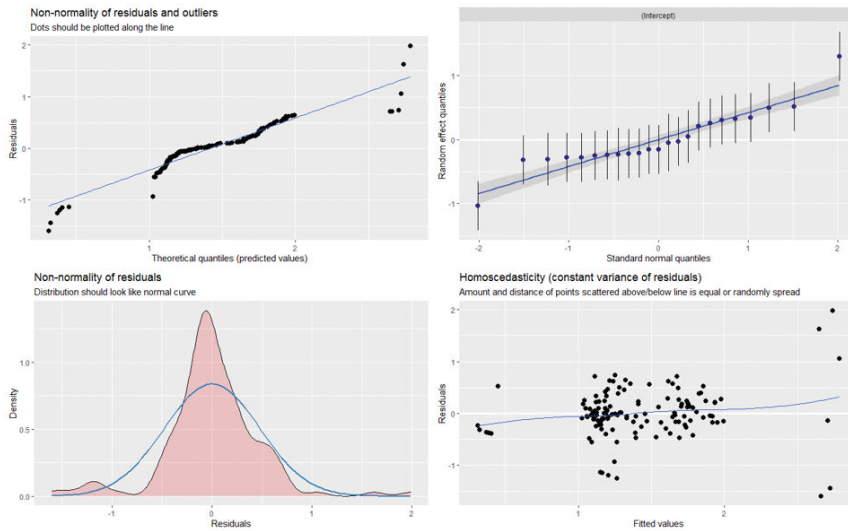
The assumptions of linearity, normality of the residuals and random effects, and homoscedasticity (*i.e.* constant variance of the residuals) were checked visually. A series of diagnostic plots were computed using the 'sjPlot' package to check these assumptions.



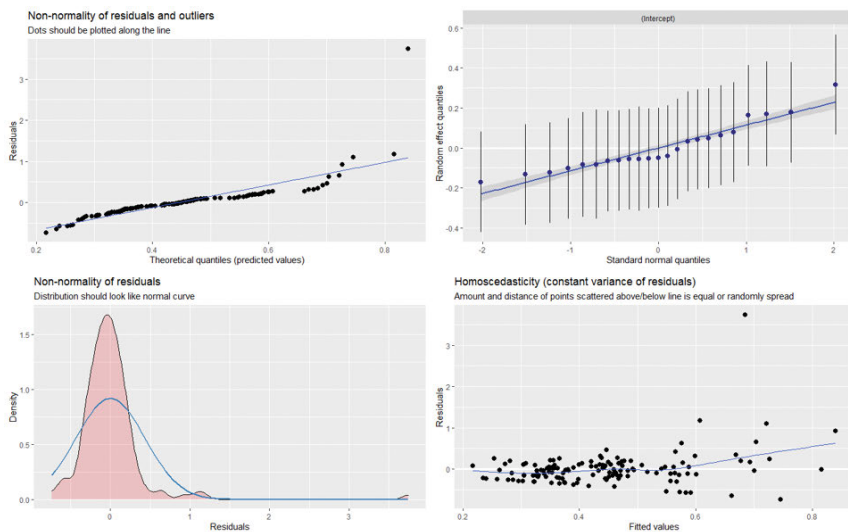
## #1 – cfDNA concentration



## #2 – CDO1 methylation level



### #3 – SOX17 methylation level



### #4 – TAC1 methylation level

*Linear mixed-effects modelling to compare the methylation levels of CDO1, SOX17, and TAC1 in cases vs. controls and explore the additional value of collecting multiple urine samples*

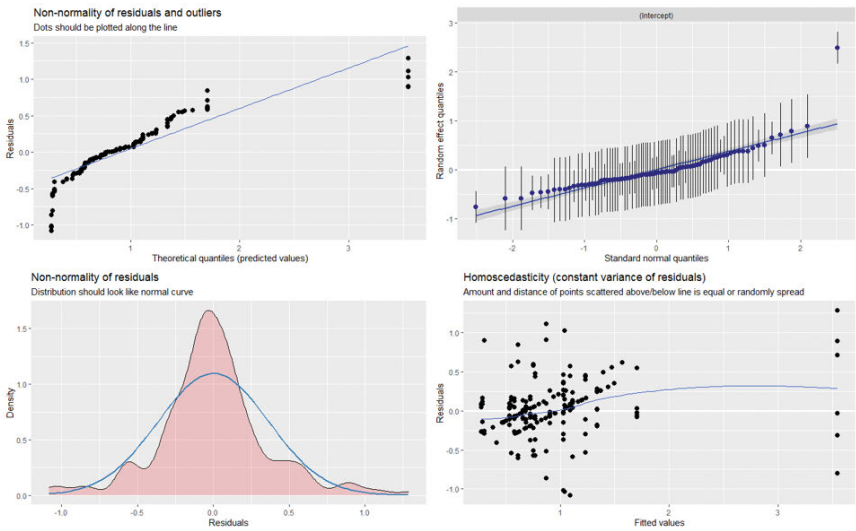
Models were fitted by backward stepwise elimination ( $p \geq 0.05$  for removal) to select fixed and random parts of the linear mixed model using the 'lmerTest' package in R.

Final models are displayed below:

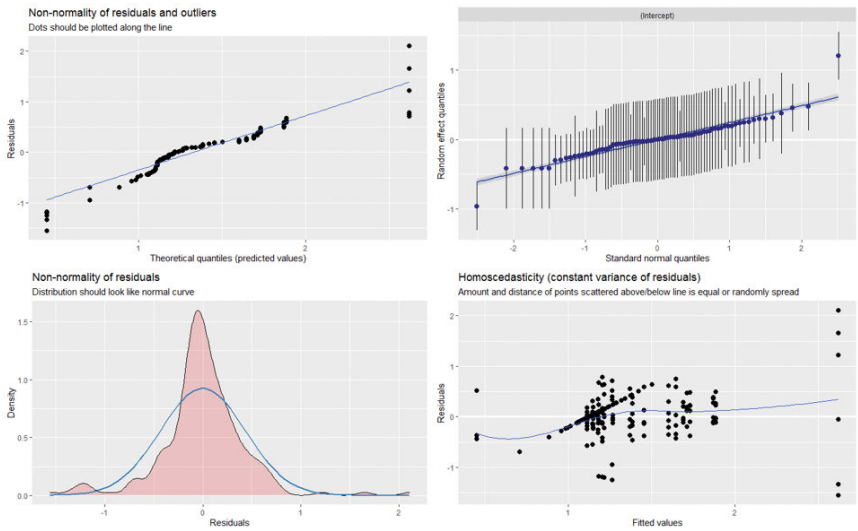
#	Response variable	Final model
5	<i>CDO1</i> methylation level	<code>lmer(CDO1sqrt ~ casecontrol + gender + (1 subject), data = URIC_R_allDT)</code>
6	<i>SOX17</i> methylation level	<code>lmer(SOX17sqrt ~ casecontrol + (1 subject), data = URIC_R_allDT)</code>
7	<i>TAC1</i> methylation level	<code>lmer(TAC1sqrt ~ casecontrol + (1 subject), data = URIC_R_allDT)</code>

Testing model assumptions

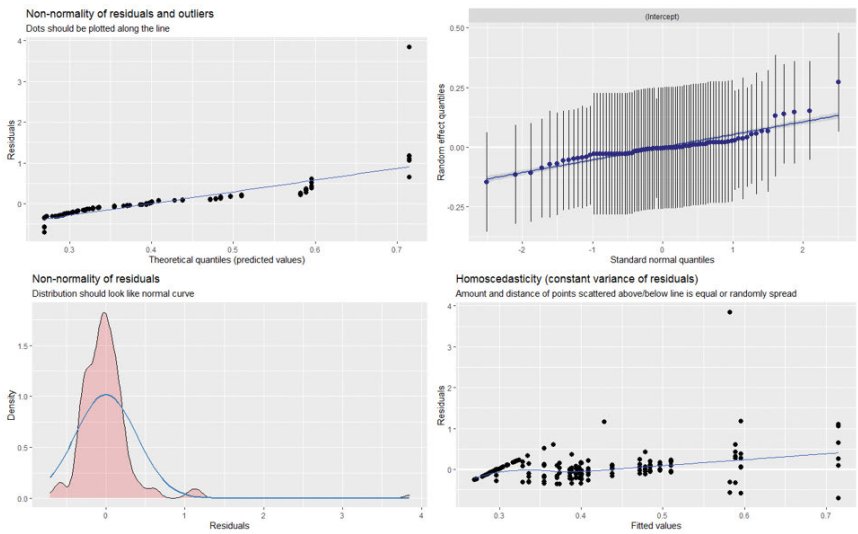
The assumptions of linearity, normality of the residuals and random effects, and homoscedasticity (*i.e.* constant variance of the residuals) were checked visually. A series of diagnostic plots were computed using the ‘sjPlot’ package to check these assumptions.



#5 – *CDO1* methylation levels in cases vs. controls

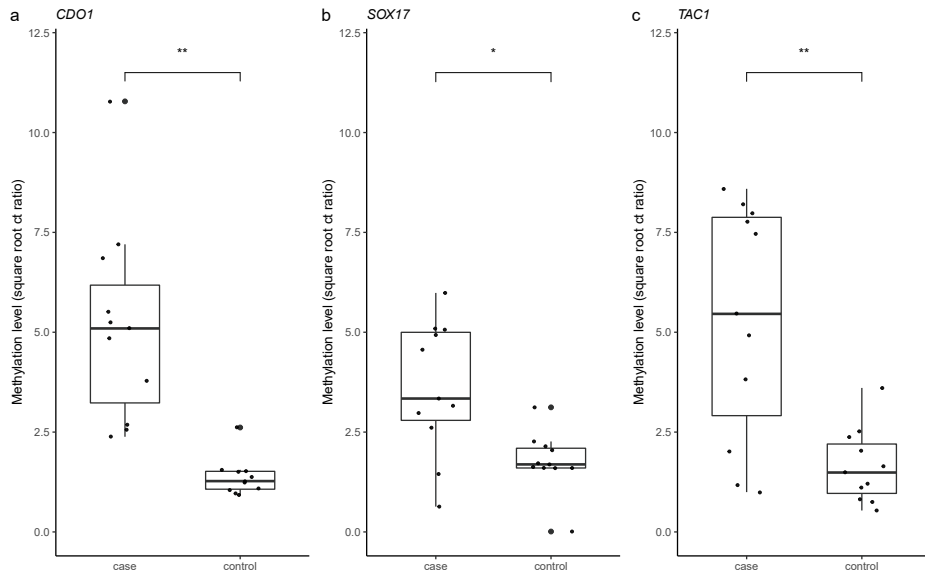


#6 – SOX17 methylation levels in cases vs. controls



#7 – TAC1 methylation levels in cases vs. controls

## Supplementary Figure



**Supplementary Figure 1:** Methylation levels of *CDO1* (a), *SOX17* (b), and *TAC1* (c) measured in 11 pairs of tumors and adjacent normal tissues from NSCLC patients. Methylation levels are normalized to the reference gene *ACTB* and presented as square root Ct ratios. Outliers are indicated by bold circles located outside the whiskers of the boxplot. Statistical significance was computed using the nonparametric paired samples Wilcoxon test. \* $p < 0.10$  (suggestive evidence), \*\* $p < 0.05$  (moderate evidence).

Supplementary Tables

Supplementary Table 1: Primer and probe sequences for methylation analysis of SOX17 by qMSP.

Gene	Forward (5' - 3')	Reverse (5' - 3')	Probe (5' - 3')	Amplicon size (bp)
SOX17	TTTGGGGCGGGGGTCG	CCCACGTCCCAATCCAACC	DFO - CCC {C}{T}{T} ACC C{C}T CCC - BHQ2	57

Locked nuclear acids are indicated by {base}. qMSP = quantitative methylation-specific PCR.

**Supplementary Table 2:** Parameter estimates of DNA methylation levels of *CD01*, *SOX17* and *TAC1* measured in NSCLC patients (n=23) and controls (n=60) according to the fitted linear mixed model.

Fixed effects	<i>CD01</i>			<i>SOX17</i>			<i>TAC1</i>		
	Estimates	95%-CI	p	Estimates	95%-CI	p	Estimates	95%-CI	p
(Intercept)	0.76	(0.48, 1.04)		1.41	(1.24, 1.59)		0.44	(0.35, 0.53)	
casecontrol [control]	-0.05	(-0.34, 0.23)	0.711	-0.26	(-0.50, -0.03)	<b>0.030</b>	-0.14	(-0.28, 0.01)	0.059
sex [male]*	0.30	(0.03, 0.57)	<b>0.031</b>						
Random Effects									
$\sigma^2$	0.19			0.24			0.17		
$\tau_{00}$ subject	0.27			0.14			0.02		
ICC	0.59			0.38			0.10		
N subject	83			83			83		
Observations**	193			193			193		

DNA methylation level estimates are presented as square root transformed ct ratios.  $\sigma^2$  = within-subject variability;  $\tau_{00}$  = between-subject variability; ICC = Intraclass Correlation Coefficient; NSCLC = non-small cell lung cancer.

\*Only *CD01* methylation levels were corrected for sex, following backward stepwise selection.

\*\*Five urine samples of patients were excluded from the analysis based on an *ACTB* Ct value of  $\geq 32$ .



# CHAPTER 8

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## SUMMARY AND GENERAL DISCUSSION

## SUMMARY

High cancer mortality rates and the rising cancer burden worldwide prioritize the development of innovative methods that facilitate the early and accurate detection of cancer. Combining patient-friendly sampling methods with reliable biomarker testing offers a method that is convenient for patients and effective in detecting cancer at a curable stage, with improved patient outcomes as an ultimate goal. This thesis assessed the feasibility of DNA methylation testing in urine as a diagnostic tool for different cancer types, including endometrial, ovarian, and lung cancer. For endometrial and ovarian cancer, the value of DNA methylation testing in self-collected cervicovaginal samples and clinician-taken cervical scrapes was also investigated.

### **Part 1: Endometrial and ovarian cancer detection in patient-friendly samples**

**Part 1** describes the detection of endometrial and ovarian cancer in urine, cervicovaginal self-samples, and clinician-taken cervical scrapes.

In **Chapter 2**, the feasibility of endometrial cancer detection in urine was evaluated. Three methylation markers (*GHSR*, *SST*, *ZIC1*), previously described for the accurate detection of cervical (pre)cancer, were measured in urine samples of endometrial cancer patients (n=42) and healthy controls (n=46). A comprehensive comparison of full void urine, urine sediment, and urine supernatant revealed that full void urine is most optimal for endometrial cancer detection. Full void urine allowed endometrial cancer detection with excellent discriminatory power with an area under the receiver operating curve (AUC) value of up to 0.95 for *GHSR*. This study was the first to demonstrate the feasibility of endometrial cancer detection in urine by DNA methylation analysis.

Given these novel findings, in **Chapter 3**, a systematic review of the literature was performed to 1) summarize previous work on endometrial cancer detection in minimally invasive sample types and 2) select which methylation markers deserve further development. A systematic search starting with 1556 relevant papers, resulted in nine eligible studies describing methylation markers for endometrial cancer detection in minimally invasive sample types, including cervical scrapes, endometrial brushes, vaginal swabs, and vaginal tampons. A total of 15 markers with a high accuracy (AUC range 0.80-0.96) were considered most interesting for further studies. We also remarked that combining methylation markers in a panel may increase test sensitivity without any impact on test specificity.

In **Chapter 4**, nine methylation markers were tested for endometrial cancer detection in paired urine, cervicovaginal self-samples, and clinician-taken cervical scrapes to

comprehensively compare their performance in different sample types. Methylation markers for endometrial cancer detection were based on both our feasibility study described in **Chapter 2** (*GHSR*, *SST*, *ZIC1*) and the systematic review described in **Chapter 3** (*ADCYAP1*, *BHLHE22*, *CDH13*, *CDO1*, *GALR1*, *HAND2*). Paired samples were collected from endometrial cancer patients (n=103) and compared with unpaired samples of healthy controls (n=317). Optimal three-marker combinations yielded a high diagnostic performance for endometrial cancer detection in urine (AUC 0.95), cervicovaginal self-samples (AUC 0.94), and clinician-taken scrapes (AUC 0.97). Diagnostic performances remained virtually equal after cross-validation and for early-stage endometrial cancer detection. The outcomes of this study demonstrated that endometrial cancer detection in home-collected samples was excellent and comparable to the diagnostic performance in clinician-taken cervical scrapes.

In **Chapter 5**, we explored the feasibility of ovarian cancer detection by molecular testing in urine, cervicovaginal self-samples, and clinician-taken cervical scrapes. Nine methylation markers were tested, which were selected from previous studies on ovarian cancer detection in cervical scrapes and plasma (*C2CD4D*, *CDO1*, *NRN1*) and cervical and endometrial cancer detection in patient-friendly sample types (*GALR1*, *GHSR*, *MAL*, *PRDM14*, *SST*, *ZIC1*). Paired samples were collected from women diagnosed with a benign (n=25) or malignant (n=29) ovarian mass and compared with unpaired samples of healthy control women (n=110). Increased methylation levels were found when comparing ovarian cancer patients with healthy controls in full void urine (*C2CD4D*, *CDO1*, *MAL*), urine supernatant (*MAL*), and cervical scrapes (*C2CD4D*, *CDO1*). Methylation levels of *GHSR* also discriminated between benign and malignant ovarian masses in the urine sediment. No elevated methylation signals were found in cervicovaginal self-samples of ovarian cancer patients. We also demonstrated that urine contains ovarian cancer-derived DNA by somatic copy number analysis. Copy number aberrations were detected in 4 out of 23 sequenced urine samples of ovarian cancer patients. This pioneering work encourages further development of urine biomarkers for ovarian cancer detection.

The outcomes of **Part 1** revealed the value of methylation analysis in patient-friendly sample types for endometrial cancer detection of all stages. Convenient modes of sample collection offer the possibility of at-home collection with high patient acceptability. This approach is clinically useful to screen patient populations at risk for endometrial cancer and to streamline who needs to undergo invasive endometrial tissue sampling. Although promising, the clinical effectiveness of this approach requires further confirmation in additional cohorts, including individuals presenting with postmenopausal bleeding and asymptomatic women at risk for endometrial cancer. The presence of ovarian cancer-derived DNA in the urine provides the first

steps toward urine-based diagnostics for ovarian cancer. Further research is needed to further explore and refine the use of urine biomarkers for ovarian cancer diagnostics.

## **Part 2: Non-small cell lung cancer detection in urine**

In **Part 2** of this thesis, the diagnostic potential of urine as a liquid biopsy for non-small cell lung cancer (NSCLC) detection was evaluated.

In **Chapter 6**, the feasibility of primary and recurrent NSCLC detection in urine by DNA methylation testing was assessed. Urine was collected from patients with non-metastatic NSCLC (n=46) and sex and age-matched controls (n=50) to assess the potential of urine for lung cancer detection. Three methylation markers (*CDO1*, *SOX17*, *TAC1*), previously described for NSCLC detection in plasma, sputum, and urine, were tested. Increased methylation levels were found for *CDO1* and *SOX17*, with a combined AUC value of 0.71 after cross-validation. We collected a postoperative urine sample from a subset of patients (n=14) to explore the potential of postoperative monitoring and showed that in 10 patients with preoperatively elevated methylation levels, reduced methylation levels were found postoperatively. This study demonstrates that urine methylation tests provide an interesting means to support primary and recurrent lung cancer diagnoses.

In **Chapter 7**, the dynamics of methylated urinary cell-free DNA (cfDNA) in NSCLC patients were evaluated to determine whether a preferred urine collection time and frequency exists. Six urine samples were prospectively collected from patients with advanced stage NSCLC (n=23) during the morning, afternoon, and evening of two subsequent days. No clear circadian pattern was found for urinary cfDNA concentrations or methylation levels across the sampling time points. While our data suggest that no preferred collection time exists, the frequency of sampling may increase the chance of detecting NSCLC in urine. Substantial variability between- and within-patients was observed and, therefore, serial sampling may increase urine test performance. The considerable biological variation of cfDNA found in this study underlines that single urine test measurements should be interpreted carefully.

The outcomes of **Part 2** demonstrate the technical feasibility of detecting NSCLC in the urine using DNA methylation markers. Further research, including larger patient cohorts and controls with benign pulmonary nodules, is needed to validate the clinical usefulness of this approach. The considerable variability between urine samples highlights the need for a more thorough understanding of cfDNA dynamics and enhancements in test development to ensure reliability. Upon further refinement, this test has the potential to serve as a valuable complementary diagnostic tool to low-dose CT screening to guide clinical decisions in patients with pulmonary nodules.

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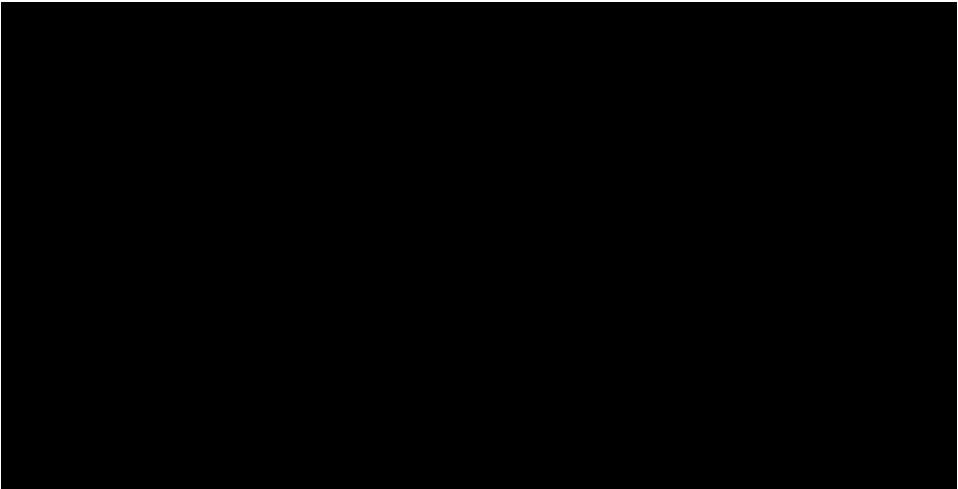
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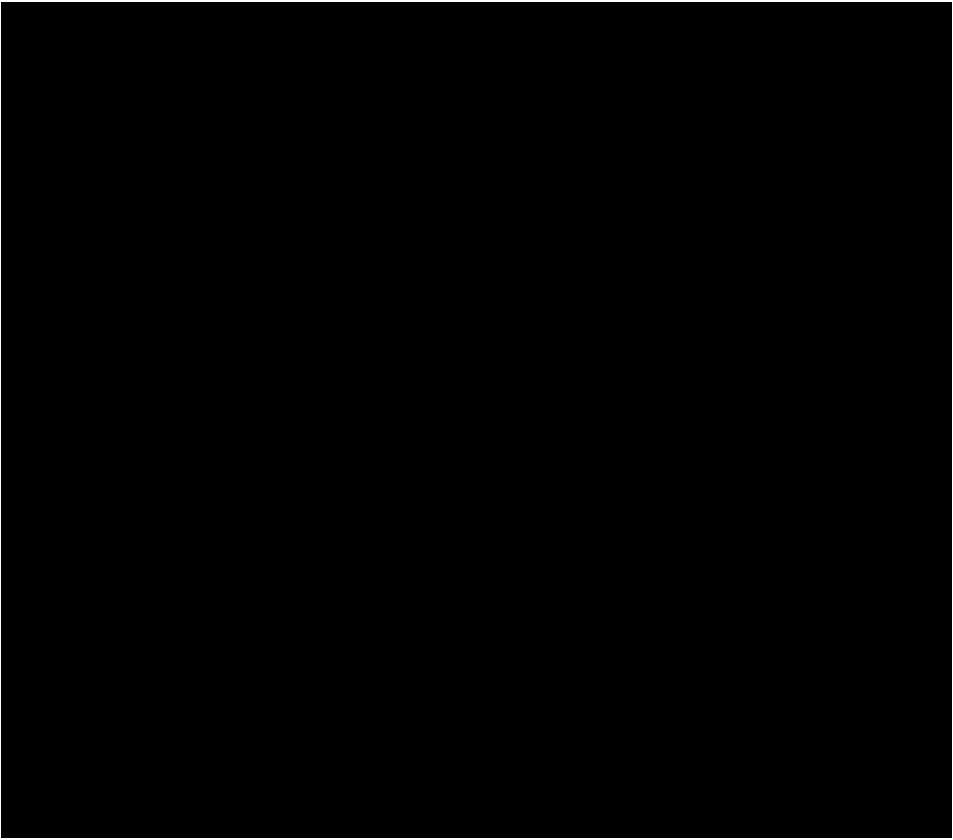
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# APPENDICES

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DUTCH SUMMARY | NEDERLANDSE SAMENVATTING

LIST OF CONTRIBUTING AUTHORS

PHD PORTFOLIO

LIST OF PUBLICATIONS

ABOUT THE AUTHOR | CURRICULUM VITAE

COVER DESIGN DESCRIPTION

ACKNOWLEDGEMENTS | DANKWOORD

## DUTCH SUMMARY | NEDERLANDSE SAMENVATTING

Het aantal patiënten met kanker neemt ieder jaar toe. Deze stijging wordt met name veroorzaakt door de groei, vergrijzing en leefstijl van de bevolking. Bij een verdenking op kanker wordt er in de meeste gevallen een stukje weefsel verzameld voor verder onderzoek. Een arts gebruikt dit om te bepalen of er sprake is van kanker. Dit soort onderzoek is vaak belastend voor patiënten en blijkt in sommige gevallen achteraf niet nodig te zijn geweest. Het is daarom wenselijk om nieuwe methoden te ontwikkelen waarmee kanker op een eenvoudige manier kan worden opgespoord. In dit proefschrift is hier onderzoek naar gedaan. Dit onderzoek draagt bij aan het verbeteren van de diagnostiek van kanker, wat niet alleen patiënten ten goede komt, maar in de toekomst ook het zorgsysteem zou kunnen ontlasten.

**Hoofdstuk 1** geeft een algemene inleiding over de huidige uitdagingen en nieuwe ontwikkelingen binnen de diagnostiek van kanker. Veel nieuwe ontwikkelingen zijn gebaseerd op het feit dat een tumor signaalstofjes kan achterlaten in de bloedsomloop. Deze signaalstofjes kunnen vervolgens in de urine belanden, zelfs als de tumor niet dichtbij de urinewegen zit. In het lab kunnen deze signaalstofjes en dus kanker worden aangetoond. Het is belangrijk om op te merken dat niet elk signaalstofje geschikt is om kanker op te sporen. In dit proefschrift hebben we het signaalstofje DNA-methylatie onderzocht. Dit signaal geeft informatie over hoe ons DNA wordt afgelezen en speelt een belangrijke rol bij de processen die zorgen voor ongeremde celgroei. Dit is een belangrijk kenmerk van kanker.

In dit proefschrift is onderzocht of een simpele urinetest gebruikt kan worden voor het opsporen van verschillende soorten kanker, waaronder baarmoederkanker, eierstokkanker en longkanker. Hiervoor is gekeken of DNA-methylatiesignalen gemeten kunnen worden in urine en voorspellend zijn voor de aanwezigheid van kanker. Voor baarmoederkanker en eierstokkanker is daarnaast ook gekeken of zelfafgenomen vaginaal materiaal en door een arts afgenomen uitstrijkjes van de baarmoedermond gebruikt kunnen worden voor het opsporen van kanker.

### **Deel 1: Het opsporen van baarmoederkanker en eierstokkanker in patiënt-vriendelijke monsters**

In het eerste deel van dit proefschrift wordt het opsporen van baarmoederkanker en eierstokkanker onderzocht in verschillende soorten patiëntvriendelijke monsters.

**Hoofdstuk 2** beschrijft of het mogelijk is om baarmoederkanker in de urine op te sporen. Om dit te onderzoeken hebben we DNA-methylering van drie genen (*GHSR*,

*SST*, *ZIC1*) gemeten in urinemonsters van zowel baarmoederkankerpatiënten (n=42) als gezonde controles (n=46). Door urine te centrifugeren is het mogelijk om deze in verschillende fracties op te delen, namelijk het urine sediment en het urine supernatant. Uit een uitgebreide vergelijking van verschillende urinefracties bleek dat alle soorten urinemonsters kankersignalen bevatten, maar dat ongecentrifugeerde urine het beste is voor het opsporen van baarmoederkanker. Methylering van *GHSR* liet een uitstekende voorspellende waarde zien (AUC 0.95). Deze voorspellende waarde is een getal tussen de 0 en 1, dat aangeeft in welke mate een test kan onderscheiden tussen gezonde personen en personen met ziekte. Hoe dichter deze waarde bij de 1 ligt, hoe beter het onderscheid tussen deze twee groepen kan worden gemaakt. Dit is de eerste studie die aantoonde dat baarmoederkanker nauwkeurig opgespoord kan worden in urine met behulp van DNA-methylering.

**Hoofdstuk 3** bouwt voort op de nieuwe bevindingen van **Hoofdstuk 2**. In dit hoofdstuk is eerder werk over het opsporen van baarmoederkanker in patiëntvriendelijke monsters samengevat. Een systematische zoekactie die begon met 1556 relevante artikelen resulteerde in negen geschikte onderzoeken. Eerder beschreven monsters waren uitstrijkjes afgenomen van de baarmoedermond en baarmoeder, vaginale swabs en vaginale tampons. In totaal werden er 15 DNA-methylatiesignalen met een hoge voorspellende waarde (AUC-bereik 0.80-0.96) beschouwd als het meest interessant voor verder onderzoek. We beschrijven ook dat de test nauwkeuriger kan worden gemaakt wanneer meerdere soorten DNA-methylatiesignalen worden gecombineerd.

**Hoofdstuk 4** beschrijft onderzoek naar negen soorten DNA-methylatiesignalen voor het opsporen van baarmoederkanker in verschillende soorten patiëntvriendelijke monsters. Hierbij hebben we urine, zelfafgenomen vaginaal materiaal en uitstrijkjes van de baarmoedermond die door een arts zijn afgenomen met elkaar vergeleken. DNA-methylatiesignalen voor het opsporen van baarmoederkanker waren gebaseerd op zowel onze studie beschreven in **Hoofdstuk 2** (*GHSR*, *SST*, *ZIC1*) als het systematische literatuuronderzoek beschreven in **Hoofdstuk 3** (*ADCYAP1*, *BHLHE22*, *CDH13*, *CDO1*, *GALR1*, *HAND2*). Gepaarde monsters werden verzameld van baarmoederkankerpatiënten (n=103) en vergeleken met ongepaarde monsters van gezonde controles (n=317). Combinaties van meerdere signalen lieten uitstekende voorspellende waarden zien voor het opsporen van baarmoederkanker in urine (AUC 0.95), zelfafgenomen vaginaal materiaal (AUC 0.94) en uitstrijkjes van de baarmoedermond die door een arts zijn afgenomen (AUC 0.97). De uitkomsten van dit onderzoek tonen aan dat het opsporen van baarmoederkanker in thuis verzamelde monsters (urine en zelfafgenomen vaginaal materiaal) veelbelovend is en vergelijkbaar is met monsters die door een arts zijn afgenomen (uitstrijkjes van de baarmoedermond).

**Hoofdstuk 5** richt zich op het opsporen van eierstokkanker in patiëntvriendelijk materiaal. Opnieuw is er gekeken naar DNA-methylatiesignalen in thuis verzamelde monsters (urine en zelfafgenomen vaginaal materiaal) en door een arts afgenomen monsters (uitstrijkjes van de baarmoedermond). In deze monsters werden DNA-methylatiesignalen van negen genen getest (*C2CD4D*, *CDO1*, *GALR1*, *GHSR*, *MAL*, *NRN1*, *PRDM14*, *SST*, *ZIC1*). Er werden gepaarde monsters verzameld van vrouwen met een goedaardig (n=25) of kwaadaardig (n=29) gezwel in de eierstokken. Deze monsters werden vergeleken met ongepaarde monsters van gezonde controles (n=110). Sommige signalen bleken voorspellend voor de aanwezigheid van eierstokkanker. Dit betrof *C2CD4D*, *CDO1* en *MAL* in ongecentrifugeerde urine, *MAL* in urine supernatant en *C2CD4D* en *CDO1* in uitstrijkjes van de baarmoedermond. Het zelfafgenomen vaginaal materiaal bleek niet geschikt voor het opsporen van eierstokkanker. Een andere belangrijke bevinding was dat de meeste DNA-methylatiesignalen ook gevonden werden in de monsters van vrouwen met een goedaardig gezwel. Dit laat zien dat deze signalen misschien niet specifiek genoeg zijn voor het opsporen van kanker. Naast DNA-methylatie is er in dit hoofdstuk ook gekeken naar afwijkingen in het gehele genoom met behulp van DNA *sequencing*. Deze complete analyse van het DNA toonde aan dat DNA afkomstig van de kwaadaardige eierstoktumor teruggevonden kan worden in de urine. De resultaten van dit hoofdstuk laten zien dat het opsporen van eierstokkanker in de urine uitdagend is, maar niet onmogelijk. Ons onderzoek vormt dan ook een basis voor verder onderzoek naar een urinetest voor eierstokkanker.

**Deel 1** toont aan dat het onderzoeken van DNA-methyleringsignalen in patiëntvriendelijke monsters veelbelovend is voor het opsporen van baarmoederkanker. Het opsporen van kanker in de urine of zelfafgenomen vaginaal materiaal is prettig voor vrouwen, omdat het verzamelen van deze monsters pijnloos is en vanuit huis gedaan kan worden. Deze aanpak zou in de toekomst nuttig kunnen zijn om patiëntengroepen met een verhoogd risico op baarmoederkanker op een laagdrempelige manier te screenen. Deze methode is veelbelovend, maar moet verder worden bevestigd in aanvullende patiëntengroepen, waaronder vrouwen met postmenopauzale bloedingen en vrouwen met een verhoogd risico op baarmoederkanker. Het is belangrijk dat de test geen ruis heeft en betrouwbaar is voor het opsporen van kanker. De aanwezigheid van DNA-methylatiesignalen en eierstokkanker-afkomstig DNA in de urine is een eerste stap in de richting van een urinetest voor het opsporen van eierstokkanker. Onze resultaten laten zien dat het waardevol is om hier verder onderzoek naar te doen. Er is meer kennis nodig om betere testen te ontwikkelen.

## Deel 2: Het opsporen van longkanker in urine

Het tweede deel van dit proefschrift staat in het teken van het opsporen van longkanker in urine. De longen bevinden zich verder weg van de urinewegen, wat het vinden van longkankersignalen in de urine uitdagender maakt.

**Hoofdstuk 6** onderzoekt of longkanker opgespoord kan worden in urine door middel van DNA-methylatiesignalen. Er werd urine verzameld van patiënten met niet-uitgezaaide longkanker (n=46) en gezonde controles (n=50). In deze monsters werden DNA-methylatiesignalen van drie genen getest (*CDO1*, *SOX17*, *TAC1*). Zowel *CDO1* als *SOX17* bleken waardevol voor het opsporen van longkanker in urine, met een gecombineerde voorspellende waarde (AUC) van 0.71. We hebben ook gekeken of terugkerende longkanker kan worden gevonden in urine. Er werd van een kleine groep longkankerpatiënten urine verzameld na de operatie. We zagen in deze groep dat DNA-methylatiesignalen opnieuw meetbaar zijn als de kanker terugkeert. Deze studie laat zien dat het mogelijk is om longkanker te detecteren in urine.

**Hoofdstuk 7** focust op de schommelingen in de hoeveelheid gemethyleerd DNA in urine gedurende de dag. Het doel van dit onderzoek was om te bepalen op welk tijdstip urine het beste verzameld kan worden. Zes urinemonsters werden tijdens de ochtend, middag en avond van twee opeenvolgende dagen verzameld van patiënten met een vergevorderd stadium van longkanker (n=23). Er werd geen duidelijk patroon gevonden voor de hoeveelheid gemethyleerd DNA gedurende de dag. Dit zou betekenen dat het niet uitmaakt op welk tijdstip urine verzameld wordt. Er werd wel aangetoond dat er grote verschillen kunnen zijn tussen urinemonsters van verschillende patiënten. Daarnaast zagen we ook dat urinemonsters van dezelfde patiënt niet altijd dezelfde testuitslag geven. Deze verschillen laten zien dat wanneer we een enkele meting in urine doen, we voorzichtig moeten zijn bij het interpreteren van de resultaten. Uit dit onderzoek komt naar voren dat het nuttig kan zijn om meerdere urinemonsters van eenzelfde patiënt te testen om tot betrouwbaardere resultaten te komen.

**Deel 2** laat zien dat het mogelijk is om longkanker in urine op te sporen. Voordat deze test uiteindelijk gebruikt kan worden, moet deze nog wel verder ontwikkeld worden. Na verdere verfijning zou deze test gebruikt kunnen worden ter ondersteuning van het screenen van mensen met een hoog risico op longkanker. Deze test zou bijvoorbeeld ingezet kunnen worden wanneer er een verdacht knobbelletje is gevonden tijdens een CT-scan, maar het onduidelijk is of deze kwaadaardig is en verwijderd moet worden.

**Hoofdstuk 8** schetst hoe patiëntvriendelijke methoden om kanker op te sporen in de toekomst gebruikt zouden kunnen worden in de praktijk. In dit hoofdstuk komen ook toekomstige uitdagingen en mogelijkheden voor de verdere ontwikkeling van deze methoden aan bod.

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2022	Scientific Data Visualization, Prof. dr. M. Boers
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2021	Medical statistics in R, Oncology Graduate School Amsterdam
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2021	How to write research papers, Taalcentrum VU Amsterdam
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2022	Supervision 2 <sup>nd</sup> year Bachelor student Biomedical Sciences, 3 months
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### Congress presentations

2023	Advances in Circulating Tumor Cells (ACTC): "Liquid biopsy and Precision Oncology: Where do we stand now?", Skiathos, Greece Oral: 'Molecular analysis for ovarian cancer detection in patient-friendly samples'
2022	European Congress on Gynecological Oncology (ESGO), Berlin, Germany Poster: 'Endometrial cancer detection by DNA methylation testing in cervical scrapes, cervicovaginal self-samples and urine'
2022	The European Association for Cancer Research (EACR) Liquid Biopsies congress, Bergamo, Italy Poster: 'Endometrial cancer detection by DNA methylation testing in cervical scrapes, cervicovaginal self-samples and urine'
2020	The European Association for Cancer Research (EACR) Liquid Biopsies congress, Digital Poster: 'Non-Invasive Detection of Endometrial Cancer by DNA Methylation Analysis in Urine'

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**Awards and prizes**

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- 2023      Travel Award, Advances in Circulating Tumor Cells (ACTC): "Liquid biopsy and Precision Oncology: Where do we stand now?", Skiathos, Greece
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- 2023      Annual PhD retreat, Oncology Graduate School Amsterdam
- 2023      Annual retreat, Cancer Center Amsterdam
- 2022      Organization of PhD skills lab: PHD = Positivity + Happiness + De-stress, Association of Amsterdam UMC PhD Candidates
- 2022      Annual retreat, Cancer Center Amsterdam
- 2022      Annual PhD student day, Oncology Graduate School Amsterdam
- 2021      Organization of PhD skills lab: Present Like a Boss, Association of Amsterdam UMC PhD Candidates
- 2021      Board member Association of Amsterdam UMC PhD Candidates
- 2020      Annual retreat, Cancer Center Amsterdam
- 2020      Annual PhD student day, Cancer Center Amsterdam
-

## LIST OF PUBLICATIONS

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\*Equal contribution.

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**Wever, B.M.M.**, Steenbergen, R.D.M. Unlocking the potential of urine for cancer detection: challenges and opportunities. *Submitted*.

## ABOUT THE AUTHOR | CURRICULUM VITAE

Birgit Margaretha Maria Wever was born on January 2 in 1996, in Oosterblokker, The Netherlands. She followed pre-university education (VWO) in Hoorn and graduated in 2014. Within the same year, she started the Bachelor Biomedical Sciences at the VU University, Amsterdam. During this Bachelor, Birgit spent six months at the University of Copenhagen in Denmark for a minor in Cell Biology. She completed her Bachelor by doing her first research internship at the Pathology department of the Cancer Center Amsterdam on *the relationship between gene-specific copy number gains and their protein expression in vulvar cancers and precursor lesions*. In 2017, she graduated Cum Laude with completion of the extracurricular Honors program. Throughout her Bachelor's degree, Birgit discovered her interest in contributing to the understanding of cancer and improving cancer care. Therefore, she started the Oncology Master at the VU University School of Medical Sciences, Amsterdam, in the same year. She did her first-year internship at the Pathology department of the Netherlands Cancer Institute, Amsterdam, on *the gene manipulation of human adenoma-derived organoids using CRISPR/Cas9*. During her second year, she wrote an extensive literature review on *the molecular basis and rationale for combining immune checkpoint inhibitors with chemotherapy in non-small cell lung cancer*, resulting in her first publication. She did her second-year internship at the Surgery department of the University of Illinois at Chicago Cancer Center in the United States on *the detection of non-small cell lung cancer in plasma and urine using epigenetic biomarkers and circulating tumor cells*. She graduated in 2019 and directly returned to her first internship position at the Pathology department of the Cancer Center Amsterdam to start her PhD trajectory on *cancer detection using patient-friendly solutions*. During her PhD, Birgit has been an active member of the Association of Amsterdam UMC PhD Candidates (ASAP) board, which strives to support the rights of all Amsterdam UMC PhD candidates on a national level and connect PhD students within the institute. She enjoyed her PhD trajectory and became even more enthusiastic and passionate about science. Therefore, she will continue her scientific journey as a Postdoctoral researcher in the liquid biopsy field at the Peter MacCallum Cancer Centre in Melbourne, Australia.



## COVER DESIGN DESCRIPTION

To me, science is like completing a puzzle without an example. Each piece of the puzzle symbolizes a new discovery. Along the way, we aim to connect these pieces to gradually reveal the bigger picture of our understanding. Unraveling the exact shape of a puzzle piece takes time and requires patience. In some cases, the form of these puzzle pieces might be surprisingly different from what we expect. The process of scientific exploration might even reshape what we thought we knew to provide a better fit with new findings. Completing a puzzle is rewarding. However, in science, there are puzzles we might never fully complete and understand. Some puzzle pieces fit well enough to advance and broaden current knowledge, but may still contain internal gaps that we cannot fill in yet.

The colors represent the different sample types used for patient-friendly cancer detection. Blue stands for the color of the brush used by clinicians to collect cervical scrape material. Pink refers to the color of the brush used by women to collect vaginal material at home. The knowledge gained from cancer detection using these brushes have laid the foundation for cancer detection in urine. The yellow puzzle pieces symbolize the ongoing process of piecing together information for urine-based cancer detection. Every new discovery gets us one step closer to completing the puzzle and creating new solutions for patient-friendly cancer detection.

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