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Highly Specific Droplet-Digital PCR Detection of Universally Methylated Circulating Tumor DNA in Endometrial Carcinoma

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BACKGROUND: No circulating biomarker is available for endometrial carcinoma (EC). We aimed to identify DNA positions universally hypermethylated in EC, and to develop a digital droplet PCR (ddPCR) assay for detection of hypermethylated circulating tumor DNA (meth-ctDNA) in plasma from patients with EC.

METHODS: DNA positions hypermethylated in EC, and without unspecific hypermethylation in tissue/cell types releasing circulating cell-free DNA in plasma, were identified in silico from TCGA/Gene Expression Omnibus (GEO) data. A methylation-specific ddPCR (meth-ddPCR) assay following bisulfite conversion of DNA extracted from plasma was optimized for detection of meth-ctDNA according to dMIQE guidelines. Performances were validated on a retrospective cohort ($n = 78$ tumors, $n = 30$ tumor-adjacent tissues), a prospective pilot cohort ($n = 33$ stage I–IV patients), and 55 patients/donors without cancer.

RESULTS: Hypermethylation of *zinc finger and SCAN domain containing 12 (ZSCAN12)* and/or *oxytocin (OXT)* classified EC samples from multiple noncancer samples with high diagnostic specificity/sensitivity [$>97\%$; area under the curve (AUC) = 0.99; TCGA/GEO tissues/blood samples]. These results were confirmed in the independent retrospective cohort (AUC = 0.99). Meth-ddPCR showed a high analytical specificity (limit of blank = 2) and sensitivity (absolute lower threshold of detection = 50 pg_{methDNA}/mL_{plasma}).

In the pilot cohort, meth-ctDNA was detected in pre-treatment plasma samples from 9/11 and 5/20 patients with advanced and non-advanced EC, respectively. 2 of 9 patients had ctDNA detected after macroscopic complete surgery and experienced progression within 6 months. No healthy donors had any copy of hypermethylated DNA detected in plasma.

CONCLUSIONS: Meth-ddPCR of *ZSCAN12/OXT* allows a highly specific and sensitive detection of ctDNA in plasma from patients with EC and appears promising for personalized approaches for these patients.

Introduction

Endometrial carcinoma (EC) is the most frequent pelvic gynecological cancer in Western countries (1). While most cases are localized in the uterus (stages I–II) at diagnosis, approximately 10–20% of patients experience relapses (2, 3), and subsequent poor prognosis (4). To identify patients with higher risk of relapse and those who could benefit from adjuvant treatments remains a challenge. *Tumor protein 53 (TP53)* mutations have been added as adverse prognostic factors justifying adjuvant chemotherapy to advanced tumor stages (stages III–IV) and nonendometrioid histologic subtypes (5). However, 59% of EC-related deaths occur in *TP53*-wild-type tumors, and 40% of patients with *TP53*-mutated carcinomas remain free of disease at 5 years even

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without chemotherapy (6). These data highlight the limitations of actual molecular characterization for prognostication, possibly related to biological and prognostic heterogeneity within molecular subgroups (7–9), and illustrate the need for new personalized prognostic tools.

Liquid biopsies have emerged in the past 2 decades as a major tool for cancer characterization and prognostic stratification (10). Detection of plasma circulating tumor DNA (ctDNA) before/after surgery of primary tumor strongly correlates with the risk of distant relapse in various cancers that are apparently localized (11, 12). Detection or quantification of ctDNA may also allow early detection of relapse and a monitoring of treatment efficacy in advanced stages (13), especially in cancers without accurate tumor circulating marker, such as EC (14).

Previous studies showed that ctDNA could be detected in plasma from patients with EC. However, these studies, mainly based on mutation detection, were limited by the mutational heterogeneity of EC (15, 16) and by technical constraints that limited analytical sensitivity (16, 17). Analyzing cancer-associated DNA methylation could be of value to overcome the mutational heterogeneity of EC (18, 19). Highly sensitive droplet-digital PCR (ddPCR) has emerged as a major tool to maximize ctDNA detection (20). Furthermore, detecting ctDNA through other targets than point mutation overcomes specificity issues related to polymerase errors in the ddPCR (21).

We report here a bioinformatical and experimental work flow allowing to identify DNA positions specifically methylated in nearly all EC, and the development of a clinically applicable highly sensitive ddPCR assay that allows robust detection and dynamic quantification of ctDNA in patients with EC.

Materials and Methods

IN SILICO DATA, PATIENT COHORTS, AND CONTROLS

In silico data. Illumina normalized methylation data from uterine (normal and cancer), lung, colorectal, breast, and ovarian cancer TCGA cohorts, and from all non-cancer TCGA samples were download from the Genomic Data Common database (22). Illumina normalized methylation data from 18 samples obtained from patients with metastatic EC were download from the Gene Expression Omnibus (GEO) database (GSE33422) (23). Illumina normalized methylation data from multiple cohorts were used to account for background methylation in blood-derived DNA, considering white blood cells (WBC) as the main provider of circulating-cell-free DNA (ccfDNA) (24). As the training dataset, GSE107205 ($n=72$ samples) and GSE85210 ($n=253$ samples) were used to identify CpG positions methylated in peripheral WBC. Eight

additional GEO dataset ($n=4399$ samples of whole blood or WBC DNA) were used for validation (Supplemental Data 1 in the online Data Supplement).

External cohorts of endometrial carcinoma samples and patients Cohort A included patients with stage I–IV EC treated at Cochin Hospital (Paris, France) (2010–2017) and was considered as an independent cohort for validation of DNA positions found in silico as universally and specifically hypermethylated in EC. Among the 125 patients reported elsewhere (25), 78 selected for a secondary analysis (transcriptome/epigenetic analyses) were included based on molecular annotation availability, formalin-fixed paraffin embedded (FFPE) tissue availability and fixation quality (26). Tumor blocks ($n=78$) were used for positive control and compared with 30 tumor-adjacent tissues (FFPE blocks without infiltrating tumor cell as per expert pathology review).

Cohort B included all patients treated in the gynecological surgery department of the Cochin Hospital between October 2019 and December 2020 and prospectively included for data collection and plasma banking. Additionally, all patients treated in the same period for stage IV or relapsed EC in the department of medical oncology were considered as a cohort of patients with advanced diseases. FFPE blocks and plasma samples were stored and provided by the Biological Resource Center and Tumor Bank Platform of Cochin Hospital (BB-0033-00023).

External cohort of healthy volunteers. Healthy donors included 20 female donors who underwent blood donation at Etablissement Français du Sang, France, in January to February 2021 and were considered as a healthy/blank control cohort. Additionally, plasma samples from 5 healthy female volunteers (2 mL, age 28–62 years) and 1 bulk plasma ($V=100$ mL) were purchased at Biopredic International (Saint Grégoire, France).

External cohort of patients with benign gynecology conditions. Thirty patients treated in the gynecological surgery department of the Cochin Hospital (2018–2020, age 22–49 years) and included in a prospective cohort studying genomic alteration of endometriosis were considered as a noncancer gynecology control cohort (27, 28): 10 were affected by deep-endometriosis, 10 by pelvic endometriosis, and 10 by nonendometriosis benign conditions (benign ovarian cysts: $n=4$, uterine myoma: $n=4$, fertility disorders: $n=2$).

Regulatory/ethical considerations (external cohorts). The study was approved by appropriate ethical committee. All patients provided written consents. Details regarding

ethical considerations and approvals are provided in [Supplemental Method 1](#).

Preanalytical considerations. Details on collection and processing of uterine and plasma samples (DNA extraction, storage) are provided in [Supplemental Method 2](#). Whole blood was collected either in 3K-EDTA or CELL-FREE DNA BCT[®] preservative tubes (StreckTM), and processed within 4 hours or 7 days, respectively, according to the manufacturers' recommendations.

Control DNA. Human genomic DNA extracted from whole blood (pool from multiple donors) (G304A, Promega) was used as negative DNA control. Enzymatically universally methylated human genomic DNA (D5011-1, ZYMO Research) was used as positive DNA control. DNA concentrations were determined after shipment using the QubitTM DNA BR Assay Kit.

Molecular characterization in external cohorts. EC molecular groups were identified according to a validated method using sequential surrogates of the TCGA classification system (29): mismatch-repair-deficient tumors (surrogate of microsatellite instable tumors), tumors with DNA-polymerase epsilon (*POLE*) mutation (ultramutated tumors), and *TP53*-mutation (tumors with high number of gene copy number alterations). Remaining tumors were classified as "no specific molecular profile" (low copy number alteration tumors). The mismatch-repair system was assessed by immunohistochemistry analysis of PMS1 Homolog 2 (PMS2) and MutS Homolog 6 (MHS6) (30). *POLE* and *TP53* mutations were identified by targeted sequencing using a custom panel. Molecular characterization of cohort A patients has been described elsewhere (25). A detailed description of molecular characterization for cohort B is provided in [Supplemental Method 3](#).

Identification of universally methylated DNA positions ([Supplemental Method 4](#)). To select methylated DNA positions that could be used as potential targets for circulating tumor DNA detection, we aimed to identify CpG positions meeting the following criteria: (i) hypermethylation of at least one target in >97.5% of EC (sensitivity), and (ii) no hypermethylation in noncancer uterine samples (specificity > 97.5%), and (iii) without background methylation level in blood-derived DNA methylation analyses.

Briefly, 2 bioinformatical pipelines were used for target identification. Pipeline A relied on a LASSO-penalized logistic regression using highly variable TCGA methylation data to identify DNA methylated positions predictive of the sample nature: cancer vs noncancer uterine samples. Pipeline B relied on combined

differentially methylated position analyses (R package *minfi*) comparing uterine cancer methylation data vs noncancer uterine samples, vs all TCGA noncancer tissues, vs peripheral WBC, and vs whole blood samples methylation data.

Finally, candidate DNA positions of the 2 pipelines were combined to select 2 positions eligible for the design of a triplex ddPCR assay (accounting for one methylation-insensitive reference gene). Candidate DNA positions hypermethylated in more than 0.5% of GEO blood-derived samples were removed ([Supplemental Data 1](#)). Internal performances of the final combination were evaluated using area under the receiver operating characteristic curve in the TCGA dataset.

Bisulfite conversion of DNA. Up to 20 μ L (or 30 ng for highly concentrated DNA samples) of DNA sample extracted from plasma, buffy coat, or tumors, were bisulfite-converted using the EZ DNA Methylation-Gold Kit (31) (ZYMO Research), according to the manufacturer's instructions. Bisulfite-converted DNA was stored up to 1 week at -20° C until ddPCR. Bisulfite-converted DNA were eluted in M-elution buffer 11 μ L. Various amount of human genomic control DNA and universally methylated control DNA were bisulfite-converted depending on the experiment. Every bisulfite-conversion batch included at least 1 well with 10 ng of human genomic DNA control and 1 well with 10 ng of universally methylated human DNA control.

ddPCR assay. The ddPCR assay was developed based on dMIQE 2020 guidelines (20). Full details describing the ddPCR assay development are provided in [Supplemental Method 5](#). Briefly, the ddPCR assay was designed as a triplex (i.e., including 3 targets). For each target, we designed forward and reverse primers (Eurofins Genomics, France), and a MGB Taqman probe[®] (Applied BiosystemsTM, UK; 3' nonfluorescent-quencher; FAMTM/VICTM-reporter dyes) aiming to amplify and detect methylated sequences of the targeted amplicons identified by bioinformatical analyses. A methylation-insensitive target on the *albumin* (*ALB*) gene was used as an internal control of the amount of DNA analyzed in each PCR well [primers and probes reported elsewhere (32)].

Statistical analyses. Categorical/binary variables were described by the number (percentage), and continuous variables by median [interquartile range (IQR)]. Associations between qualitative/continuous variables were analyzed using Student's *t*-test/Wilcoxon test/ANOVA, as appropriate. Associations between continuous variables were analyzed using linear regression.

Associations between categorical/binary variables were assessed using chi-square/Fisher's exact test. Differential gene expression analyses were performed using the EdgeR R package.

Results

ENDOMETRIAL CARCINOMA UNIVERSAL METHYLATION SIGNATURE

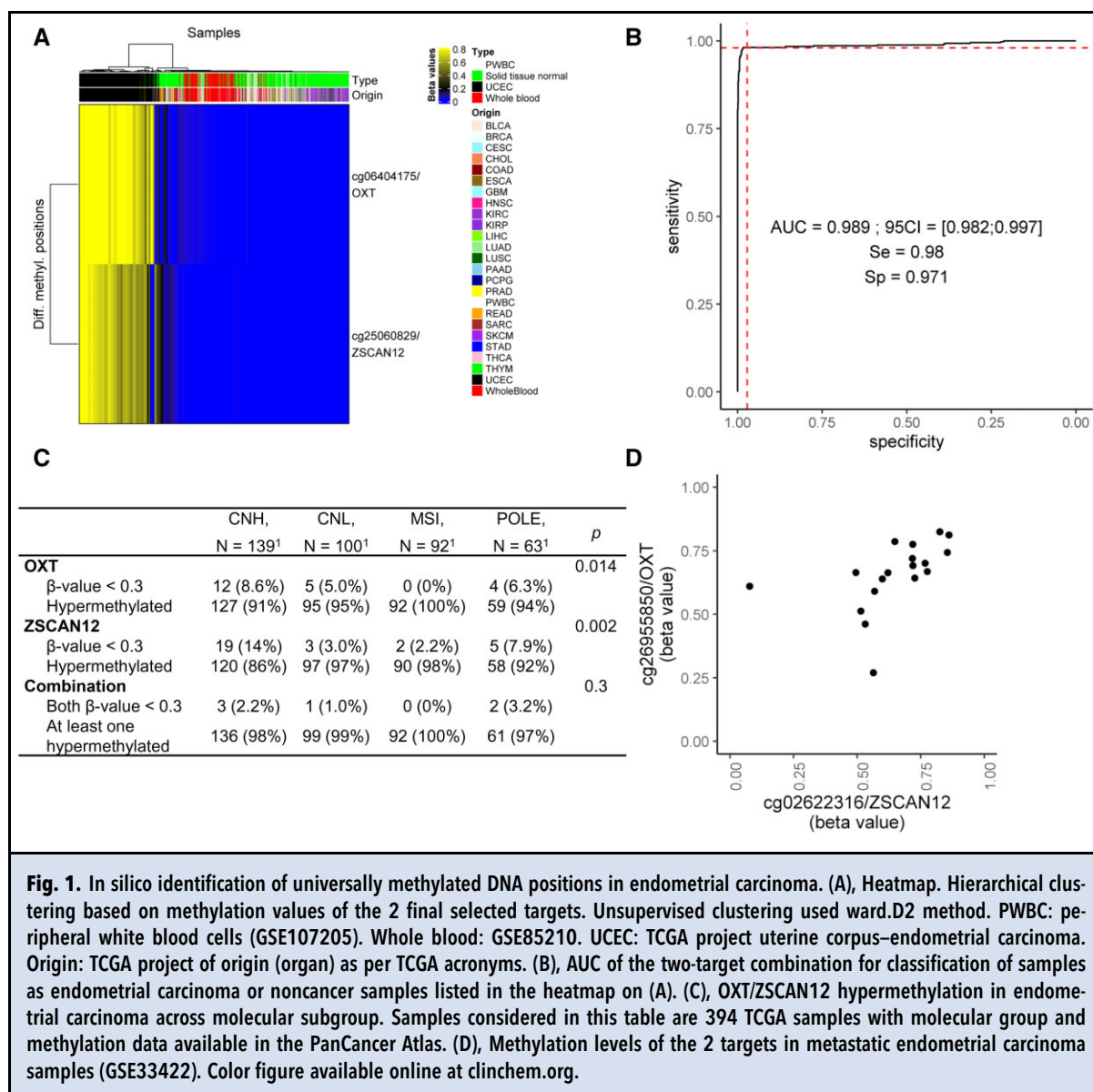
In silico identification of the universal methylation signature. Three DNA methylated positions were identified as potential candidate positions hypermethylated in EC samples (Supplemental Methods). Among the 3 identified targets, one showed background methylation levels in blood DNA methylation analyses (Illumina HM450 probe ID cg18782604—nearest gene: *SIM1*—Supplemental Data 1) and was excluded. The 2 other DNA methylated positions were identified as located on *OXT* and *ZSCAN12* promoters. Combining these 2 targets accurately classified EC samples from noncancer samples [area under the curve (AUC) = 0.99] (Fig. 1, A and B). High methylation levels were observed regardless of EC molecular subgroup (Fig. 1, C). A classification threshold in methylation levels [β -value > 0.3 as reported previously (33)] allowed classification of EC samples from other samples with diagnostic sensitivity/specificity of 0.98/0.97. High methylation levels were observed in metastatic samples from patients with EC (Fig. 1, D). *OXT/ZSCAN12* also showed hypermethylation in other cancers, mainly in gynecological tumors (breast and ovarian cancers): 591 (98%) ovarian cancers showed *OXT* hypermethylation while only 32 (5%) showed *ZSCAN12* hypermethylation (Supplemental Fig. 1).

Genomic features of the universal methylation signature. TCGA data analyses confirmed these 2 positions as belonging to hypermethylated regions in EC (Supplemental Fig. 2, A–D). *ZSCAN12* gene expression was lower in EC samples than in noncancer uterine tissues. Conversely, *OXT* showed similarly low expression levels in noncancer uterine tissues and EC samples (Supplemental Fig. 2, E and F). The DNA methyltransferase 3 Beta gene (*DNMT3B*, major de novo DNA methyltransferase) showed high gene expression levels in almost all samples when compared with noncancer uterine samples, supporting a wide de novo DNA methylation in EC (Supplemental Fig. 2, J). Finally, the limited subset of EC samples with low levels of *ZSCAN12* and *OXT* methylation were samples with a low genome-wide methylation burden (Supplemental Fig. 2, I). Together, these results support that the hypermethylation of *ZSCAN12/OXT* observed in most EC could be part of a genome-wide hypermethylation phenotype.

ddPCR assay for detection of universally hypermethylated tumor circulating DNA Detailed description of the developed ddPCR assay is provided in Supplemental Tables 1 and 2 and Supplemental Figs. 3–5. Analytical specificity was estimated for each target as a limit of blank of 2 droplets based on 78 replicates of negative genomic DNA control (Supplemental Data 2). False-positive droplet detection was independent of the amount of DNA analyzed (Supplemental Fig. 6, A). No false-positive droplet was detected from healthy donor buffy coat DNA ($n = 20$), healthy donor plasma DNA ($n = 25$), and from plasma DNA from patients with benign gynecology conditions ($n = 30$) (Supplemental Fig. 6, B–D). Median ccfDNA concentration for plasma controls was 7.3 ng/mL (IQR 5.0–9.0) (Supplemental Fig. 7). Gathering the results of the 153 negative controls (replicates of negative genomic DNA control, all buffy coat DNA, plasma DNA), no sample showed double-false-positive droplet (i.e., for both *OXT* and *ZSCAN12*).

Analytical sensitivity was estimated in silico by a limit of detection of 6 droplets (Supplemental Data 2). We further assessed experimentally a lower threshold of detection (Supplemental Fig. 8). After bulk bisulfite conversion of negative genomic DNA control and 100% enzymatically methylated positive control (>100 ng of DNA treated in bisulfite conversion for optimal yield), post-conversion serial dilutions of positive control DNA in negative control DNA were performed (from 1:1 to 1:1000). ddPCR detected 0–3 copies of methylated targets for an absolute input of methylated DNA control estimate of 10 pg (pre-bisulfite-conversion fluorometric quantification—QubitTM) (Supplemental Fig. 8, A). The bisulfite-conversion-ddPCR process detected 3–13 copies of methylated targets for an absolute input of methylated DNA control of 50 pg in the bisulfite conversion (each dilution replicate being individually treated by bisulfite conversion before ddPCR) (Supplemental Fig. 8, B). Finally, after dilution of methylated DNA control in plasma before extraction, 2–4 copies of each methylated target were detected for 50 pg of methylated DNA control. These findings support an absolute lower threshold of detection of 50 pg of hypermethylated DNA in plasma samples (Supplemental Fig. 8, C).

Validation of the endometrial carcinoma universal methylation signature. ddPCR analysis confirmed the high performances of combining *OXT/ZSCAN12* methylation levels to classify EC samples from tumor-adjacent tissues in the external validation cohort A (AUC = 0.99, $n = 78$ patients, $n = 30$ tumor-adjacent tissues) (Fig. 2, Supplemental Table 3). Only one patient was found with a tumor without *OXT/ZSCAN12* hypermethylation [*TP53* mutation with variant allele frequency (VAF) = 35% on tumor DNA]. Clinical-

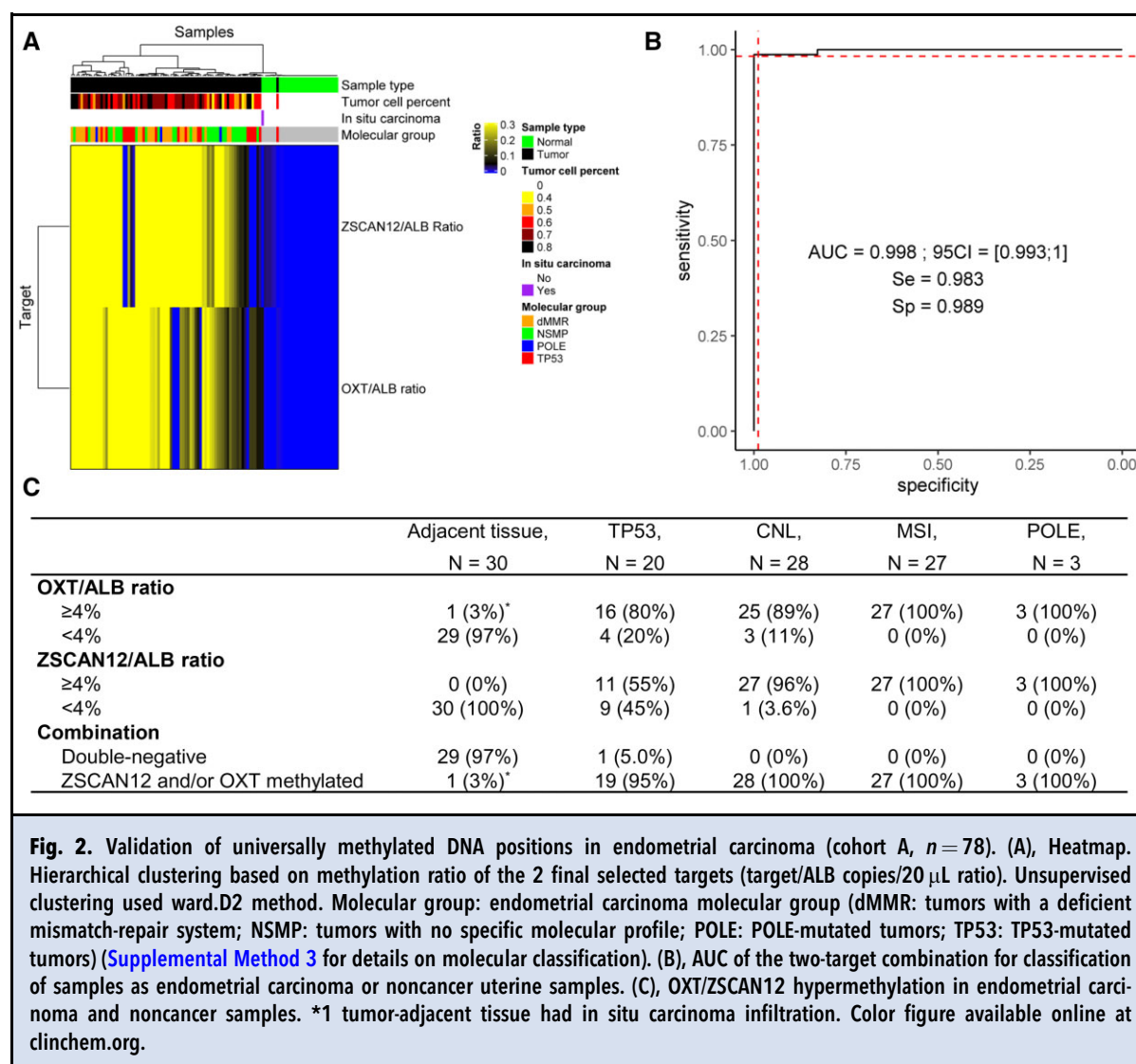


pathological review confirmed its uterine origin. An unusual aggressive history was observed with bone/central nervous system metastatic, chemo-resistant, relapse (death 2 months after relapse). Among nontumor blocks, 5 samples showed low methylation ratio <4%, possibly due to minimal tumor cell contamination. Higher proportion of epithelial cells or adenomyosis were not associated with higher *OXT/ZSCAN12* methylation levels, supporting these 2 targets as associated with cancer rather than with the epithelial cell type (Supplemental Data 3).

Gathering results from analytical sensitivity/specificity analyses and from the validation cohort led to the establishment of a decision tree for analyzing patient

plasma samples (Fig. 3). Briefly, in this decision tree, any plasma with 1–2 droplets detected for both targets, or any plasma with more than 2 droplets of at least 1 target were considered with putative or confirmed ctDNA detection, respectively.

Detection of circulating tumor DNA by methylation-specific ddPCR assay. Thirty-three patients with EC were included in the prospective pilot cohort study B (Flow chart in Supplemental Fig. 9). The median cfDNA concentration was 15.3 ng/mL (IQR 10.6–18.6, Supplemental Fig. 7). ctDNA was detected in 14/31 (45%) patients for whom the first plasma was collected before surgery or chemotherapy. ddPCR detected ctDNA



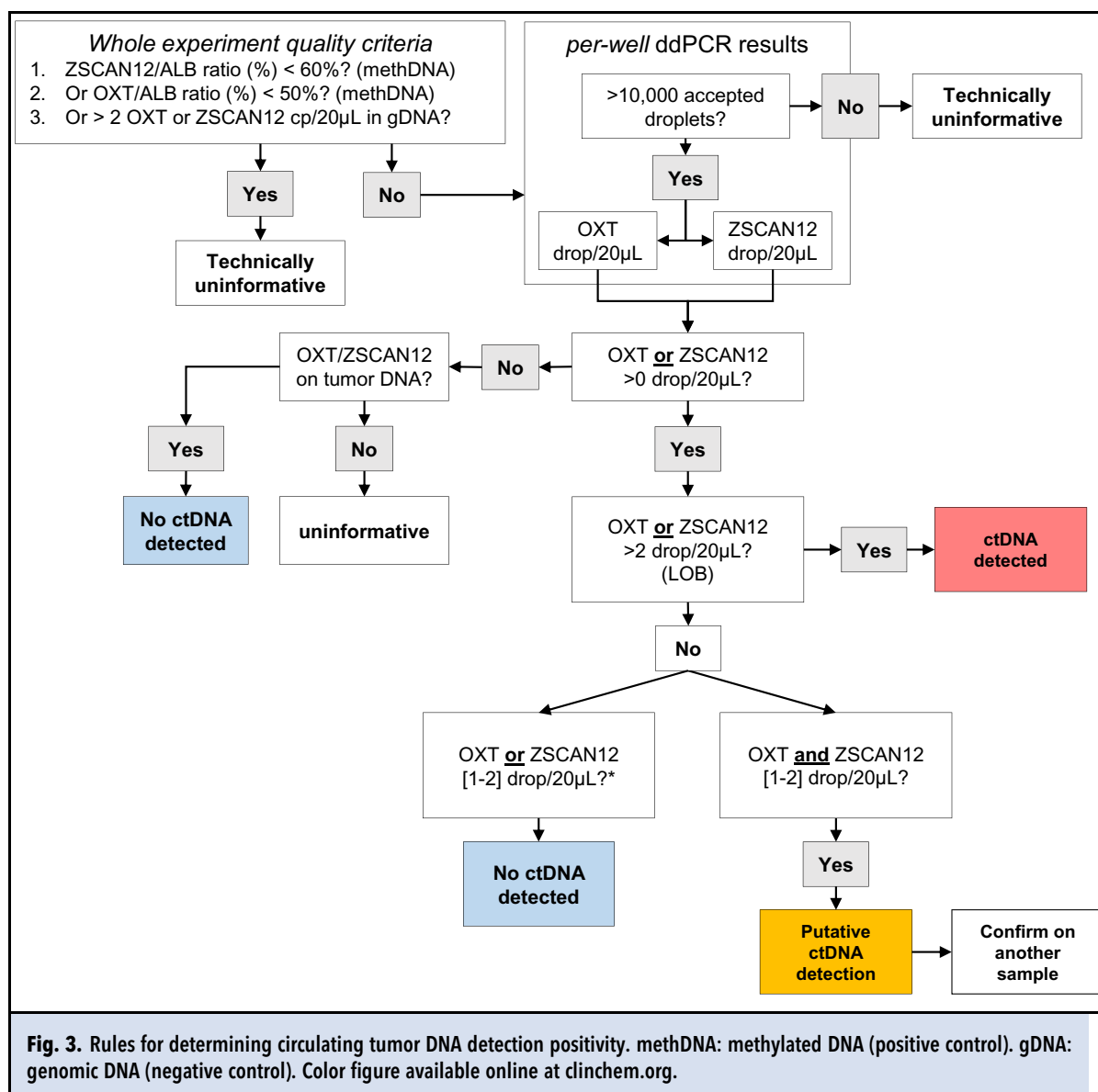
from stage I to stage IV cancers and across all histological types, grades, and molecular subgroups (Table 1, Fig. 4, A). ctDNA was detected in 2/15 stage I patients who had tumors with lymphatic/vascular space involvement.

Nine out of 11 patients with stage IV or relapsed cancer had ctDNA before any treatment. Among the 2 other patients who had no ctDNA detected (Fig. 4, A), patient #3 had a 22-mm pelvic relapse after a slow evolution during 2 years, and patient #6 did not show OXT/ZSCAN12 hypermethylation on tumor. Patient #6 tumor was a stage IV clear-cell carcinoma with a TP53 p. Arg175His mutation (VAF = 56% on tumor DNA; VAF = 4% on ccfDNA—analyzed by targeted sequencing, Supplemental Method 3). This patient exhibited a poor outcome similar to the patient previously identified in cohort A with an unmethylated tumor (death 1 month after initial diagnosis).

Additionally, 9 patients had a plasma sample collected after macroscopically complete surgery, among whom 2 had ctDNA detected (stage III and IV tumors) and experienced tumor progression at 4 and 1 month. Conversely, none of the 7 other patients experienced relapse/progression within 6 months (stage I: $n = 3$, stage III: $n = 1$, stage IV: $n = 3$).

Four patients had serial plasma samples available after a baseline plasma sample with ctDNA detected. ddPCR allowed quantitative and dynamic ctDNA detection consistent with patient outcomes (Fig. 4, B).

ddPCR analysis of buffy coat DNA from patients with ctDNA confirmed that methylated DNA detected did not originate from WBC (Supplemental Fig. 10, A). High reproducibility was observed in ccfDNA detection yield by ddPCR after bisulfite conversion (Supplemental Fig. 10, B1). The ALB (copies/20 μ L) detection yield



was similar between ccfDNA samples and methylated DNA control (Supplemental Fig. 10, B2). All samples without ctDNA detected were considered negative at ddPCR analytical sensitivity $\leq 0.2\%$ (target/ALB). All samples reached a theoretical individual absolute lower limit of detection estimated as ≤ 17.5 pg/mL of ctDNA (Supplemental Fig. 10, C).

Discussion

Despite the wide heterogeneity of EC, we identified 2 hypermethylated DNA positions shared by nearly all tumors. Conversely, *OXT* and *ZSCAN12* were unmethylated in multiple noncancer cells/tissues, such as

WBC, whole blood, and solid organs that potentially contribute to ccfDNA release (24). Indeed, the consideration of highly-vascularized organs (kidney, thyroid, brain, liver) allowed us to account for unspecific DNA methylation changes present in endothelial cells and therefore in ccfDNA. The high diagnostic sensitivity in EC and the high specificity vs tissues releasing ccfDNA allowed identifying hypermethylated targets without biological background noise for ctDNA detection in plasma. The ddPCR assay allowed highly sensitive detection and absolute quantification of ctDNA in all patients with macroscopic visceral/peritoneal disease and in a subset of patients with stage I–III EC, particularly in case of substantial lymphatic/vascular space

Table 1. Association between circulating tumor DNA (ctDNA) detection and tumor features.

Characteristic	ctDNA detection ^a , n = 14 ^b	No ctDNA detection, n = 19 ^b	P-value ^c
Age at diagnosis	72 (64, 78)	70 (64, 77)	0.8
Histological type			>0.9
Endometrioid	8 (57%)	11 (58%)	
Serous	4 (29%)	6 (32%)	
Carcinosarcoma	1 (7.1%)	1 (5.3%)	
Clear cell	1 (7.1%)	1 (5.3%)	
Histological grade			0.5
Low grade	5 (36%)	10 (53%)	
High grade	9 (64%)	9 (47%)	
Molecular group			0.9
dMMR	2 (14%)	1 (5.3%)	
POLE	2 (14%)	2 (11%)	
TP53	5 (36%)	8 (42%)	
NSMP	5 (36%)	8 (42%)	
Sample timing (first sample)			-
Before chemotherapy/surgery	14 (100%)	17 (89%)	
After surgery	-	2 (11%) ^d	
FIGO stage at sampling			0.008
IA	2 (14%)	6 (32%)	
IB	0 (0%)	7 (37%)	
II	0 (0%)	1 (5.3%)	
IIIA	1 (7.1%)	0 (0%)	
IIIC1	1 (7.1%)	1 (5.3%)	
IIIC2	1 (7.1%)	0 (0%)	
Advanced/relapse	9 (64%)	4 (21%)	
Uterine mass	9 (64%)	16 (84%)	0.2
LVSI	8 (80%)	2 (12%)	<0.001
Unknown	4	2	
Peritoneum invasion	4 (29%)	1 (5.3%) ^d	0.14
Visceral/bone metastasis	6 (43%)	0 (0%)	0.003
Pelvic/para-aortic nodes	5 (36%)	2 (11%)	0.11
tDNA OXT methylation	14 (100%)	16 (84%)	0.2
tDNA ZSCAN12 methylation	13 (93%)	16 (84%)	0.6

^aIncluding 1 patient with putative ctDNA detection (one droplet of ZSCAN12 and OXT).

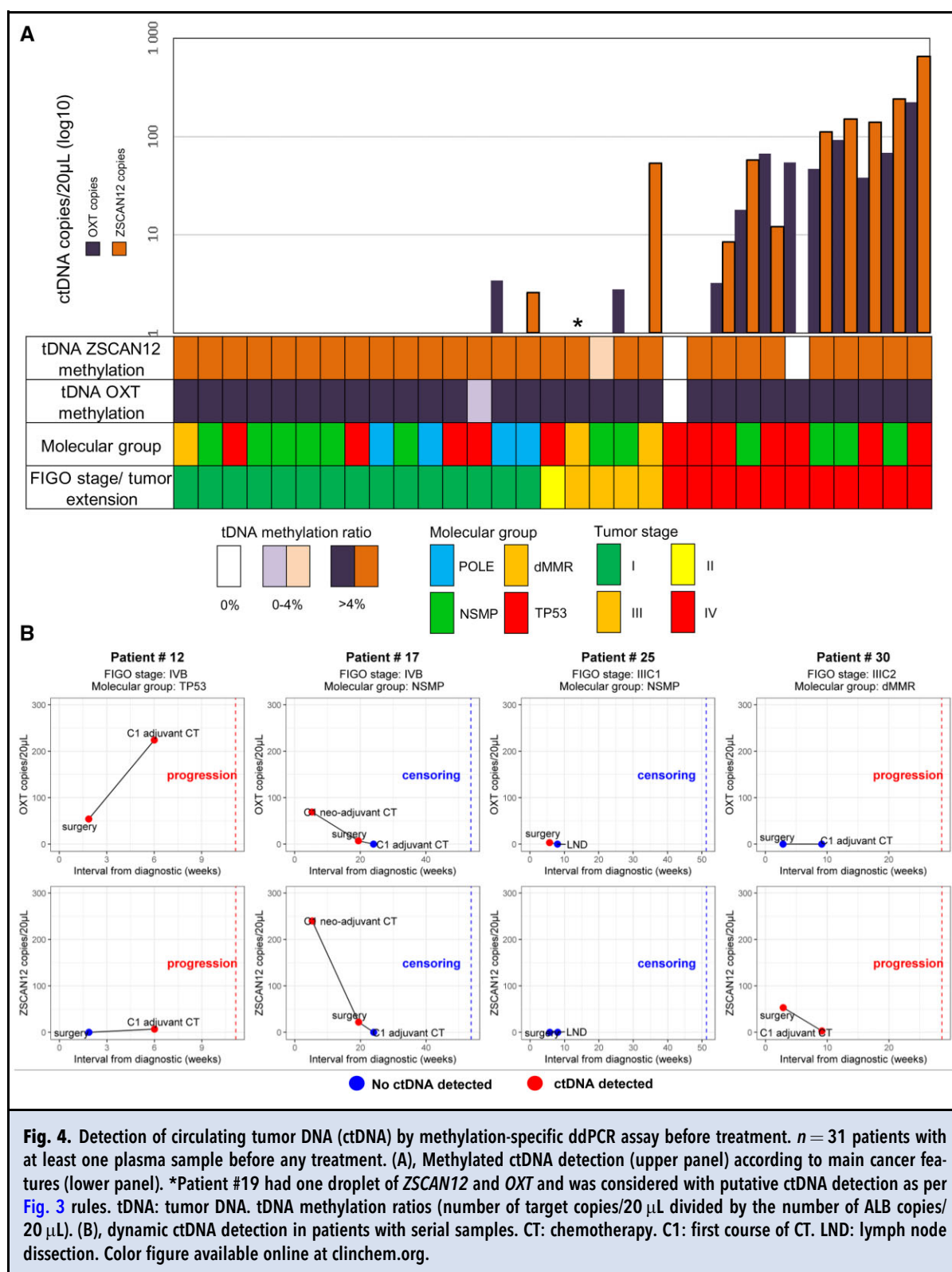
^bStatistics presented: n (%) and median (interquartile).

^cStatistical tests performed: Fisher's exact test.

^dPatients #9 and #10 had first plasma available after initial surgery. FIGO: International Federation of Gynecology and Obstetrics (FIGO) v.2010 staging system.

^ePatient #6 with a double-unmethylated tumor. Metastatic sites considered in this table are anatomic sites affected by macroscopic lesions as per surgical exploration and/or imaging. LVSI: substantial lymphatic or vascular space involvement. tDNA: tumor DNA.

POLE: POLE-mutated tumors. dMMR: mismatch-repair deficient tumors. NSMP: no specific molecular profile. TP53: TP53-mutated tumors. (Supplemental Method 3 for details on molecular classification). NACT: neoadjuvant chemotherapy. CT: chemotherapy. L1/2/4: indicate the number of the chemotherapy line at plasma collection (all samples at first course).



involvement. ctDNA was detected across all histological types, stage, and molecular groups. Importantly, ctDNA was detected after surgical management only in the subset of patients who experienced subsequent early tumor relapse/progression.

Breast, ovarian, and EC share genomic features, which is consistent with a shared hypermethylation pattern (22). *ZSCAN12* belongs to a family of transcription factors involved in the regulation of multiple key hallmarks of cancers (34). Its silencing by promoter methylation appears consistent with the neoplastic transformation. Conversely, *OXT*, a gene encoding the oxytocin/neurophysin I prepropeptide (35), showed constitutively low expression levels in uterine and ovarian tissues, as previously described (35, 36). Its promoter hypermethylation in EC could occur as a “silent” or “passenger” methylation of a gene constitutively silenced by other processes related to chromatin remodeling (33). This “silent/passenger” methylation could be associated with the wide increase in expression level of *DNMT3*. Interestingly, other neuropeptides have been found as widely hypermethylated in other tumor types, such as *NPY* in colorectal cancer (32).

A number of issues have been found in ddPCR assay development, leading to actualization of the dMIQE guidelines in 2020 (20), strictly followed in the present work. In addition to general recommendations, our work points to specific methodological parameters to consider for the development of methylation-specific ddPCR assays for ctDNA detection. First, relying on multiple in silico genome-wide methylation analyses of WBC DNA and normal tissue DNA allowed a high analytical ddPCR specificity, supporting the reliability of ctDNA detection even at very low fractions. The enrichment of primers/probes sequences in CpG could have also contributed to the ddPCR specificity. Second, beyond limit of blank/limit of detection, we experimentally identified an absolute lower threshold of detection, allowing us to confidently consider a sample as with or without ctDNA.

In addition to previous findings (15, 16), we report evidence that methylation-specific-ddPCR could allow (i) dynamic and absolute quantification of ctDNA, regardless of the amount of ccfDNA, (ii) ctDNA detection after initial management for minimal residual disease assessment, and (iii) universal ctDNA detection across all molecular subgroup, histological types, and stage. Beyond plasma DNA, the high diagnostic specificity of *OXT/ZSCAN12* could find application in urine or cervical scrapings (37), particularly for the purpose of cancer screening. The low frequency of ctDNA detection in stage I cancers is consistent with the expectedly very low risk of distant relapse in this setting. Innovations in ccfDNA extraction or bisulfite conversion may lead to future improved detection performance.

Some limitations should be mentioned. Despite the use of a bisulfite-conversion method with high DNA recovery (31), the detection of ctDNA can be impaired by the fragmentation of ccfDNA due to the conversion temperature, and by the inherent fragmentation of ctDNA itself (38). New enzymatic processes have been developed and showed improved performances for methyl-sequencing (39). Whether this innovation would improve ctDNA detection by ddPCR remains to be confirmed. Knowing the minimal achievable absolute lower limit of detection remains uncertain. Hence, the 100 copies of *ALB* detected from 1 ng of ccfDNA (Supplemental Fig. 10, B) indicate a theoretical loss of detection of approximately 37% due to the bisulfite conversion and/or the constitutive ccfDNA fragmentation. Considering the *ALB* primer/probe amplifying only the sense DNA strand, 160 copies would be expected (40). Preanalytical heterogeneity between cancer patients and controls may have introduced bias, despite the amount of *ALB* copies/20 μ L analyzed in controls allowed to reach a median of <0.37% analytical sensitivity. Finally, our results show that an important parameter limiting analytical sensitivity is the yield of ccfDNA extraction itself. Overall, the technical lower threshold of detection established at 50 pg of methylated DNA control supports that even the detection of very low fraction of methylated target is indicative of a large amount of circulating methylated target.

In conclusion, we report a bioinformatical and technical framework allowing the development and validation of a highly analytically sensitive and specific ddPCR assay for ctDNA detection in plasma from patients with EC. This represents a promising approach for quantitative and dynamic ctDNA detection all along the therapeutic management of patients with EC, including minimal residual disease assessment, particularly for patients with stage III–IV EC.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

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