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TUMOR MARKERS AND SIGNATURES

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Noninvasive detection of microsatellite instability in patients with endometrial cancer

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Abstract

The analysis of mismatch repair proteins in solid tissue is the standard of care (SoC) for the microsatellite instability (MSI) characterization in endometrial cancer (EC). Uterine aspirates (UAs) or circulating-DNA (cfDNA) samples capture the intratumor heterogeneity and provide a more comprehensive and dynamic molecular diagnosis. Thus, MSI analysis by droplet-digital PCR (ddPCR) in UAs and cfDNA can provide a reliable tool to characterize and follow-up the disease. The UAs, paraffin-embedded tumor tissue (FFPE) and longitudinal plasma samples from a cohort of 90 EC patients

Abbreviations: cfDNA, circulating free DNA; CRC, colorectal cancer; ctDNA, circulating tumor DNA; CTX, carboplatin-paclitaxel; ddPCR, droplet-digital PCR.; dMMR, deficient mismatch repair; EC, endometrial cancer; EEC, endometrioid endometrial cancer; FFPE, formalin-fixed paraffin-embedded; IHC, immunohistochemistry; ITH, intratumor heterogeneity; ITP, immunotherapy; LoB, Limit of Blank; LoD, Limit of Detection; MSI, microsatellite instability; MSS, microsatellite stable; NEEC, non-endometrioid endometrial cancer; PD, progression disease; pMMR, proficient mismatch repair; RD, residual disease; RTX, radiotherapy; UA, uterine aspirate; VAF, variant allelic frequency.

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were analyzed using ddPCR panel and compared to the SoC. A high concordance (96.67%) was obtained between the analysis of MSI markers in UAs and the SoC. Three discordant cases were validated as unstable by ddPCR on FFPE samples. Besides, a good overall concordance (70.27%) was obtained when comparing the performance of the ddPCR assay on UAs and cfDNA in high-risk tumors. Importantly, our results also evidenced the value of MSI analysis to monitor the disease evolution. MSI evaluation in minimally invasive samples shows great accuracy and sensitivity and provides a valuable tool for the molecular characterization and follow-up of endometrial tumors, opening new opportunities for personalized management of EC.

KEYWORDS

cfDNA, ctDNA, disease monitoring, endometrial cancer, liquid biopsy, microsatellite instability, uterine aspirate

What's new?

The analysis of mismatch repair proteins in solid tumor tissue is the current standard of care for microsatellite instability (MSI) characterization in endometrial cancer. Our study demonstrates the accuracy and advantages of MSI analysis by droplet-digital PCR in uterine aspirates and plasma samples from endometrial cancer patients. The new approach allows comprehensive tumor genotyping without the need for invasive procedures and improves the rate of MSI detection. The assay also has the capacity of monitoring tumor evolution in both localized and metastatic disease by assessing MSI markers. The approach could be easily implemented into the clinic as a follow-up tool.

1 | INTRODUCTION

Microsatellite instability (MSI) represents a clinically relevant molecular alteration in endometrial cancer (EC) and other tumors.[1] Microsatellites are short-tandem repeats observed in coding and noncoding regions throughout the human genome.[2,3] DNA polymerases are prone to introduce errors in these regions, generating mismatched DNA strands. Tumors with a deficiency in any of the main proteins of the DNA mismatch repair (MMR) pathway are characterized by the presence of MSI.[4]

Moreover, the MMR deficiency resulting from germline mutations or epigenetic alterations in any of the MMR genes, as well as deletions in the *EPCAM* gene, are the principle cause of Lynch syndrome and its variants (Muir-Torre or Turcot syndromes).[5] Approximately 20% to 30% of sporadic ECs are characterized by MSI, mainly caused by somatic promoter hypermethylation and silencing of *MLH1* gene[6] while defective MMR pathway due to Lynch syndrome accounts for 2% of ECs.[7]

Although the diagnosis of MMR-deficiency/MSI in ECs is recommended as an initial step in Lynch syndrome screening algorithms, today this particular molecular feature has also therapeutic implications as predictive biomarker for various anti-PD1 therapies across different tumor types, particularly in colorectal and endometrial cancers.[8,9] Moreover, MSI represents a well-established prognostic biomarker associated in ECs with a better prognosis.[10]

MMR-deficiency can be detected by MSI analysis and/or immunohistochemical (IHC) characterization of the four MMR proteins. IHC has been used as the standard technique in numerous institutions since the loss of MMR protein expression is normally homogeneous and easy to detect.[11] However, despite the concordance between the MMR proteins and the MSI assessment has demonstrated to be robust, some centers combine both tests, since some discordances have been described, mainly due to false positive or negative cases after the IHC analysis.[12]

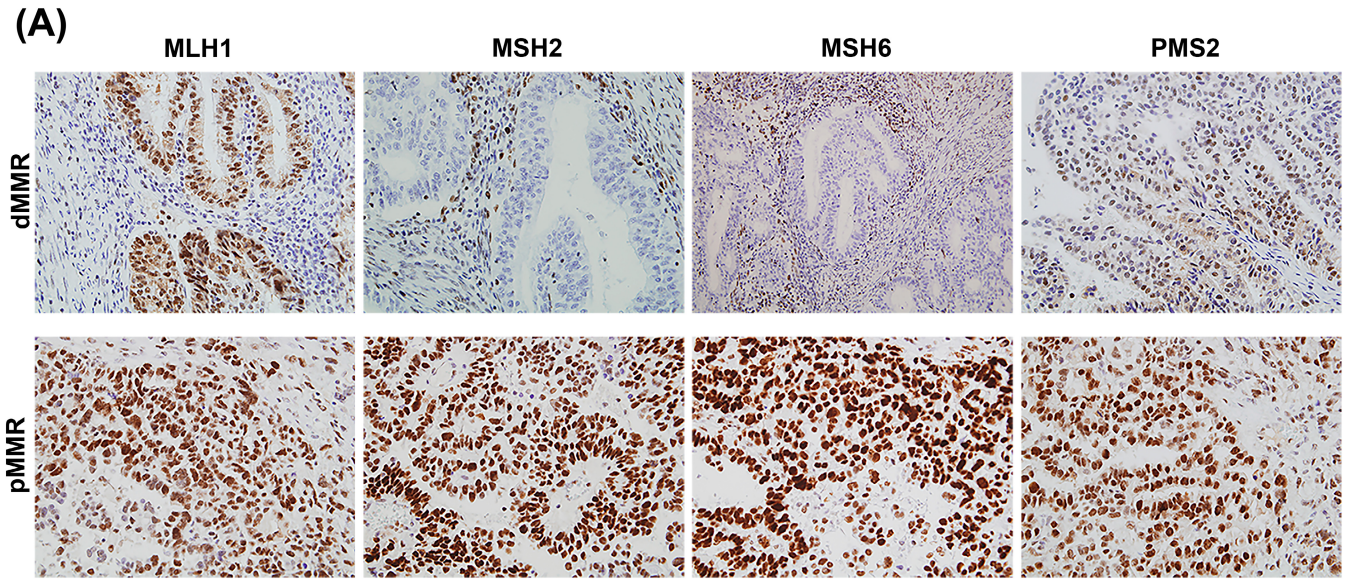
Traditionally, MMR/MSI deficiency is interrogated in solid tissue samples with the limitation of analyzing only a snapshot of the tumor diversity, which is relevant especially in the context of advanced EC.[13,14] Our group has previously demonstrated the value of the genomic characterization of the uterine aspirates (UAs) to capture the intratumor heterogeneity (ITH) and identify potential targeted therapies to treat EC patients.[15] Besides, ctDNA monitoring has shown to be feasible and informative in the context of EC, representing a promising tool to characterize the genetic landscape of endometrial tumors within their temporal evolution.[15,16]

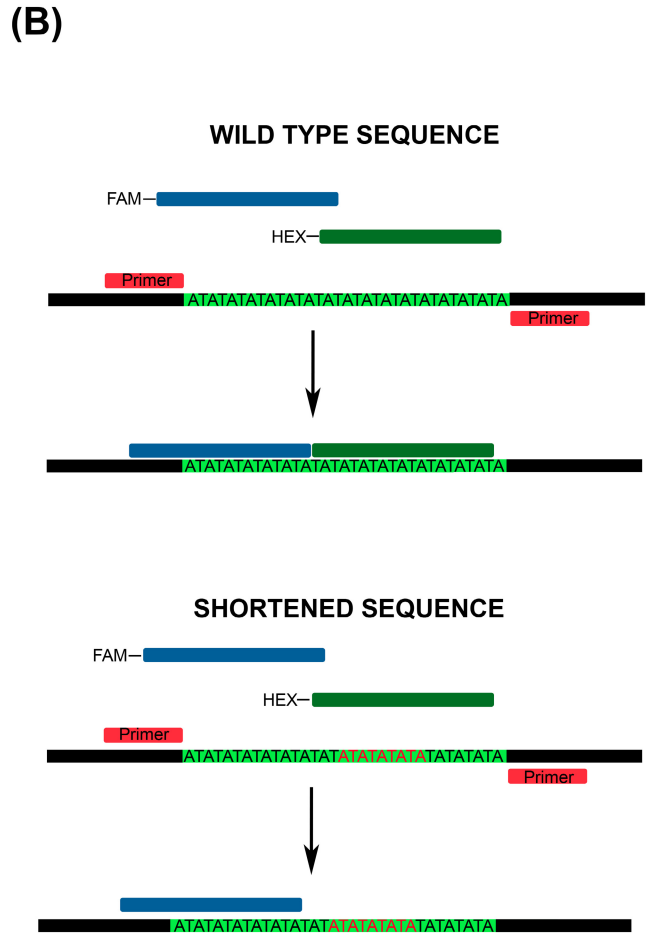
With the goal of translating the MSI testing into minimally invasive scenarios and improving the current diagnostic and monitoring tools, in the present study we sought to validate the analysis of MSI in UAs and cfDNA obtained from patients with EC through the application of a high-sensitive droplet digital PCR (ddPCR) assay.

2 | METHODS

2.1 | Patient inclusion and samples collection

A total of 90 patients with EC were recruited between January 2018 and June 2021 at the Gynecology Department of Vall





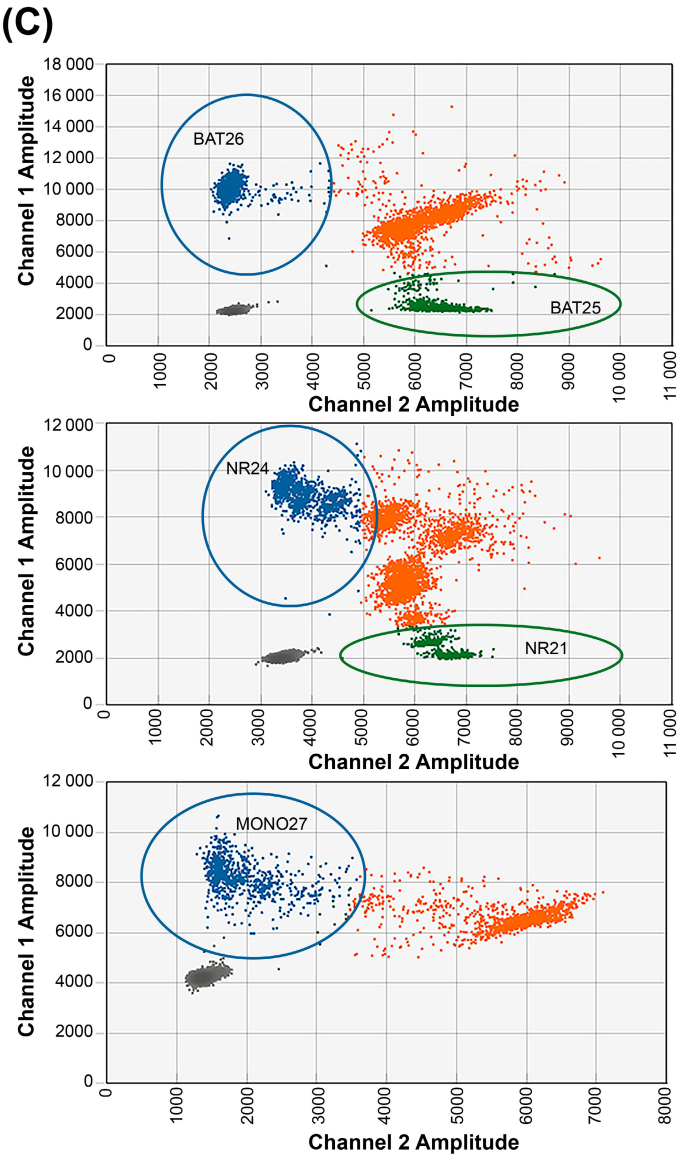


FIGURE 1 Legend on next page.

d'Hebron University Hospital (Barcelona, Spain), the MD Anderson Cancer Center (Madrid, Spain) and the University Clinical Hospital of Santiago de Compostela (Santiago de Compostela, Spain). Patients were included in the study if the following criteria were met: (a) Patients were diagnosed with endometrial carcinoma of any histology (excluding sarcomas or carcinosarcomas). (b) Patients were over 18 years old. (c) Patients signed informed consent. (d) The status of the MMR proteins was known. (e) There was availability of UA. (f) Patients were not undergoing antitumoral treatment at the time of sample collection. (g) Patients did not have any nonbenign tumor within the last 5 years before the sample collection.

All UAs were collected at surgery using a Cornier canula and processed as previously described.[15] Peripheral blood samples were collected at surgery and during the patients' follow-up (every 6 months until recurrence/progression of the disease) using CellSave Preservative tubes (Silicon Biosystems Inc, Huntingdon Valley, PA). Plasma was isolated by a two-step centrifugation. Formalin-fixed paraffin-embedded (FFPE) tumor tissue resected after hysterectomy was obtained from the Pathology Departments of the participating hospitals. FFPE samples were selected by the pathologist as of being representative of the tumor of origin.

2.2 | Immunohistochemistry characterization

The immunohistochemistry characterization of all samples was performed at the Pathology Services from the hospitals involved in the study. Sections (4 μm thick) from formalin-fixed paraffin-embedded (FFPE) tumor tissue were automatically stained in a Dako Omnis immunostainer (Dako-Agilent, Santa Clara, CA). Briefly, the immunohistochemical protocol included the following steps: (a) heat-induced epitope retrieval solution at high pH (Dako-Agilent) for 20 minutes at 97°C; (b) incubation with ready to use FLEX primary monoclonal antibodies (Dako-Agilent) which recognize MLH1 (mouse antibody, clone ES05), MSH2 (mouse antibody, clone FE11), MSH6 (rabbit antibody, clone EP49) and PMS2 (rabbit antibody, clone EP51) for 20 minutes; (c) mouse and rabbit linker (Dako-Agilent) for 10 minutes each; (d) EnVision FLEX/HRP (Dako-Agilent) for 20 minutes; (e) 3,3′-diaminobenzidine tetrahydrochloride chromogen solution (Dako-Agilent) for 5 minutes and (f) EnVision FLEX hematoxylin (Dako-Agilent) for 5 minutes. Nuclear negativity for MLH1, MSH2, MSH6 or PMS2 in tumor cells implies a genetic or genomic alteration of the corresponding DNA mismatch repair genes (Figure 1A). Adjacent normal glands and surrounding lymphocytes were employed as internal positive controls.

2.3 | Cell lines culture

The endometrial cancer cell lines HEC-1-A (RRID:CVCL_0293) and AN3-CA (RRID:CVCL_0028) were maintained in a humidified atmosphere at 37°C and 5% CO_2. HEC-1-A and AN3-CA were cultured in Dulbecco's Modified Eagle's Medium-Nutrient Mixture F-1 Ham2 (Sigma-Aldrich, St. Louis, MO) supplemented with 1% penicillin-streptomycin (Gibco, Waltham, MA) and 10% fetal bovine serum (Gibco, Waltham, MA). All experiments were performed with mycoplasma-free cells. All human cell lines have been authenticated using STR (or SNP) profiling within the last 3 years.

2.4 | DNA extraction

DNA isolation from the cell cultured pellets was performed using the QIAamp DNA Mini and Blood (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. The DNA and RNA from the UA were obtained using RecoverAll Total Nucleic Acid Isolation Kit (Thermo Fisher) following the manufacturer' conditions. After extraction, a cleaning with Gene JET RNA cleanup and Concentration Micro Kit (Thermo) was done. The quantification of DNAs and RNA were performed using NanoDrop 2000 Spectrophotometer and Qubit 3.0 Fluorometer using Qubit BR Assay Kit (Life Technologies). For the analysis of fusions, 200 ng of RNA was used for the cDNA synthesis using SuperScript IV VILO Master Mix (Invitrogen). DNA from plasma samples was extracted with the QIAamp DNA Circulating Nucleic Acid Kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions All cfDNA extractions were performed using the same volume of plasma (5 mL) and the concentration was normalized based on the extraction volume and the elution volume. Samples were stored for a maximum of 6 months before the cfDNA was isolated. DNA from FFPE samples was isolated using the AllPrep DNA/RNA FFPE Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. All DNA samples were quantified using the Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA) and stored at −20°C until use.

2.5 | Next-generation-sequencing analysis

The UAs were subjected to targeted sequencing the Oncomine Comprehensive Panel v3 (Thermo Fisher, Pleasanton, CA) according to the manufacturer's instructions. Out of the 60 patients, 35 were already published.[15] The sequencing coverage and quality statistics for each new sample are summarized in Table S1.

FIGURE 1 (A) Representative images of immunohistochemistry staining for the mismatch repair proteins MLH1, MSH2, MSH6 and PMS2 (×60) in one case MMR proficient (pMMR down image) and one case MMR deficient (dMMR, up image). (B) Schematic representation of BioRad's MSI determination panel, two probes recognize the sequence of interest, one marked with FAM and one with HEX, whenever there is a reduction in the sequence, one of the probes will not be able to attach. Therefore, identifying the altered sequences with only one probe. (C) Results of the MSI assay (assessment of the mononucleotide repeats BAT26, BAT25, NR24, NR21 and Mono27) by ddPCR on the UA obtained from the patient with the dMMR tumor described in A

Briefly, 10 ng of both DNA and cDNA from each uterine aspirate were used for library assembly in a multiplex PCR in an AB 2720 Thermal Cycle (Life Technologies) following the manufacturer' conditions. For PCR, a total of 18 and 20 cycles were performed. After PCR amplification, primary primers were partially digested using FuPa reagent (Thermo Fisher). For the identification of each analyzed sample was used Ion P1 Adapter and Ion Xpress Barcode X which were combined and used for the amplicon ligation (22°, 30 minutes; 72°, 10 minutes; 10°, hold). Libraries were purified and then quantified using the Ion Library TaqMan Quantitation Kit and ViiA 7 system. For this purpose, libraries were diluted (1:50) to reach a concentration within the range of the *Escherichia coli* DH10B Control Library standards. This Control Library is a validated, prequantified, ready-to-use standard that was serially diluted (from 6.8 to 0.00068 pM), generating a standard curve for the qPCR. Relative concentration of the sample libraries to the Control Libraries were obtained from the qPCR analyses. Template preparation and enrichment were performed with the Ion S5 XL system. Libraries were diluted to reach a final concentration of 100 pM in nuclease-free water. Libraries with different barcodes were combined with the following ratio: 4 mL from DNA library and 1 mL from cDNA library. Next, diluted libraries were mixed with template-positive Ion Sphere Particles (ISPs) and Ion S5 enzyme mix (ThermoFisher) to perform an emulsion PCR following the manufacturer's protocols and finally, the enrichment was carried out on the Ion OneTouch 2. Targeted massive sequencing was performed using S5 sequencer[17] (ThermoFisher) following manufacturer's protocols. A range of six libraries (RNA and DNA) were run in 540 chips (ThermoFisher). Duplicates were analyzed for 10% of the samples and found to yield equivalent results. For the bioinformatics analysis, alignment to the Hg19 human reference genome and variant calling were performed with Torrent Suite Software v.15.1 (Life Technologies, Santa Clara, CA). All samples were sequenced and analyzed in comparable conditions. Variants with a Phred quality score field value <100 were considered as low-quality variants. The prediction of genomic variant effects on protein function was performed with the PROVEAN Genome Variants tool (http://provean.jcvi.org/index.php) and Alamut Visual Plus. Variants with possibly damaging or deleterious consequences, as predicted by at least one of the PROVEAN predictors, were considered to be of interest and were visually checked with Integrative Genomics Viewer (IGV) v.2.3.40, Broad Institute. Variants with a global minor allele frequency above 0.05 were considered as single nucleotide polymorphisms and were rejected (data from dbSNP, [http://www.ncbi.nlm.nih.gov/SNP/]).

2.6 | ddPCR analysis

The MSI status was interrogated in UAs, cfDNA and FFPE samples with the MSI multiplex assay (Bio-Rad, CA) and run on the QX-200 ddPCR system (Bio-Rad, CA) using TaqMan chemistry. The assay consists in two probes competing for the same target sequence. Depending on the alteration one or the other probe is out-competed, indicating MSI. It can be distinguished between mutant (shortened) and wild-type alleles if the length difference is at least two base pairs in the microsatellite repeat region, therefore it does not require the usage of a paired normal sample (Figure 1B). All assays were run in triplicates with at least 30 ng of DNA being analyzed per sample, and, to assure their performance, the analytical sensitivity was estimated using serial dilutions of DNA obtained from HEC-1-A and AN3-CA cell lines in a background of WT DNA, with variant allelic frequencies (VAFs) ranging from 100% to 0.1%. The Pearson correlation between the estimated and experimental mutant copies detected was calculated to determine the sensitivity of the assay (Figure S1A; Pearson's $R \geq .99$; P-value <.001). An analytical LoB was established at 0.03% and a LoD of 0.1% for all assays according to the approved guidelines.[18]

PCR was performed with the ddPCR Supermix for probes (Bio-Rad, Santa Rosa, CA). The sample was partitioned into a median of 50 000 droplets (run in triplicates) in an automated droplet generator (Bio-Rad, CA), according to the manufacturer's instructions. Emulsified PCR reactions were run on 96-well plates on a C1000 TouchTM thermal cycler (Bio-Rad, CA) by incubating the plates at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds; 55°C for 60 seconds; and 98°C for 10 minutes. The temperature ramp increment was 2.5°C/s for all steps. Plates were read on a Bio-Rad QX-200 droplet reader with Bio-Rad's QuantaSoft v1.7.4 software to quantify the number of droplets positive for mutant DNA, wild-type DNA, both and neither. Analysis was performed manually by two independent molecular biologists according to the following guidelines: a minimum of 30 000 positive droplets across wells were required for a valid assay, and a minimum of five, single FAM-positive or HEX-positive droplets with no positive events in the WT control were required to consider samples as mutated (Figure 1C).

2.7 | Statistical analysis

Statistical analysis was conducted in R (R Core Team, 2020) and figures were generated using ggplot2[19] and GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA). Kappa Cohen analysis was used to evaluate the concordance between the results obtained in UAs (ddPCR), FFPE tissues (IHC and ddPCR) and cfDNA (ddPCR). Wilcoxon's signed-rank test was used to evaluate the differential VAFs between the UA and FFPE. The Pearson correlation test was performed to determine the relationship between quantitative experimental and clinical variables. The Spearman correlation test was performed to determine the relationship between experimental non-parametric variables. A P-value <.05 was set as the level of statistical significance.

3 | RESULTS

3.1 | ddPCR-based analysis of MSI in uterine aspirates and FFPE tissue improves the instability detection in comparison with IHC study

The MSI status was analyzed on the UAs of 90 patients with EC using a multiplex ddPCR assay which interrogates the status of five

TABLE 1 Clinico-pathological features of the EC cohort included in the study

Feature	pMMR, n = 35 (38.89%)	dMMR, n = 55 (61.11%)	Total, n = 90 (100.00%)
Time of diagnosis			
First diagnosis	33 (94.28%)	54 (98.18%)	87 (96.67%)
Recurrence	2 (5.71%)	1 (1.82%)	3 (3.33%)
Histology			
Endometrioid	21 (60.00%)	48 (87.27%)	69 (76.67%)
Non-endometrioid	14 (40.00%)	7 (12.73%)	21 (23.33%)
Grade			
G1	7 (20.00%)	11 (20.00%)	18 (20.00%)
G2	9 (25.71%)	20 (36.36%)	29 (32.22%)
G3	19 (54.29%)	24 (43.64%)	43 (47.78%)
FIGO stage			
I	17 (48.57%)	31 (56.36%)	48 (53.33%)
II	4 (11.43%)	12 (21.82%)	16 (17.79%)
III	12 (34.29%)	9 (16.36%)	21 (23.33%)
IV	2 (5.71%)	2 (3.64%)	4 (4.44%)
na	0 (0.00%)	1 (1.82%)	1 (1.11%)
Myometrial infiltration			
<50%	8 (22.86%)	22 (40.00%)	30 (33.33%)
≥50%	27 (77.14%)	33 (60.00%)	60 (66.67%)
Risk of recurrence			
Low/intermediate risk	10 (28.57%)	23 (41.82%)	33 (36.67%)
High risk	25 (71.43%)	32 (58.18%)	57 (63.33%)

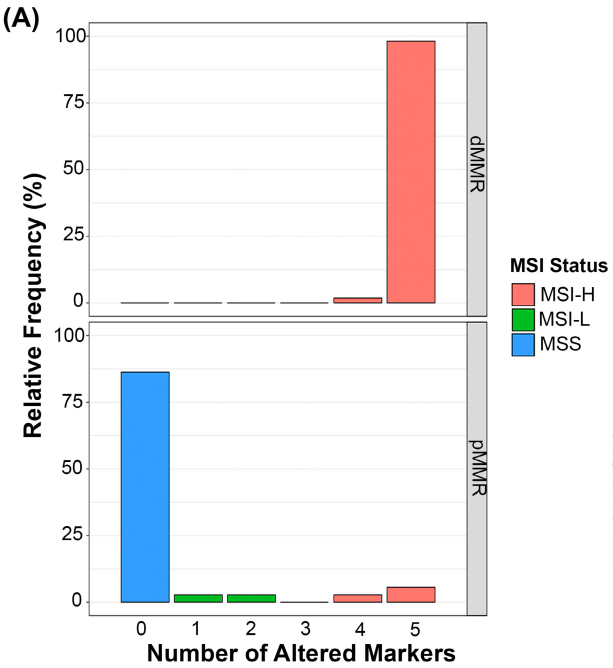
Abbreviations: dMMR, deficient mismatch repair proteins; pMMR, proficient mismatch repair proteins.

mononucleotide markers (BAT26, BAT25, NR24, NR21 and Mono27) (Table 1). The cohort included low to high risk of recurrence, Grades 1 to 3, FIGO I to IV patients (Table 1), being most of the cases endometrioid (EEC) tumors (76.6%), Grade 3 (47.8%) and high risk of recurrence (63.3%). Patients were classified as either low, intermediate or high risk of recurrence, being the patients with non-endometrioid (NEEC) histology, EEC histology Grade 3 with FIGO stage IB-IV or EEC Grades 1 and 2 with FIGO stages II to IV classified as of high risk; EEC with Grade 3 FIGO stage IA or EEC with Grades 1 and 2 FIGO stage IB, as intermediate and EEC with Grades 1 and 2 and FIGO stage IA as of low risk of recurrence, according to the ESGO/ESTRO/ESP guidelines.[20]

Cases with at least three markers altered were classified as MSI high (MSI-H), those with one or two markers altered as MSI low (MSI-L) while stable cases (MSS) were those without any marker altered.[21-24] Thus, after the analysis, we identified 64.45% (58/90) of the cases as MSI-H, 2.22% (2/90) as MSI-L and 33.33% (30/90) as MSS. All the questioned markers showed similar levels of variant allelic fractions (VAFs) within samples, except for the mononucleotide repeat NR21 that showed a lower level of alteration in most of the cases (Wilcoxon Signed-Rank test: *P*-value ≤.001; Figure S2A).

Importantly, when the results obtained with the MSI test in the UAs were analyzed based on the SoC, the MMR proteins status by IHC in solid biopsies, 91.42% (32/35) of pMMR cases showed <3 MSI markers altered (5.71%, 2/35 MSI-L and 85.71%, 30/35 MSS) while three pMMR cases (8.58%, 3/35) were classified as MSI-H. Of note, all patients with dMMR showed three or more MSI markers altered (55/55) (Figure 2A,B). Therefore a high global agreement of 96.67% (Kappa Cohen = 0.93) was obtained between the MSI results in the UAs and the MMR proteins status in tissue (Figure 2D). Indeed, when analyzing the cohort based on clinical variables, the agreement was over 91% (Kappa Cohen >0.81) in all the clinico-pathologic groups (Figure 2D).

Additionally, to further validate the assay, DNA from FFPE tumor tissue was also analyzed using the ddPCR assay and the results were compared to those obtained on UAs samples with the same assay. A global concordance of 100% was reached (Kappa Cohen = 1.00) between both samples, including the three discordant cases (MSI-H in UAs and pMMR with the IHC) found when comparing the MSI panel and the proficiency of the MMR proteins (Figure 2C). Of note, one of the discordant cases had focal expression of PMS2 and MLH1 in the IHC analysis, indicating that molecular heterogeneity could explain this discordant case. In the other two cases an abnormal preserved antigenicity could be behind the false negative for MSI. Therefore, by analyzing the MSI status in UAs or FFPE tissues with the ddPCR assay we were able to reclassify three cases determined as stable by the SoC, showing the advantage of the ddPCR strategy in comparison with the IHC. Moreover, the mean VAF of the MSI markers was



(B)

UA (ddPCR)	FFPE (IHC) dMMR	FFPE (IHC) pMMR
MSI-H	55	3
MSI-L/MSS	0	32

Overall agreement: 96.67%
Positive agreement: 94.83%
Negative agreement: 100.00%

(C)

UA (ddPCR)	FFPE (ddPCR) MSI-H	FFPE (ddPCR) MSI-L/MSS
MSI-H	41	0
MSI-L/MSS	0	20

Overall agreement: 100.00%
Positive agreement: 100.00%
Negative agreement: 100.00%

(D)

Molecular Classification	n	Agreement	Kappa	95% CI
Risk of Recurrence				
Low/Intermediate risk	33	96.96%	0.93	[0.78-1.00]
High risk	57	96.49%	0.93	[0.83-1.00]
Histology				
Endometrioid	69	95.65%	0.89	[0.78-1.00]
Non-Endometrioid	21	100.00%	1.00	[1.00-1.00]
Grade				
G1-G2	47	100.00%	1.00	[1.00-1.00]
G3	43	93.02%	0.86	[0.70-1.00]
FIGO Stage				
FI-FII	64	98.44%	0.96	[0.89-1.00]
FIII-FIV	25	92.00%	0.84	[0.63-1.00]
Myometrial Infiltration				
≤50%	30	100.00%	1.00	[1.00-1.00]
>50%	60	95.00%	0.90	[0.79-1.00]
Overall Concordance	90	96.67%	0.93	[0.85-1.00]

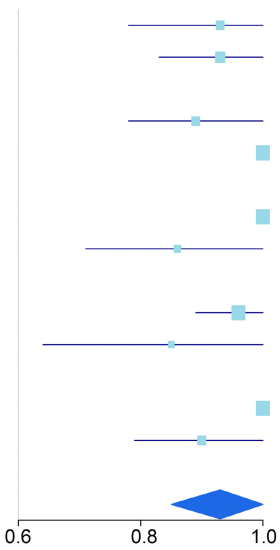


FIGURE 2 (A) Distribution of the number of altered mononucleotide repeats identified by ddPCR in the UAs of the cohort of 90 EC patients based on the MMR status, determined by IHC in tissue samples. All dMMR cases were classified as MSI-H using the ddPCR assay while three MSI-H UAs were classified as pMMR by IHC. (B) Concordance table between the UAs analyzed by ddPCR and the FFPE analyzed by IHC. (C) Concordance table between the UA analyzed by ddPCR and the FFPE analyzed by ddPCR. (D) Concordance analysis comparing the results obtained after the MSI-panel assessment on UAs and the FFPE analyzed by IHC accordingly to the different clinic-pathologic characteristics. The concordance was analyzed with the Kappa-Cohen statistics

significantly higher in the UAs (Wilcoxon Signed-Rank test: *P*-value ≤.05), except for the case of NR21, suggesting the presence of a higher tumoral content in the UA compared to the FFPE tumor tissue (Figure S2A). Furthermore, the VAF levels of each mononucleotide marker found in the UA showed a good correlation with its corresponding FFPE sample (Figure S2A,B; Spearman's test: $R \geq .6$, *P*-value <.001).

3.2 | VAFs of the five MSI markers correlates with the VAFs of point mutation identified by targeted sequencing in the UAs

In addition to the analysis of the five MSI markers, in 60 patients (26 MSI-L/MSS and 34 MSI-H) of the cohort we had data regarding the genomic landscape of the UAs (Table S2) obtained after their

analysis with targeted sequencing as previously published by our group.[15] Importantly, in 8 of the 34 MSI-H patients a mutation in *MLH1* (3/8), *MSH2* (3/8), *PMS2* (1/8) or *MSH6* (1/8) was found, indicating a genomic origin of the MMR deficiency in these 8 cases and a possible epigenetic regulation in the remaining cases. In fact, the methylated status of *MLH1* was studied and defined as positive in three of the cases without mutation in the MMR genes.

Besides, genes enriched in MSI-H tumors included *PTEN* (21.7%), *PIK3CA* (13.8%), *ARID1* (9.2.%), *KRAS* (7.2%) and *TP53* (5.9%) as the most frequently altered genes. Those enriched in MSI-L/MSS tumors included *PIK3CA* (15.2%), *PTEN* (12.4%), *TP53* (11.4%), *PIK3R1* (7.6%) and *ARID1* (6.7%) (Figure S3A,B). Although PI3K-AKT pathway mainly supports the impact of the common alterations between the two groups the alteration of *RAS* family genes has been previously associated with MSI-H endometrial tumors, as also shows our cohort of patients.[25]

Additionally, to determine if the levels of the MSI markers and if the variants identified after sequencing correlate and equally represent the tumor content of the samples, we compared the VAFs value of each nucleotide marker in the UA and the average VAF value of the pathogenic variants using in both cases the same DNA input (Figure S3C). A strong correlation was found between BAT26 and BAT25 and the NGS analysis (Spearman's test: $R \geq .6$, P-value $\leq .01$), a moderate correlation with NR24 (Spearman's test: $R = .5$, P-value $\leq .05$) and no correlation was found with NR21 and Mono27 (Spearman's test: $R \leq .4$, P-value $\leq .05$). Although the comparison of different technologies such as the ddPCR and NGS render different VAFs, even when analyzing the same variant, these results agree with the fact that alterations in MSI markers, normally represent an early event during EC development.

3.3 | cfDNA represents a valuable sample to evaluate the MSI status and monitor the disease evolution

The cfDNA was isolated from plasma samples of 59 patients and analyzed for its potential usage as a diagnostic and monitoring tool. First, the LoD of the assay was determined using dilutions of DNA from endometrial cancer cell lines with known pathogenic mutations and MSI. Our study determined a LOD = 0.1% VAF and a good correlation between the MSI markers and point mutations, as we also observed when analyzing the UAs (Figure S1A,B). Importantly, in 47 of the 59 samples, we had data regarding the presence of detectable ctDNA based on the presence of patient-specific point mutations or CNVs, previously identified on the UAs.[15]

Of note, most of the plasma samples (57/59) analyzed were collected at surgery and the remaining samples were collected at the time of tumor progression (2/59) (Table S3). When considering the threshold of ≥3 markers altered to define MSI-H cases, the overall agreement between the results on the UAs and the cfDNA was 50.85% (Kappa Cohen = 0.21; Figure S4A). Of note, the concordance agreement was higher in patients with a more aggressive phenotype: high-risk of recurrence (70.27%; Kappa Cohen = 0.45); high myometrial infiltration (61.11%; Kappa Cohen = 0.32); non-endometrioid histology (73.33%; Kappa Cohen = 0.44) and patients previously classified as positive for ctDNA (n = 18) based on the presence of patient-specific somatic point mutations or CNV (76.47%; Kappa Cohen = 0.5; Figure S4C).

We also focused on the detection rate in patients classified as MSI-H (n = 43) based on the ddPCR panel applied in UAs, showing that 60.46% had ≥1 marker altered when interrogating the corresponding cfDNA (Figure 3A). Furthermore, when patients were categorized based on their clinic-pathologic features, a clear correlation between the percentage of detection and the more aggressive phenotypes was found. Thus, 70.83% of patients with high risk of recurrence showed at least one mononucleotide marker altered and three mononucleotide markers altered in 54.16%, respectively (Figure 3A). Overall, higher positivity was detected in patients with high myometrial infiltration, high grade, NEEC histology and higher FIGO stages (Figure 3A). Of note, these ctDNA detection percentages were higher than previously reported in the literature in nonmetastatic EC.[15,26-29] Besides, patients with high-risk tumors showed higher levels of VAFs although the cfDNA levels were comparable between low and high-risk tumors (Figure S5A,B). In addition, a good correlation between the VAF of point mutations, CNVs and the MSI markers (n = 8, Figure S4B) was found.

Furthermore, to assess the potential of the MSI assay to monitor the disease evolution, longitudinal samples from 15 patients were analyzed. Of note, 6 patients had a pMMR/MSS tumor profile and were included to evaluate the potential shift to MSI during the disease evolution. All 6 patients were negative for the presence of ctDNA and remained as MSS in all longitudinal samples, including the four progressions. From the remaining 9 patients, classified as MSI-H, 5 were negative during the longitudinal analysis while 4 progressed after surgery, being the MSI markers detectable after disease progression in all cases (Table S4). Patient no. 1 underwent surgery in 2019, and received postsurgery carboplatin-paclitaxel combined therapy and radiotherapy. Nevertheless, a lung progression was detected by CT-scan 20 months later. Patient was then treated with chemotherapy but showed only partial response with a final tumor progression at brain level. Two MSI markers were detectable after this second progression (Figure 3B). Patient no. 2 was operated and 9 months later showed peritoneal progression and was treated with chemotherapy. As shown in Figure 3C, although the levels at surgery for the MSI markers and two specific point mutations in *CTNNB1* and *PIK3CA* were negative, they increased at progression disease and decrease when treatment started. A lung progression was detected after 4 months of treatment and the MSI markers increased again. In the case no. 3 the levels of ctDNA became detectable using the MSI assay 6 months after surgery, becoming more prominent at 12 months post-surgery, indicating an early progression of the disease which was confirmed by image (Figure 3D). Finally, patient no. 4, was initially diagnosed and underwent surgery in 2020; however, a vaginal lesion was detected shortly after surgery. The patient was treated with chemotherapy and radiotherapy but showed a bad response with the appearance of an abdominal lesion, after the second surgery the patient was subjected to Pembrolizumab treatment; however, as seen

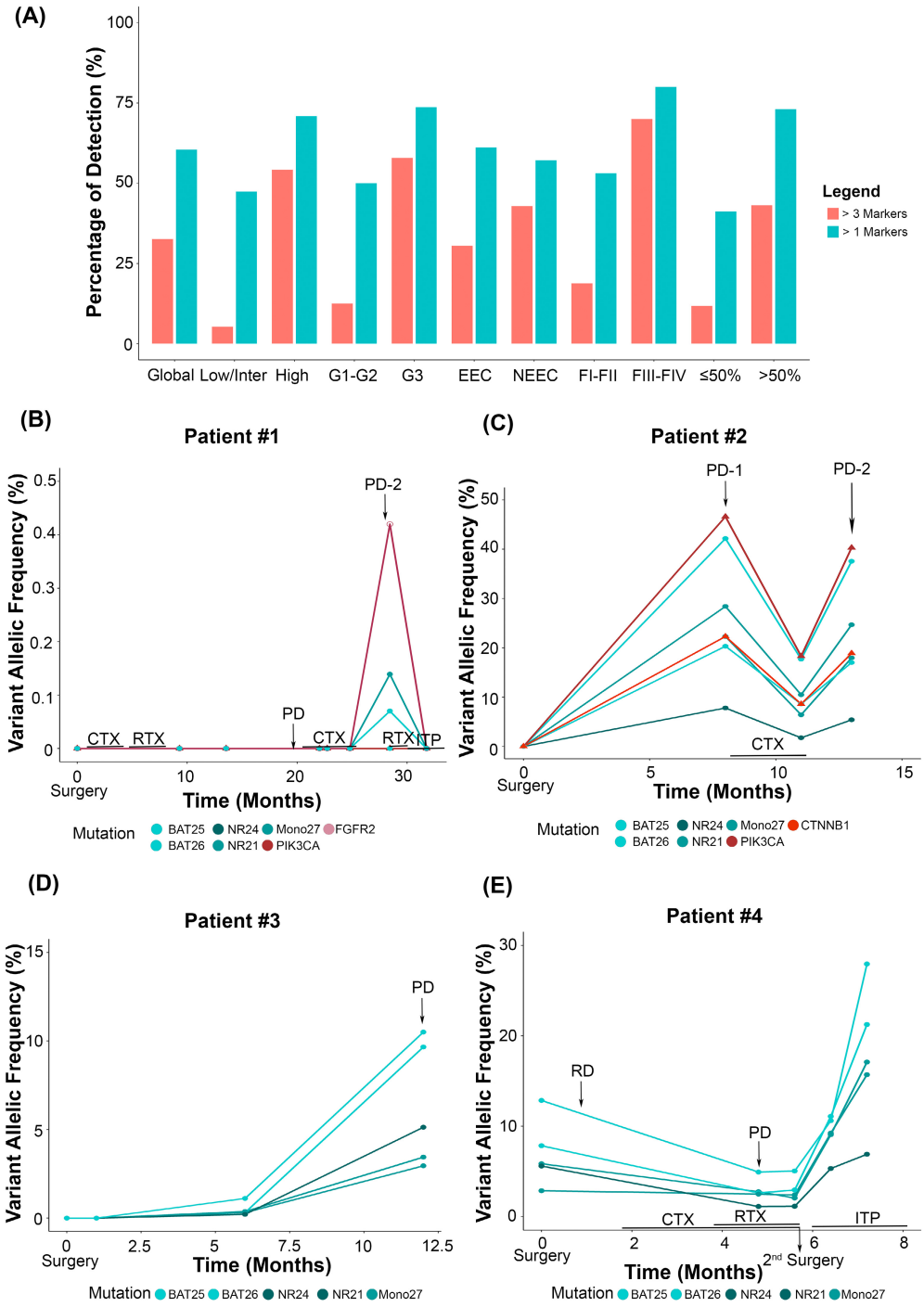


FIGURE 3 (A) Percentage of detection of MSI markers altered on the cfDNA isolated from plasma in 40 patients classified as MSI-H based on the UAs analysis and according to the tumor characteristics. At least three MSI markers altered (red) and one altered (green) were used as the threshold. (B) ctDNA kinetics in patient no. 1 showcasing the value of the MSI assay to detect progression disease. (C) ctDNA dynamics in patient no. 2 mirrored the tumor evolution in response to therapy. (D) ctDNA evolution in patient no. 3 showed potential to detect early progression. (E) ctDNA levels in patient no. 4 evidence the potential of the MSI assay to detect residual disease and progression. CTX, chemotherapy treatment; ITP, immunotherapy; PD, progressive disease; RD, residual disease; RTX, radiotherapy treatment

by the ctDNA kinetics the patient showed no response to treatment. The MSI markers were positive in all samples collected after surgery indicative of persistent disease (Figure 3E).

4 | DISCUSSION

MSI determination is becoming crucial in solid tumors, as it favors the hereditary screening and provides valuable information for the tumor prognosis and immunotherapy selection.[8,30] In fact, results of KEYNOTE-028 (NCT02054806) demonstrated that pembrolizumab was active in advanced PD-L1 positive EC patients progressing after standard therapy[31] providing robust data supporting the use of immunotherapy in EC.

Current strategies for EC diagnosis and molecular characterization are based on the analysis of solid tumor tissue at surgery[32] with the limitations inherent to this sample. Our study was novel to apply a highly sensitive approach, based on ddPCR, to interrogate the MSI status in UAs. The commercial assay employed includes five nucleotide markers (BAT25, BAT26, NR21, NR24 and MONO27) which has been previously applied in EC and other tumors with high accuracy[23] and sensitivity, since they can detect as little as 2 bp difference without

requiring paired normal sample.[33] To validate our approach, the UAs from a total cohort of 90 patients with EC were interrogated using the ddPCR assay, being 64.44% classified as MSI-H after the analysis. In 8 of the 34 of these MSI-H tumors we found a genetic cause of the MSI status, being the remaining cases probably associated to epigenetic regulation, in line with the percentages expected of sporadic and Lynch-associated MSI tumors.[34,35] Importantly, this patients' cohort was enriched in MMR deficient tumors with the purpose to technically validate our analytic approach, therefore, the results obtained should be interpreted taking into account that in a real-world cohort the rate of MSI tumors will be about the 30%.[35] The cohort is also enriched in high-risk tumors since they have a greater clinical interest as potential future recurrent cases that could be treated and monitored based on our non-invasive approach. To interrogate the accuracy of our approach the results obtained with the MSI panel in the UAs were compared to the MMR status determined by IHC in the corresponding solid biopsy, and a high global agreement (96.67%) was observed between both strategies for all the patients, showing the great value of UAs for the MSI testing in EC. We only observed three discordant cases, classified as pMMR by the IHC and as MSI-H by the ddPCR-based approach. The correlation between the MSI and MMR assessment is normally high; however, about 3% to 11% of MSI-H tumors can show conventional MMR staining and localization due to preserved antigenicity and compensation mechanisms.[12,36] Although these discrepancies are relatively low, there may be different clinical behaviors between the group characterized by MSI testing and the group identified by IHC. We confirmed the MSI-H status of these three discordant cases by applying the ddPCR assay in FFPE tissues, evidencing the additional value of assessing MSI to characterize EC; moreover, significantly higher VAFs were found in the UA when compared to the FFPE. Our study demonstrated a total concordance of the MSI assessment in solid tissue samples and UAs, supporting the use of UAs for the MSI determination, as a highly sensitive, fast, cost effective and noninvasive diagnostic strategy for the accurate molecular stratification of EC patients. Although our workflow includes the UAs collection at surgery, this sample could be obtained by the gynecologists without the need of special care and provides relevant information to confirm the diagnostic and surgical management of the patient. For example, in a recent work, a small NGS panel including 60 genes and 17 microsatellite markers was successfully applied to liquid-based cytology specimens obtained for EC screening and the corresponding FFPE tissues in seven patients, showing the same MSI profile.[37] Although MMR/MSI alteration is normally an early event in EC generation, mosaicism has been described in the expression of MMR proteins in about 3% to 5% of the tumors[13,38] together with MSI status shifts during tumor progression.[14] The analysis of the MSI panel in the UAs can have a special value in these cases.

To complement our noninvasive approach, we also applied the test on cfDNA isolated from plasma samples. Our study allowed us to explore the feasibility to monitor MSI markers in longitudinal samples during tumor evolution. Notably, we were able to detect at least one of the MSI markers altered in 60.46% of the cases classified as MSI-H in the UAs which can be considered as indicators of the ctDNA content. These results are really promising, taking into consideration that we applied the test to a cohort of nonmetastatic patients, who normally have low levels of ctDNA.[15] Actually, Silveira et al employed another ddPCR-based strategy (which covers BAT-26, ACVR2A and DEFB105A/B) in plasma samples from a cohort of six patients with advanced EC, being 50% of the cases classified as unstable based on the cfDNA analysis.[29] Therefore, our data supports the great potential of this technology to be applied in advanced and nonadvanced tumors candidates for immunotherapy in which the primary tumor or the metastasis can be inaccessible, such as pancreatic, gastric, prostate or ovarian cancer.[39] NGS based strategies have also been recently applied on cfDNA to characterize MSI in different tumor types and showed the same benefit from immunotherapy in patients classified as MSI-H in tissue samples and cfDNA.[40] Although this strategy has the advantage of providing more information regarding the genomic landscape of the tumor, including a higher number of MSI loci, it is less sensitive than ddPCR-based assays.[41-43]

In addition to providing a more complete view of tumor heterogeneity, the opportunity to interrogate the status of MSI in cfDNA of EC patients represents a valuable tool to monitor tumor evolution without the need to have a broad knowledge about each patient-specific genetic alteration, making the analysis ctDNA more cost-effective and less time-consuming than other strategies. In fact, we monitored the MSI panel in plasma samples from 15 EC patients, including four patients who showed tumor progression. One of these cases was characterized by an early progressive disease, being the MSI markers detectable prior to the disease recurrence, and reflecting the tumor response to treatment and its fast evolution after recurrence. Interestingly, a second case also showed an increment of the MSI markers 6 months after surgery and only few months later was considered under clinical and radiological progression. Additionally, the MSI panel positivity, although lower, was detected 6 months after the tumor resection in a patient with residual disease who was treated with immunotherapy after progression. Besides, no MSI positivity was found in the longitudinal samples of patients with a good postsurgery evolution. These data reflects the potential of the MSI assessment by ddPCR in cfDNA to anticipate the disease recurrence and monitor the tumor evolution as it has been recently shown in other tumor types such as CRC.[29] Therefore, our results, together with previous preliminary data obtained by NGS,[44] support that cfDNA analyses assessing the MSI status could open new opportunities for a personalized follow-up and immunotherapy selection in EC patients and other immune-responsive tumors.

In summary, our study is pioneer in demonstrating the accuracy of MSI evaluation in UAs from EC patients using a highly sensitive and robust ddPCR-based assay. This approach allows a comprehensive tumor genotyping without the need for invasive procedures and can be easily implemented for the clinical diagnostic of EC. Furthermore, our results also demonstrate the value of the assay to interrogate the MSI status on cfDNA from EC patients, opening new options to make a dynamic characterization of this therapeutic-relevant molecular alteration and to monitor the tumor evolution in the context of both localized and metastatic solid tumors candidates to receive immunotherapy. These promising results obtained in a cohort enriched in MSI-H tumors represent the basis to a further validation in larger cohort of patients and longitudinal samples.

AUTHOR CONTRIBUTIONS

Carlos Casas-Arozamena: Conceived and designed the study; contributed to the collection of samples and clinical data; performed the DNA extractions; performed the UA/FFPE/cfDNA analysis; performed the statistical analysis; wrote the article. **Cristian Pablo Moiola:** Contributed to the collection of samples and clinical data; performed the DNA extractions. **Ana Vilar:** Contributed to the collection of samples and clinical data. **Marta Bouso:** Was responsible for the pathologic analysis. **Juan Cueva:** Contributed to the collection of samples and clinical data. **Silvia Cabrera:** Contributed to the collection of samples and clinical data. **Victoria Sampayo:** Contributed to the collection of samples and clinical data. **Efigenia Arias:** Contributed to the collection of samples and clinical data. **Alicia Abalo:** Contributed to the collection of samples and clinical data. **Ángel García:** Was responsible for the pathologic analysis. **Ramón Manuel Lago-Lestón:** Performed the DNA extractions; performed the UA/FFPE/cfDNA analysis. **Sara Oltra:** Performed the NGS analysis. **Eva Díaz:** Performed the NGS analysis. **Juan Ruiz-Bañobre:** Contributed to the collection of samples and clinical data. **Rafael López-López:** Conceived and designed the study; contributed to the collection of samples and clinical data. **Gema Moreno-Bueno:** Performed the NGS analysis. **Antonio Gil-Moreno:** Contributed to the collection of samples and clinical data. **Eva Colás:** Contributed to the collection of samples and clinical data. **Miguel Abal:** Conceived and designed the study. **Laura Muinelo-Romay:** Conceived and designed the study; performed the statistical analysis; wrote the article. The work reported in the article has been performed by the authors, unless clearly specified in the text. All authors provided critical feedback and helped guide the research, analysis and article. All authors have approved the final article.

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CONFLICT OF INTEREST

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DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The study was approved by the corresponding Research Ethics Committees (Galician Research Ethics Committee—reference number 2017/430, Vall d'Hebron Research Ethics Committee—reference number PRAMI276-2018) and conducted in accordance with the guidelines for Good Clinical Practice and the Declaration of Helsinki. All patients provided written informed consent before enrolment.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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