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SHORT REPORT

Tumor Markers and Signatures

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High performance of the DNA methylation-based WID-qEC test for detecting uterine cancers independent of sampling modalities

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Abstract

Endometrial cancer (EC) is the most prevalent gynaecological cancer in high-income countries and its incidence is continuing to rise sharply. Simple and objective tools to reliably detect women with EC are urgently needed. We recently developed and validated the DNA methylation (DNAme)-based women's cancer risk identification—quantitative polymerase chain reaction test for endometrial cancer (WID-qEC) test that could address this need. Here, we demonstrate that the stability of the WID-qEC test remains consistent regardless of: (i) the cervicovaginal collection device and sample media used (Cervex brush and PreservCyt or FLOQSwab and eNAT), (ii) the collector of the specimen (gynaecologist- or patient-based), and (iii) the precise sampling site (cervical, cervicovaginal and vaginal). Furthermore, we demonstrate sample stability in eNAT medium for 7 days at room temperature, greatly facilitating the implementation of the test into diagnostic laboratory workflows. When applying FLOQSwabs (Copan) in combination with the eNAT (Copan) sample collection media, the sensitivity and specificity of the WID-qEC test to detect uterine (i.e., endometrial and cervical) cancers in gynaecologist-taken samples was 92.9% (95% confidence interval [CI] = 75.0%–98.8%) and 98.6% (95% CI = 91.7%–99.9%), respectively, whilst the sensitivity and specificity in patient collected self-samples was 75.0% (95% CI = 47.4%–91.7%) and 100.0% (95% CI = 93.9%–100.0%), respectively. Taken together these data confirm the robustness and clinical potential of the WID-qEC test.

Ojone Illah, Malcolm Scott and Elisa Redl contributed equally to this study.

KEYWORDS
collection modalities, DNA methylation, early detection, endometrial cancer

What's new?

Subjective diagnostic tests with modest accuracy, such as cytology or ultrasound, are currently used to assess the likelihood of uterine cancer in women with abnormal bleeding. Here, the authors show that the DNA methylation-based women's cancer risk identification—quantitative polymerase chain reaction test for endometrial cancer (WID-qEC) test they have previously developed has high sensitivity and specificity in detecting uterine cancers in symptomatic women irrespective of the sample collection device and medium, sample collector and precise sampling site. Furthermore, they demonstrate the compatibility of the WID-qEC test used with the Copan sampling system with established diagnostic laboratory workflows, confirming the robustness and clinical potential of the WID-qEC test.

1 | INTRODUCTION

Endometrial cancer (EC) is the most common gynaecological malignancy in high-income countries. Recently, a sharp rise in the incidence of EC subtypes associated with poor prognoses has been observed and is partly responsible for increasing EC mortality rates.[1–4] A delay in diagnosing EC reportedly lowers overall survival rates, with stronger implications for patient outcomes compared to other cancers.[5] More than 90% of women with EC present with abnormal uterine bleeding (AUB), yet only 0.33% and of pre-[6] and 3% peri-/post-menopausal[7] patients with AUB are eventually diagnosed with EC.

Transvaginal ultrasound is the current gold standard to triage women with AUB. Local and/or national guidelines exist to define sonographic endometrial thickness thresholds for performing histological procedures in peri/postmenopausal women with AUB; however, these guidelines are inconsistent. For instance, the German S3 guidelines and the European Society for Medical Oncology recommend histological assessments in women with >3 mm endometrial thickness,[8,9] whereas the United States and the United Kingdom suggest thresholds of >4 or >5 mm.[10]

Other diagnostic procedures to triage women with AUB, such as cervicovaginal cytology, have substantial shortcomings: (i) cytology shows an unacceptably low (45%[11]) sensitivity for EC detection, (ii) samples for assessment of cytological changes must be obtained by healthcare professionals at specific, anatomically well-defined regions and (iii) the microscopic assessment of cells is subjective and—at least for cervical screening—the quality of cytological assessment has started to deteriorate.[12]

Recently, we developed[13] and validated[7,13,14] the WID-qEC test, a real-time PCR-based assay which detects methylation in genetic alleles of *ZSCAN12* and *GYPC*. In the latest prospective, consecutive cohort study, the WID-qEC test outperformed ultrasound in the detection of EC.[7] Furthermore, the WID-qEC identified two cases where hysteroscopy or curettage-histology was negative, but a subsequent diagnosis of EC was made based on hysterectomy- or metastases-histology.[7]

In the current study, we aimed to test the dependence of WID-qEC performance on (i) the choice of sample collection device and associated sample media (Cervex brush and PreservCyt or FLOQSwab and eNAT), (ii) the sample collector (gynaecologist or the patient themselves) and (iii) the precise sampling site (cervical, cervicovaginal and vaginal). Furthermore, to investigate compatibility with diagnostic laboratory workflows, we assessed the stability of our test using FLOQ swabs in eNAT medium stored for 7 days at room temperature.

2 | MATERIALS AND METHODS

Cervicovaginal samples were collected at the University College London Hospital using two different collection systems after obtaining signed informed consent from all patients. The two collection systems comprised the Cervex brush (Rovers Medical Devices, cat #70671-001) combined with PreservCyt (ThinPrep, Hologic Inc., cat #70098-002) collection medium, and the FLOQSwab (Copan, cat #552C.80 PB) used alongside eNAT (Copan, cat #608C) collection medium. Samples were collected according to standard operating procedures and manufacturers' protocols. All samples were either gynaecologist- (gyn-) or patient- (self-) collected.

In one subset of volunteers ($n = 16$), the samples were gynaecologist-collected following two different collection sequences, with the FLOQSwab/eNAT (Copan) sample taken before the Cervex brush/PreservCyt (ThinPrep) sample or vice-versa (Figure 1A). PreservCyt samples were stored at room temperature until DNA extraction. eNAT samples were stored at −20°C. A 0.5 mL aliquot of the sample was subjected to immediate DNA extraction after thawing the sample, whilst the residual sample was stored at room temperature (RT) for 7 days ahead of DNA extraction (Figure S1A).

Samples from the second subset of volunteers ($n = 96$) were collected solely using the Copan system. Respective patients were invited to provide one self-sample followed by a clinician taken gyn-sample. The Copan gynaecologist sample was taken after the insertion of a speculum (without or with minimal amount of lubricant) from the posterior vaginal fornix and the cervix (Figure 2A). All eNAT samples were stored at −20°C and half of the volume was subjected to immediate DNA extraction after thawing.

The volunteers' characteristics and clinicopathological features are provided in Table S1. Control volunteers (i.e., women without an

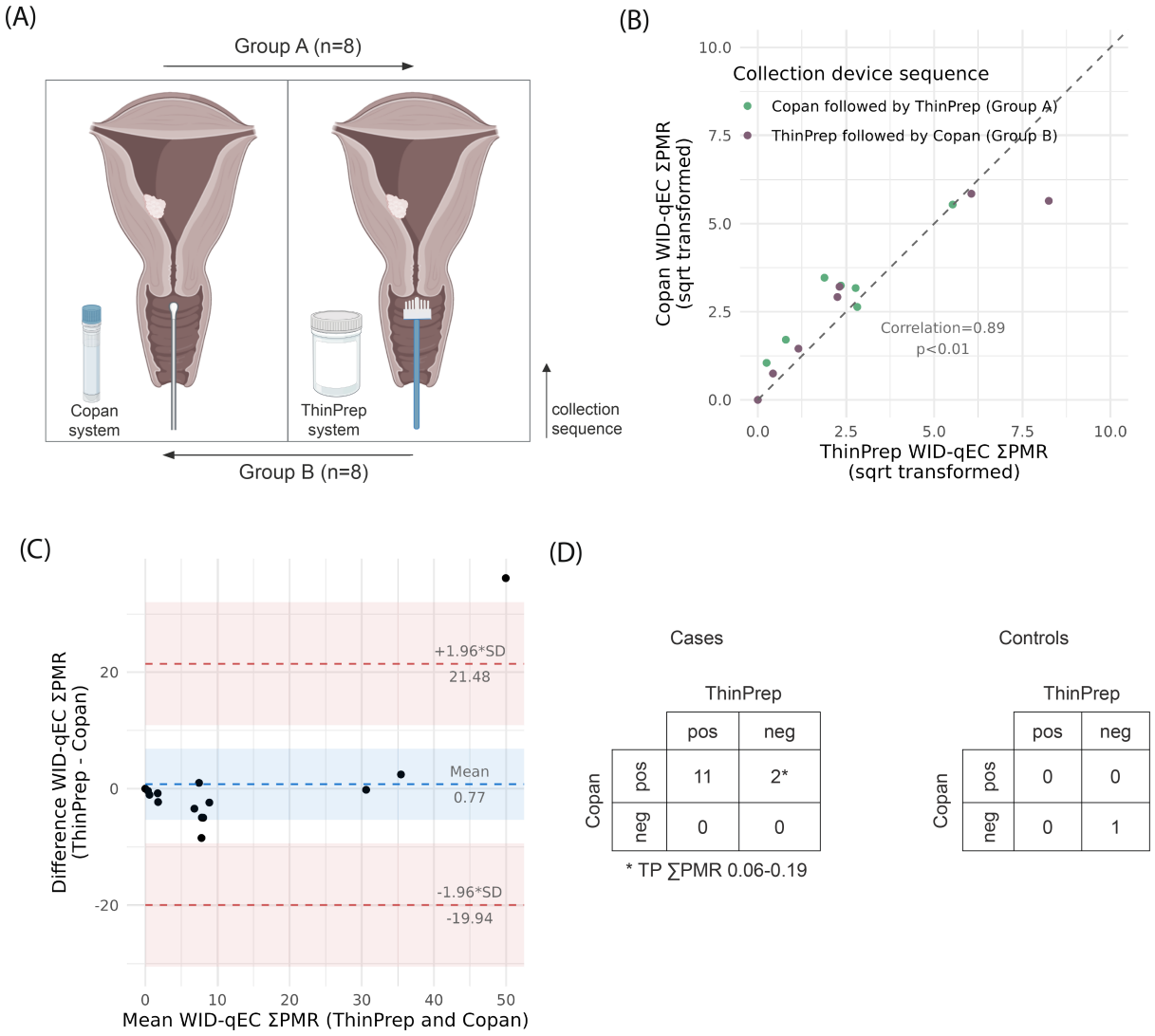

FIGURE 1 Comparison of ThinPrep (TP) and Copan collection system. (A) Graphical outline displays collection sequence of gyn-collected samples using TP and Copan systems in two experimental groups, group A ($n = 8$) and group B ($n = 8$). (B) Correlation based on the square root transformed WID-qEC ΣPMR between both groups, (C) Bland–Altman plot comparing both collection methods, and (D) table of test outcomes based on a predefined WID-qEC ΣPMR ≥0.3 cutoff. Note that test results for both the TP and Copan systems were available for 14/16 volunteers due to two test failures. PMR, percentage of fully methylated reference; sqrt, square root; WID-qEC, women's cancer risk identification—quantitative polymerase chain reaction test for endometrial cancer.

active endometrial or cervical cancer) and cases (women with an active invasive endometrial or invasive cervical cancer) were recruited from gynaecology outpatients and from gynaecological oncology clinics. Controls included women with several benign pathologies and a few patients with non-cervical and non-endometrial malignancies and this is reflected in the younger age of these women (Table S1).

DNAme-specific, quantitative real-time PCR (MethyLight) analysis was performed as previously described.[7] The final test result (WID-qEC ΣPMR) is defined as the sum of the percentage of fully methylated reference (PMR) values of the two regions assessed (*ZSCAN12* and *GYPC*). In short, cervicovaginal DNA was extracted using the Mag-Bind Blood & Tissue DNA HDQ 96 Kit (Omega Bio-tek, cat #M6399-01) on a Hamilton Microlab® STAR™ liquid handling platform as per the manufacturer's protocol. DNA was normalised to 10 ng/μL and bisulfite modified using the EZ-96 DNA Methylation-Lightning Kit (Zymo Research, cat. #D5033) as per the manufacturer's protocol. Bisulfite-modified DNA was amplified using the Luna Universal Probe qPCR Master Mix (NEB, cat. #M3004G) and primer-probe sets as described.[13] All qPCR reactions covering the two target regions *ZSCAN12* and *GYPC* as well as the reference region *COL2A1* were performed in technical duplicates. qPCR reactions were run on the QuantStudio 7 Pro (Applied Biosystems) and results were further extracted via the Design & Analysis Software 2.5.0 (Applied Biosystems). The PMR values at each target locus were calculated by dividing the *TARGET*:*COL2A1* input amount ratio (derived using the *COL2A1* standard curve) of a sample by the *TARGET*:*COL2A1* input amount ratio of gBlocks Gene Fragments DNA (equivalent to fully methylated DNA) and multiplying by 100.[13]

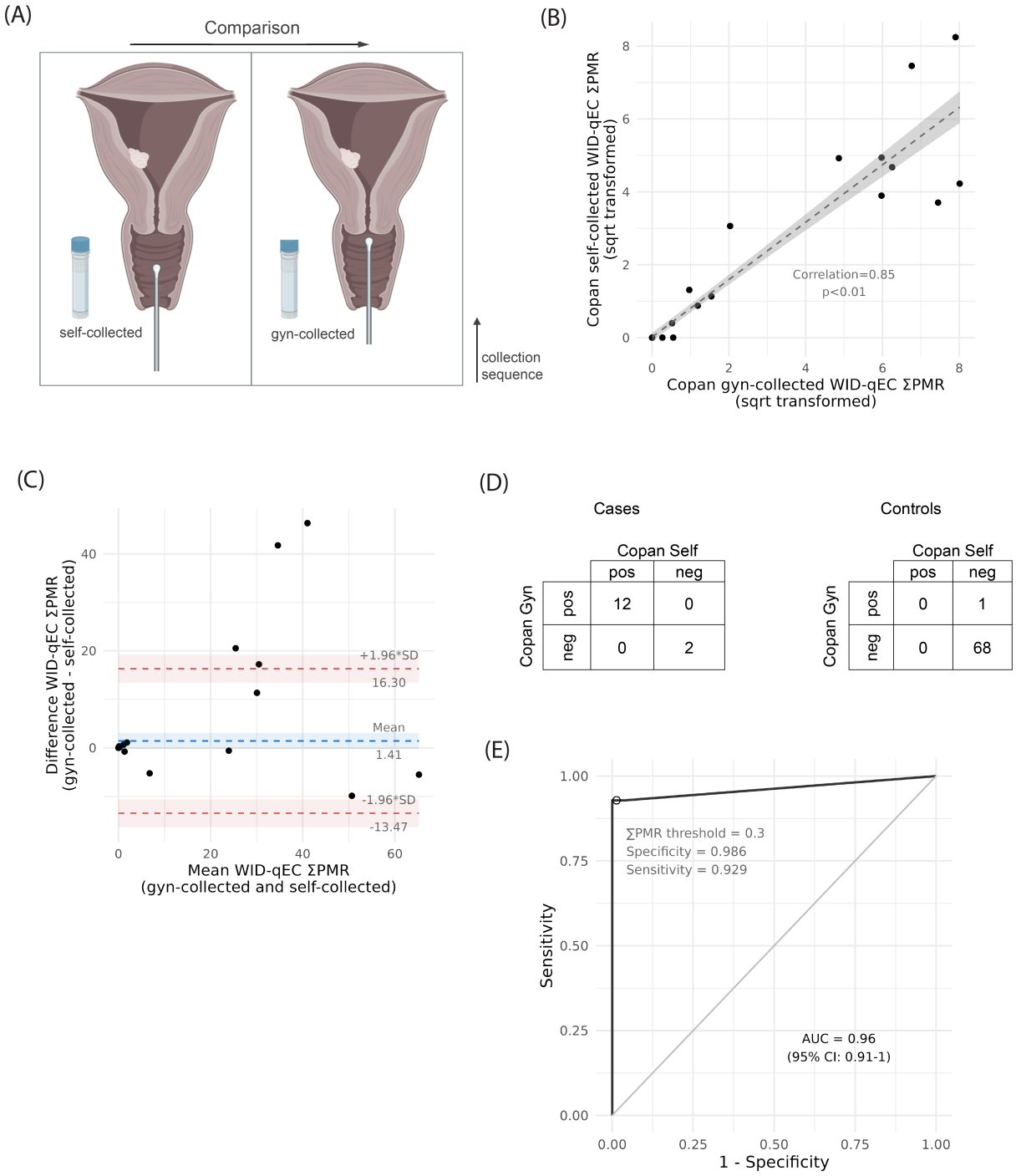

FIGURE 2 Comparison of Copan samples collected by the patient (self) or by a gynaecologist (gyn). (A) Graphical outline of sample collection sequence of self- and gyn-collected Copan samples. (B) Correlation based on the square root transformed WID-qEC ΣPMR from both sampling methods. (C) Bland–Altman plot comparing both methods. (D) table of test outcomes based on a predefined WID-qEC ΣPMR ≥0.3 cutoff. (E) Receiver under the operating characteristic curve (ROC) for all gyn-collected Copan samples. Note that ΣPMR values for both gyn- and self-collected samples were available for 83/96 volunteers. PMR, percentage of fully methylated reference; sqrt, square root; WID-qEC, women's cancer risk identification—quantitative polymerase chain reaction test for endometrial cancer.

WID-qEC results were analysed applying the a priori defined[7] ΣPMR of 0.3, with positive results defined as ΣPMR ≥0.3.

Amongst all 238 samples analysed (i.e., Cervex and Copan), 15 (6.3%) did not lead to a conclusive test result (Table S2). This was due to insufficient DNA or to high reference gene *COL2A1* Ct values ($n = 12$) or to the fact that in the duplicate analysis, the Ct value of one duplicate target gene was consistently above the limit of detection. In a clinical setting, resampling would be required for these 15 women.

All statistical analyses were carried out in R version 4.3.2. Correlations and corresponding p-values were calculated using the cor.test function in the stats R package, version 4.3.2. Comparisons between groups were made using the t.test function in the stats R package. Receiver operating characteristic curves, areas under the curve, and corresponding 95% confidence intervals (CIs) were generated using the pROC package, version 1.18.2. Sensitivity and specificity, including 95% CIs, were calculated according to the Wilson method using the prop.test function in the Rstats package, version 4.3.2. Bland–Altman plots, where the difference between two methods is plotted against the mean, were used to assess the agreement between two measurement methods.[15]

3 | RESULTS AND DISCUSSION

Conventional cervical collection devices, like the ThinPrep system, include brushes to mechanically remove cells and mucus from the cervix. In contrast, swabs compatible with self-sampling, such as the FLOQSwab from Copan, often involve a gentler collection approach, primarily absorbing cervicovaginal fluid. Whereas liquid-based cytology systems usually have a large volume of fluid in which the brush contents are released and a sedimentation step is required, small volumes of nucleotide-stabilising medium such as eNAT into which the swab content is released can be directly used for DNA extraction. These small-volume devices facilitate automation and are preferred for use in diagnostic laboratory workflows.

Results from our first group of volunteers were evaluated to determine whether WID-qEC results were affected by use of the cervical (Cervex brush and PreservCyt, ThinPrep samples) or cervicovaginal (FLOQSwab and eNAT, Copan samples) collection systems. The first experimental subgroup of 16 patients covered 15 active EC cases and one without an active cancer. All 16 patients were randomly assigned to two experimental groups, group A ($n = 8$) and group B ($n = 8$). Two cervicovaginal smear samples (ThinPrep and Copan) were obtained from all patients, with the two groups differing in sample collection sequence. In group A, a Copan sample followed by a ThinPrep sample was obtained by a gynaecologist from all patients. The test failed for one of the Copan samples. In group B, the sampling sequence was reversed with a ThinPrep sample followed by a Copan sample (Figure 1A). The test failed for a single Copan sample. Both failed Copan samples were active EC cases that showed an inconclusive PCR result in technical replicates and would have required re-extraction or re-sampling. Since this was not possible, samples from these two women were excluded from further analysis. We observed a strong correlation in the WID-qEC ΣPMR between samples collected with the Copan and the ThinPrep systems (correlation coefficient = .89, $p < .01$ after square root transformation; based on 14 samples with available ΣPMR values for both collection systems; Figure 1B). A Bland–Altman plot shows that the mean difference between both groups is close to 0 (mean = 0.77 in terms of absolute ΣPMR values; Figure 1C).

The WID-qEC test was positive in all Copan samples from women with an EC (13/13) (i.e., WID-qEC ΣPMR ≥0.3) (Figure 1D). 2/13 cancer case samples collected with the ThinPrep system had a negative WID-qEC test with a ΣPMR >0 but <0.3. Reasons for these failures may include the fact that one of these women had a very small (stage IA) highly differentiated (grade 1) cancer and the other was in group A (Copan swab taken first), potentially reducing the amount of leftover tumour DNA at the cervix. The patient with no EC had a negative WID-qEC test result (WID-qEC ΣPMR = 0) in both sample types (Figure 1D).

Due to its detergent- and guanidine thiocyanate-based formula, eNAT homogenously lyses cell membranes, prevents bacterial proliferation, inactivates nucleases and stabilises nucleic acids. The absence of formaldehyde or methanol and the small volume (1 mL) enable the use of the entire sample for automated fast DNA isolation protocols. To determine whether eNAT sample storage at room temperature potentially impacts WID-qEC results, we re-extracted DNA from the 14 above-described gyn-collected Copan samples with available WID-qEC results after storing an aliquot of all samples at RT for 7 days (Figure S1A). The test failed for a single stored sample, meaning that ΣPMR values for stored and non-stored samples were available for 13 volunteers. Importantly, no relevant storage effect on WID-qEC ΣPMR levels was observed. The correlation coefficient of the WID-qEC ΣPMR of samples processed immediately and after storage was 0.91 ($p < .01$ after square root transformation; Figure S1B). The mean difference between samples processed immediately after thawing and stored samples is close to zero (mean = −0.47 in terms of absolute ΣPMR values; Figure S1C). Sample storage had no impact on test positivity (Figure S1D), demonstrating DNA methylation stability in eNAT medium.

Our data support the use of the Copan system (FLOQSwab/eNAT) for the WID-qEC test. WID-qEC results on cervicovaginal samples from patients sampled with Copan and ThinPrep collection systems are strongly correlated; DNA extraction is easily automated; and samples are stable in WID-qEC downstream analyses (i.e., bisulfite conversion and real-time PCR reaction) following short-term storage at room temperature. Furthermore, the use of Copan devices affords an opportunity for patient-friendly self-sampling.

Sample self-collection for cervical screening is now offered routinely in some countries, such as Sweden. These efforts improve population coverage and attendance among under-screened and hard-to-reach women.[16] Self-collection could further improve cancer screening programs in vulnerable populations, including racial or ethnic minorities, LGBTQI persons, immigrants or socioeconomically disadvantaged people.[17] Annual EC screening starting at the age of 30 or 35 years is recommended in women with Lynch syndrome. Notably, self-sampling could significantly improve active surveillance opportunities also for these women.[18] Our studies and conversations with patients also suggest that some women at least welcome the option to provide a self-collected sample. Here, we wanted to assess whether vaginal self-sampling using FLOQSwabs was feasible and comparable to gyn-collected FLOQSwab samples obtained directly from the area to which the endometrial effluent is typically drained (i.e., top part of the vagina around the cervix) (Figure 2A). Importantly, we assumed that vaginal self-samples were likely taken from the mid-vagina. FLOQSwab samples were directly released into a DNA stabilising collection fluid (eNAT).

TABLE 1 Sensitivity and specificity of the WID-qEC test based on Copan gyn- or self-collected samples. We assessed the WID-qEC test with a predefined threshold (ΣPMR ≥0.3).

Characteristics	Copan gyn	Copan self
EC/CC cases, *n*	28	16
Cancer-free controls, *n*	74	74
Sensitivity % (95% CI)	92.9% (75.0%–98.8%)	75.0% (47.4–91.7%)
Specificity % (95% CI)	98.6% (91.7%–99.9%)	100.0% (93.9–100.0%)
PPV %[a]	68.0%	100.0%
NPV %[a]	99.8%	99.2%

Note: The PPV and NPV have been calculated based on an estimated 3% cancer prevalence.
Abbreviations: 95% CI, 95% confidence interval; CC, cervical cancer; EC, endometrial cancer; NPV, negative predictive value; PMR, percentage of fully methylated reference; PPV, positive predictive value; WID-qEC, women's cancer risk identification—quantitative polymerase chain reaction test for endometrial cancer.
[a]Estimated values, based on assumed population prevalence of 3%.

Samples were collected from $n = 96$ volunteers. A total of $n = 90$ had self-collected test results available, $n = 88$ had gyn-collected results available, $n = 83$ had both available and $n = 1$ had neither available. Test result unavailability was due to test failures (see Section 2) with the exception of two volunteers in which one of the samples was not taken. The WID-qEC ΣPMR of self- or gyn-collected samples showed high correlation (correlation coefficient = .85, $p < .01$ after square root transformation; Figure 2B). A Bland–Altman plot shows that the mean difference between both sampling methods is also close to 0 (mean = 1.41 in terms of absolute ΣPMR values; Figure 2C), although there were two outliers. With a single exception, WID-qEC test outcomes (positive or negative based on a ΣPMR threshold of ≥0.3) were identical across gyn- and self-collected Copan samples (Figure 2D). The two cancer cases with a negative WID-qEC test result were both grade 1 endometrioid ECs, stage IA.

Finally, we assessed the WID-qEC performance (using the predefined[7] ΣPMR threshold of ≥0.3 to call a sample positive) to detect active endometrial or cervical cancers based on Copan samples ($n = 28$ cancer cases and $n = 74$ samples from women without an active endometrial or cervical cancer after omitting volunteers with missing test results). For gyn-collected samples, the AUC was 0.96 (95% CI = 0.91–1.00; Figure 2E), the sensitivity was 92.9% (95% CI = 75.0%–98.8%), and the specificity was 98.6% (95% CI = 91.7%–99.9%) (Table 1). For self-collected samples, the sensitivity was 75.0% (47.4%–91.7%) and the specificity was 100.0% (95% CI = 93.9%–100.0%). Based on recently published data from a case/control setting,[7] we estimated a population cancer prevalence of 3% to calculate the predictive values. The PPV estimate was 68% and 100% and the NPV estimate was 99.8% and 99.2% for gyn- and self-samples, respectively (Table 1). These findings suggest that gyn-collected FLOQSwab samples demonstrate performance levels that are at least equivalent to those using Cervex brushes.[7] Furthermore, this indicates that Copan self-samples may be an adequate alternative for women who do not want to undergo speculum examinations.

For this particular study we cannot provide detailed information on the ethnicity of the volunteers, but the majority of women in this cohort were white, reflective of the general population in the United Kingdom. Yet, ongoing work will assess the performance of the WID-qEC in black women.

Overall, we demonstrate that the WID-qEC test shows high sensitivity and specificity in detecting uterine cancers in symptomatic women irrespective of the collection device and fluid (Cervex brush and PreservCyt or FLOQSwab and eNAT), the sample collector (clinician or patient), or the precise sampling site. Furthermore, we show the compatibility of the WID-qEC test using the Copan sampling system with diagnostic laboratory workflows, including sample collection, shipment and downstream analysis.

AUTHOR CONTRIBUTIONS

Conceptualization: Martin Widschwendter, Adeola Olaitan, Nicola MacDonald and Adam Rosenthal. Formal analysis: James E. Barrett, Lena Schreiberhuber and Martin Widschwendter. Investigation: Ojone Illah, Malcolm Scott and Elisa Redl. Methodology: Ojone Illah, Malcolm Scott, Elisa Redl, Lena Schreiberhuber, James E. Barrett, Chiara Herzog, Charlotte D Vavourakis, Allison Jones, Iona Evans, Dan Reisel, Dhivya Chandrasekaran, Kostas Doufekas, Ioannis Kotsopoulos, Radha Graham, Nicola MacDonald and Rupali Arora. Writing—original draft: Martin Widschwendter. Writing—review and editing: all authors. The work reported in the paper has been performed by the authors, unless clearly specified otherwise in the text.

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CONFLICT OF INTEREST STATEMENT

J.E.B., C.H., E.R., A.J., I.E. and M.W. are inventors of WID-qEC-related patent applications. J.E.B., C.H. and M.W. are shareholders of Sola Diagnostics GmbH. Sola Diagnostics GmbH holds an exclusive licence to the intellectual property that protects the commercialisation of the WID-qEC test. All the other authors do not have a conflict of interest.

DATA AVAILABILITY STATEMENT

All the data that support the findings of this study are available in Table S2. Further information is available from the corresponding author upon request.

ETHICS STATEMENT

The study was a sub-study of the FORECEE (4C) program, which has ethical approval from the UK Health Research Authority (REC 14/LO/1633). All volunteers provided a written informed consent.

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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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