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Improving Specificity for Ovarian Cancer Screening Using a Novel Extracellular Vesicle–Based Blood Test

Performance in a Training and Verification Cohort

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The low incidence of ovarian cancer (OC) dictates that any screening strategy needs to be both highly sensitive and highly specific. This study explored the utility of detecting multiple colocalized proteins or glycosylation epitopes on single tumor-associated extracellular vesicles from blood. The novel Mercy Halo Ovarian Cancer Test (OC Test) uses immunoaffinity capture of tumor-associated extracellular vesicles, followed by proximity-ligation real-time quantitative PCR to detect combinations of up to three biomarkers to maximize specificity and measures multiple combinations to maximize sensitivity. A high-grade serous carcinoma (HGSC) case-control training set of EDTA plasma samples from 397 women was used to lock down the test design, the data interpretation algorithm, and the cutoff between cancer and noncancer. Performance was verified and compared with cancer antigen 125 in an independent blinded case-control set of serum samples from 390 women (132 controls, 66 HGSC, 83 non-HGSC OC, and 109 benign). In the verification study, the OC Test showed a specificity of 97.0% (128/132; 95% CI, 92.4%–99.6%), a HGSC sensitivity of 97.0% (64/66; 95% CI, 87.8%–99.2%), and an area under the curve of 0.97 (95% CI, 0.93–0.99) and detected 73.5% (61/83; 95% CI, 62.7%–82.6%) of the non-HGSC OC cases. This test exhibited fewer false positives in subjects with benign ovarian tumors, non-ovarian cancers, and inflammatory conditions when compared with cancer antigen 125. The combined sensitivity and specificity of this new test suggests it may have potential in OC screening. *(J Mol Diagn 2024, ■: 1–20; https://doi.org/10.1016/j.jmoldx.2024.09.001)*

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Ovarian and tubal cancers (OC) remain the most fatal of all gynecological cancers, with nearly 20,000 new cases of OC and >12,700 deaths in the United States in 2024 and >300,000 new cases and >200,000 deaths worldwide.[1,2] OC is the fifth leading cause of cancer death among women in the United States, representing 4% of cancer deaths overall and affecting women of all ethnic backgrounds.[3] Most OC mortality occurs in women with high-grade serous cancer (HGSC), an estimated 22% of whom have germline mutations in the *BRCA1/2* genes.[4,5] The 5-year survival rate for localized OC is 92.4%, whereas only 31.5% of patients with OC diagnosed with distant metastases survive beyond 5 years (National Cancer Institute, Surveillance, Epidemiology, and End Results Program, *https://seer.cancer.gov/statfacts/html/ovary.html*, 2013 to 2019, last accessed August 18, 2023).[4] Besides disease biology, non-specific symptoms, physician suspicion of ovarian cancer over other differential diagnoses, and minimization of symptoms by patients also contribute to delayed diagnosis, with only 18% of women diagnosed with localized disease (National Cancer Institute, Surveillance, Epidemiology, and End Results Program, *https://seer.cancer.gov/statfacts/html/ovary.html*, 2013 to 2019, last accessed August 18, 2023).[6] This highlights the need for better tests for early detection of OC when the cancer is still localized.[5]

Despite its low prevalence, the high mortality rate of OC has catalyzed significant research into candidate screening modalities that may yield mortality benefits associated with early detection. The two candidate screening methods that have received the most attention are transvaginal ultrasound (TVUS) imaging and serum cancer antigen 125 (CA125). These screening modalities have been evaluated in large randomized, controlled trials (RCTs) in the United States, Japan, and the United Kingdom.[7–17]

Screening using TVUS alone has not proven effective. There was no difference in stage at diagnosis or deaths due to OC in the annual TVUS arm of the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) RCT compared with the no screening arm. In addition, 50 women per 10,000 screens underwent unnecessary surgery,[12] highlighting the specificity challenge associated with imaging-based diagnostic modalities for detection of OC. Most HGSC arises from the fallopian tubes,[18] but studies evaluating the sensitivity of TVUS have reported that, even in expert hands, fallopian tubes could not be imaged in 23% of healthy women.[19]

In the Prostate, Lung, Colorectal, and Ovarian cancer screening RCT,[7,8] screening using serum CA125 in combination with TVUS did not detect ovarian and tubal cancers at an earlier stage compared with the no screening control arm. However, in the multimodal screening arm of UKCTOCS,[10–15] using longitudinal serum CA125 interpreted by the Risk of Ovarian Cancer Algorithm (ROCA) with TVUS and repeat CA125 as second-line tests, there was a significant decrease in advanced stage HGSC compared with the no screening arm. Sensitivity for invasive epithelial OC diagnosed within 1 year of the test was 87% and specificity was 99.8%. Despite this, false positives, due in part to benign ovarian tumors, remained high; and 14 women per 10,000 screens underwent unnecessary surgery. Crucially, neither RCT demonstrated a mortality benefit associated with OC screening. As a result, screening is not recommended for women at average risk for developing OC.[20] These data underline the continuing unmet need for improved early-stage diagnosis of OC in the general population to improve survival.

Prospective studies involving women at elevated risk of developing OC due to germline mutations or family history have also used the multimodal ROCA screening approach, measuring CA125 concentrations at 3- to 4-month intervals and monitoring longitudinal CA125 profile combined with second-line TVUS.[21–24] These studies demonstrated larger decreases in advanced-stage disease at diagnosis. However, given that they were not RCTs, it was not possible to ascertain whether there was an associated mortality benefit. For women with genetic risk of OC,[25] a risk-reducing salpingo-oophorectomy after completion of childbearing or a decision to not conceive naturally is recommended by clinical practice guidance documents from the American College of Obstetricians and Gynecologists, the Society of Gynecologic Oncology, the National Comprehensive Cancer Network (United States), as well as the European Reference Networks on Genetic Tumour Risk Syndromes[26] and in recent guidance from the National Institute for Health and Care Excellence UK (*https://www.nice.org.uk/guidance/indevelopment/gid-ng10225*, last accessed September 16, 2024). It is acknowledged but not

Key Points

- This study demonstrates that a novel ovarian cancer (OC) blood test using colocalized membrane-associated biomarkers on extracellular vesicles can detect OC, and especially the most common and lethal histotype (high-grade serous carcinoma), with high sensitivity and specificity.
- The use of combinations of biomarkers significantly decreased false positives from benign gynecological tumors compared with using cancer antigen 125 alone.
- The Mercy Halo Ovarian Cancer Test (OC Test) works with samples collected in either standard EDTA plasma or serum blood collection tubes.
- This OC Test performance offers the potential for significant improvement over existing approaches used for the early detection of OC and suggests that this test may be useful in average and/or high-risk OC screening. Future studies will explore this in asymptomatic populations.

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specifically recommended in the current US Preventive Services Task Force screening guidance for OC.[20]

A blood-based screening test must meet key requirements to be considered suitable for the early detection of OC. First, the analyte targeted by the test must be present in circulation in sufficient quantities from small, early-stage tumors to permit detection, and must have sufficient stability to support analytical measurement following blood collection. Second, the analyte must be tumor specific. High levels of both sensitivity and specificity are required to screen for cancers with lower incidence rates, such as OC. Even the use of multimodal screening using ROCA and second-line TVUS, which combined two tests with orthogonal methods (eg, serum biomarker plus imaging) and achieved 99.8% specificity, is not an adequate solution. This is because women who receive positive results from OC screening for a potential early-stage tumor cannot undergo a less invasive biopsy, as this carries the risk of spreading the cancer to the peritoneum. Instead, they must proceed directly to surgical removal of the ovaries and fallopian tubes under general anesthesia, a procedure that comes with significant risks.

Extracellular vesicles (EVs) offer a unique analyte for diagnostic tests given their abundance, stability, and representation of the genomic and proteomic content from the cell of origin.[25,26] The high plasma EV concentration of approximately 10^{10} EVs per mL[27] and estimated tumor-associated EV shedding rates per cubic millimeter of tumor volume[28] make EVs an abundant source of tumor-associated biomarkers to target in cancer screening assays designed for detection of smaller, early-stage tumors.[27–30] We have previously described a novel assay design for EV detection that uses the presence of multiple colocalized biomarkers on extracellular particles to differentiate between healthy and cancer samples.[31] The studies described here characterize a Mercy Halo Ovarian Cancer Test (OC Test) that exhibits sensitive and specific detection of HGSC using this novel design. The test is suitable for use in serum and plasma and offers the potential for significant improvement over existing diagnostic modalities for the early detection of OC.

Materials and Methods

Ovarian Cancer Test Design and Biomarker Selection

Development of the OC Test began with a computational biomarker discovery approach. First, gene expression data from The Cancer Genome Atlas[32] and the Genotype-Tissue Expression[33] projects were analyzed to identify OC-associated surface biomarkers that were likely to be overexpressed on OC EVs. The resulting list of 124 computationally derived biomarkers was further refined by human curation using the UniProt database[34] to narrow the list to 52 membrane-associated biomarkers with an extracellular domain that could be used to capture EVs. Literature review and compiled data from Vesiclepedia (*http://microvesicles.org*, last accessed January 17, 2024), PhosphoSitePlus (*https://www.phosphosite.org/homeAction.action*, last accessed January 17, 2024), and cancer glycosylation databases[35–37] were used to add another 11 candidate biomarkers.

Genes with correlated overexpression in individual OC cases in The Cancer Genome Atlas database were used to predict combinations of two or three protein biomarkers that might be used to distinguish OC cases from healthy controls. Antibodies for use in the capture and detection steps of the assay were screened and selected, as previously described.[31] This was followed by extensive vetting of 300 unique combinations of two or three biomarkers based on their ability to differentiate the assay background signal for healthy individuals from the signal for early-stage OC cases. The second-level consideration for biomarker selection was separation of the signal from benign ovarian tumors from true cancer cases.

A final group of combinations and biomarkers that exhibited the best test-panel performance was chosen for the OC EV-based test (the OC Test) reported here. The optimized OC Test is composed of five biomarkers [bone marrow stromal antigen-2/tetherin,[38] folate receptor α,[39] mucin-1 (MUC-1),[40] mucin-16/CA125 (MUC-16),[40,41] and sialylated Thomsen–nouveau antigen (sTn)[42,43]], which are all membrane-associated surface biomarkers known to be overexpressed by OC relative to healthy tissues. The test panel is composed of three combinations of these five biomarkers designed to distinguish HGSC from both benign ovarian tumors and healthy controls.

Bone-marrow stromal antigen-2 is a type II transmembrane tetherin protein composed of four domains.[34] It is expressed on the apical side of cells, mainly on B cells, and regulated by both extrinsic and intrinsic stimuli, (eg, cytokines, interferons). Bone-marrow stromal antigen-2 may independently regulate both primary tumor growth and metastasis. Hypomethylation in cancer leads to its overexpression, affecting cancer progression, including cell adhesion, anchorage-independent growth, survival, primary tumor growth, invasion, and metastasis.[38]

Folate receptor-α, a member of the human folate-binding protein family, is a glycosylphosphatidylinositol (GPI)-anchored cell-surface glycoprotein encoded by the *FOLR1* gene.[34] It mediates cellular responses to folate, including cell division, proliferation, and tissue growth. Protein expression is lowest in normal ovarian tissue, higher in benign ovarian tumors, and highest in malignant tumors.[39]

MUC-1 is a heavily glycosylated protein found on the apical surface of epithelial tissues and plays a role in mucus formation and lubrication of mucosal-epithelial surfaces.[34] It interacts with a variety of signaling pathways associated with cancer and is known to be hypoglycosylated in cancer. The cleaved domain (cancer antigen 15.3) has been used as a serum biomarker for breast, lung, and ovarian cancer.[40]

MUC-16 is the largest mucin and second-longest human protein.[40] It is a type I transmembrane protein with one membrane-spanning domain and a cytoplasmic tail. The extensively glycosylated (N- and O-glycosylation) N-

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terminal domain consists of approximately 60 tandem repeats that are rich in serine and threonine residues with extensive O-linked glycosylation clusters.[34] MUC-16 is well known to be overexpressed in ovarian, pancreatic, lung, and breast cancer, and the cleaved domain (CA125) is the most commonly used serum biomarker for OC detection.[40,41]

sTn is a truncated *O*-glycan containing sialic acid α-2,6 linked to N-acetyl galactose on surface proteins, including mucins.[42,43] It is generated by disrupted *O*-glycan processing in cancer cells and affects cell adhesion, cellular recognition, and cell signaling. Increase in sialylation is associated with adverse outcome and poor prognosis in patients with cancer. It is the target for the OC therapeutic SGN-STNV, currently in clinical trials. This anti-sTn antibody targets and binds to sTn expressed on tumor cells and, following internalization of SGN-STNV and release of MMAE, binds to tubulin, and inhibits microtubule polymerization, resulting in G_2/M phase cell cycle arrest and apoptosis in sTn-expressing tumor cells.[42]

Ovarian Cancer Test Protocol

The OC Test protocol was optimized and performed as previously described.[31] Briefly, EVs are enriched from human plasma or serum by size-exclusion chromatography. Then, the size-exclusion chromatography–enriched EVs are captured in solution with magnetic bead–antibody conjugates targeting a specific surface biomarker. Next, immunoaffinity-captured EVs are incubated with detection antibodies conjugated to complementary double-stranded DNA probes. The double-stranded DNA oligonucleotides contain single-stranded overhangs that ligate only when in proximity to a complementary probe to generate a template for PCR. Finally, the abundance of the detection biomarkers captured on the EVs is read out using real-time quantitative PCR. Figure 1 illustrates the basic test concept for the specific detection of EVs containing colocalized biomarkers. Between the time of the training study and the verification study, the size-exclusion chromatography used as the first purification step (qEVoriginal 70 nm; Izon Science, Arundel, QLD, Australia) was replaced with a newer version (Gen2 qEVoriginal 70 nm), which showed equivalent performance.

CA125 ELISA Protocol

The Human CA125/MUC16 Quantikine enzyme-linked immunosorbent assay (ELISA) kit (catalog number DCA 125; R&D Systems, Minneapolis, MN) was used to measure the CA125 level in all EDTA plasma and serum samples. The VarioSkan Lux (catalog number VL0000D0; Thermo Fisher Scientific, Waltham, MA) was used to measure the resultant OD of the plate at 450, 540, and 570 nm. Blinded human plasma and serum samples as well as Lyphochek Tumor Marker Plus low, medium, and high CA125 control sera (catalog number 548X; Bio-Rad, Hercules, CA) were diluted 1:10 in 1× phosphate-buffered saline, pH 7.4 (Gibco catalog number 10010023; Thermo Fisher Scientific). Samples were run in duplicate following the manufacturer's instructions, and values were reported as the mean of the duplicates. If the measured human plasma CA125 concentration exceeded the detection range of 5 to 320 U/mL, as determined from the standard curve generated using the standards provided with the kit, the human plasma samples were diluted 1:100 and reprocessed.

Assay Linearity

Healthy K_2EDTA plasma was collected from 11 women with no history of cancer under a WCG Institutional Review Board–approved protocol for collection of large-volume blood samples from healthy donors (number 20212722). Each donor provided up to 15 tubes of plasma, which was collected using 10 mL K_2EDTA tubes (catalog number 366643; Becton Dickinson, Franklin Lakes, NJ). After collection of the K_2EDTA plasma from each donor, each tube was inverted several times and centrifuged at the collection site to separate the plasma from the blood cells. The plasma collected from each donor was pooled separately, and all aliquots were then frozen and stored at −80°C. All samples were tested for their background signal with the three OC Test combinations and with the CA125 ELISA, as described above. Patient samples that demonstrated low test background were used to generate the normal-plasma pool for the linearity study and to generate the multilevel controls that were used for the training and verification studies.

The normal-plasma pool was used to make the dilutions used for the linearity study. Four different replicates of the highest concentration sample in the dilution series were made by spiking in EVs that had been previously isolated from conditioned media obtained from the culture of the COV413A human epithelial serous ovarian carcinoma cell line (used under license from the European Collection of Authenticated Cell Cultures) and quantitated on the Spectrodyne nCS1 particle counter (Signal Hill, CA), as previously described.[31] Each of these spiked replicates underwent its own 11-point fourfold dilution series (one part spiked plasma diluted with three parts unspiked, normal pooled plasma). Unspiked plasma was used as the lowest concentration sample. Each of the samples from the four-replicate dilution series was run separately in duplicate through the entire test process. Samples prepared in this manner were expected to cover the real-time quantitative PCR C_T range of approximately 20 to approximately 40. The dilution series was prepared four times and then each sample was run in duplicate (eight replicates) in a single assay plate.

Full Process Control Preparation

On the basis of the results from the linearity study, five-level controls were targeted at 3.2×10^8 EVs per well, 8×10^7

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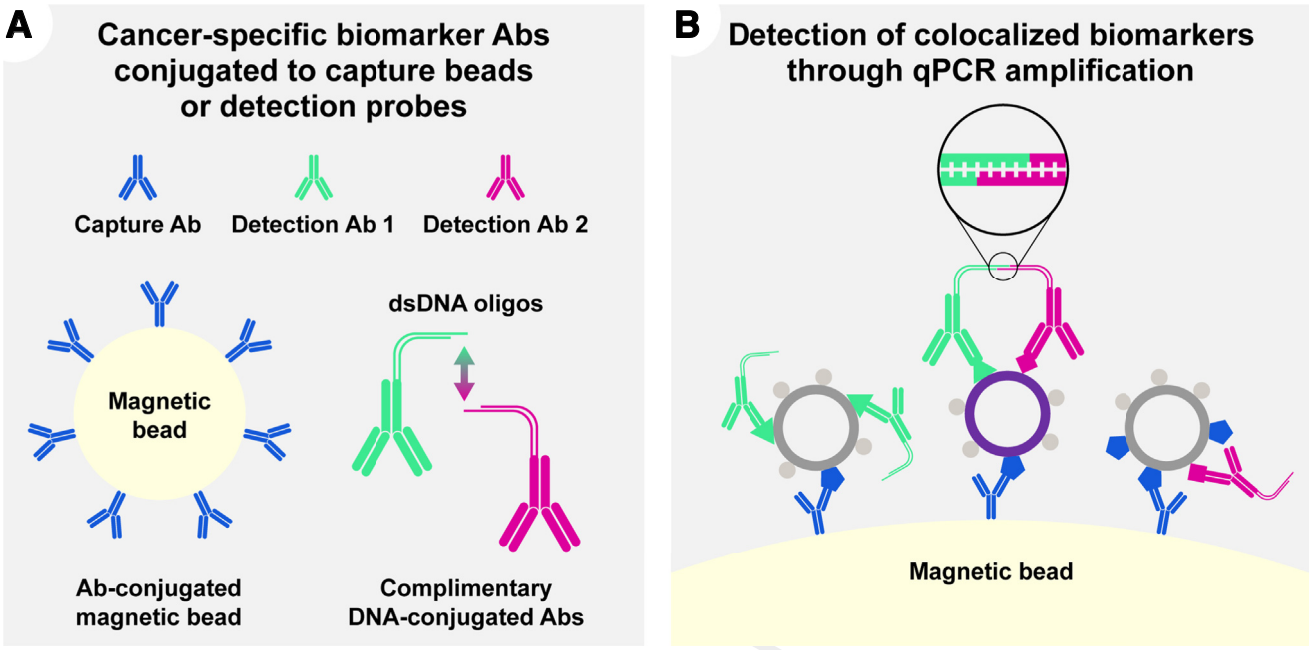

Figure 1 Overview of a biomarker combination design. **A:** Antibodies (Abs) are conjugated to magnetic beads (capture antibody) or double-stranded DNA (dsDNA) oligonucleotides (detection antibodies). **B:** After immunoaffinity capture, the extracellular vesicles are incubated with the dsDNA detection antibodies. Their single-stranded overhangs ligate only when in proximity to the complementary probe on a second antibody and are then quantitated using TaqMan PCR. qPCR, real-time quantitative PCR.

EVs per well, 2×10^7 EVs per well, 5×10^6 EVs per well, and unspiked plasma background to cover the range of OC Test scores expected from clinical samples. The spiked plasma pool for each of the controls was prepared separately, split into single use aliquots in cryovials, and then frozen and stored at −80°C.

Reproducibility Assessment

This reproducibility evaluation was designed according to Clinical Laboratory Standards Institute guidance, and the resulting data were analyzed as outlined in that guidance document.[44] The control data from the 16 days of the training study were analyzed to establish the mean, SD, and %CV for each of the five OC Test and the three CA125 ELISA controls. The results from these runs were used to establish the acceptance range for each control for use in the verification study (Supplemental Tables S1–S3). The same reagent lots, operators, and assay equipment were used when each of the studies were run, but some reagent lots and operators differed between the training and the verification studies.

Serum and Plasma Matrix Equivalency

The OC Test was initially developed using K_2/K_3EDTA plasma samples to select the optimal biomarker combinations for HGSC sensitivity and specificity. However, it is desirable to be able to also use serum as an alternate sample matrix for this test, as many biorepositories have residual banked serum following measurement of serum CA125. The paired serum and EDTA plasma samples used to assess matrix equivalency between EDTA plasma and serum were selected to cover the range of expected values from the OC Test and included 22 HGSC cases (4 early-stage and 18 late-stage cases), 7 samples from patients with type 2 diabetes, and 18 healthy controls. The paired serum and EDTA plasma from the HGSC and type II diabetes cases were purchased from ProteoGenex (Inglewood, CA), who sourced them from Moscow, Russia. Clinical annotation of the samples was provided by ProteoGenex. The healthy donor plasma and serum samples were collected under the same WCG Institutional Review Board–approved protocol (number 20212722) described above. All serum samples were collected in serum separator tubes (Becton Dickinson; catalog number 367988) and were allowed to stand for 30 to 120 minutes at room temperature before centrifugation for 10 minutes at 2000 × *g*. The serum was divided into 1-mL aliquots in 2-mL cryovials, and then frozen and stored at −80°C.

A confirmatory matrix equivalency study was also performed using donor-matched serum and K_3EDTA plasma samples selected from within the verification study cohort described below.

Training and Verification Study Design

The OC Test was run on a training set of K_2EDTA plasma samples that included stage I, II, and III OC cases as well as samples from healthy controls, and women with benign ovarian tumors, nonovarian cancers, and inflammatory

Table 1 Histopathology in Women Included in the Training and Verification Studies

Histologic category	Training study ($N = 534$) *N*	Verification study ($N = 401$) *N*
HGSC	**89**	**67**
Stage I	17	13
Stage II	35	7
Stage III	37	40
Stage IV	-	7
Non-HGSC ovarian cancer	**0**	**85**
Borderline serous	-	20
Invasive epithelial non-HGSC		
Clear cell	-	21
Endometrioid	-	15
Low-grade serous	-	6
Mixed	-	3
Mucinous	-	15
Synchronous	-	5
Healthy controls	**124**	**138**
Benign adnexal tumors	**192**	**111**
Adenofibroma	24	-
Cyst	16	-
Cystadenofibroma	7	-
Cystadenoma	51	-
Endometriosis/endometriotic cyst	13	6
Fibroma	15	11
Leiomyoma	13	-
Mucinous/mucinous other	-	24
Normal adnexa	13	-
Serous/serous mucinous/serous other	-	60
Teratoma/dermoid cyst	9	10
Other	31	-
Nonovarian cancers	**87**	**0**
Bladder (urothelial carcinoma)	14	-
Breast (infiltrating ductal carcinoma)	12	-
Breast (infiltrating lobular carcinoma)	3	
Colorectal adenocarcinoma	14	-
Endometrial adenocarcinoma	14	-
Non–small-cell lung carcinoma	15	-
Pancreatic adenocarcinoma	15	-
Inflammatory conditions	**42**	**0**
Crohn disease	7	-
Endometriosis	7	-
Diabetes type 2	7	-
Pancreatitis	7	-
Rheumatoid arthritis	7	-
Ulcerative colitis	7	-

HGSC, high-grade serous carcinoma.

conditions (Tables 1 and 2). The goal was to generate an algorithm for combining the results from these three combinations into a single OC Test score that could be used to accurately distinguish cancer from no cancer for each sample. Finally, the OC Test and classifier algorithm were used to test and call cancer/no cancer for an independent set of blinded, high provenance verification samples.

Each sample included in the training and verification study was accessioned and scored for hemolysis, icterus, and lipemia using standard Clinical Laboratory Standards Institute notation.[45] The vast majority of the samples were of normal appearance (score of <3) with only one sample with an elevated lipemia score in the training study and two samples with an elevated lipemia score in the verification study. The absence of grossly hemolyzed samples was expected because sample hemolysis was an exclusion criterion used by all sample providers.

Training Study Samples

Plasma aliquots were shipped frozen on dry ice from the collection site, biobank, or commercial vendor to Mercy BioAnalytics and were then stored at −80°C. All samples were de-identified before shipment. Full sample annotation for the training study samples can be found in Supplemental Table S4.

The 124 healthy donor plasma samples were collected after approval by the WCG Institutional Review Board (number 20212722). All individuals participating in the healthy collection study provided written informed consent, and the study complied with the Health Insurance Portability and Accountability Act and the Declaration of Helsinki. Blood from each healthy donor, recruited from prequalified collection sites, was collected using a 10-mL collection volume K_2EDTA tube (catalog number 366643; Becton Dickinson). After collection, each tube of K_2EDTA plasma was inverted six to eight times, centrifuged at the collection site (1500 RCF at room temperature for 15 minutes) within 60 minutes of collection to separate the plasma from the other blood components. The plasma was divided into 1-mL aliquots in 2-mL cryovials, and then frozen and stored at −80°C. These samples were used to determine the normal reference range for the OC Test.

The 89 HGSC K_2EDTA plasma samples were sourced from two academic biobanks, the Ontario Tumour Bank (Toronto, ON, Canada) and the Ovarian Cancer Research Program (OVCARE; Vancouver BC, Canada). The 192 benign adnexal mass K_2EDTA plasma samples were all from OVCARE. Clinical annotation of the samples was provided by the biobanks. Informed consent for tissue collection and research use was obtained from all patients in the Ontario Tumour Bank biobank, and participating health care institutions comply with all existing Canadian federal, provincial, and institutional requirements pertaining to the participation of patients in research as well as the collection and use of research biospecimens and accompanying clinical data (*https://ontariotumourbank.ca/about/oversight*, last accessed January 22, 2024). The OVCARE biobank is registered and certified under the Canadian CTRNet Biobank Program (*https://biobanking.org/canreg/view/83*, last accessed January 22, 2024).

Table 2 Demographic Information of the Women Included in the Training and Verification Studies

Variable	Training study (N = 534)					Verification study (N = 401)			
	High-grade serous ovarian cancer	Benign tumors	Nonovarian cancers	Inflammatory conditions	Healthy controls	High-grade serous ovarian cancer	Other ovarian cancers	Benign tumors	Healthy controls
N	89	192	87	42	124	67	85	111	138
Age, years									
Median	ND	64	63	44	51	64	66	65	64
Mean	ND	63	62	49	50	65	65	64	65
<31	0	4	0	3	21	0	0	0	0
31–40	0	8	1	15	17	0	0	0	0
41–50	3	22	11	7	23	0	3	0	0
51–60	17	43	23	8	30	21	24	39	44
61–70	29	55	33	5	19	27	34	45	68
71–80	19	39	16	3	13	17	21	23	26
>80	21	21	3	1	1	2	3	4	0
Ethnicity									
White	-	-	87	42	112	64	85	105	136
Black	-	-	0	0	10	-	-	-	-
Asian	-	-	0	0	0	-	-	-	-
Non-White*						1	-	-	1
Unknown	89	192	0	0	2	2	-	6	1
Sample source									
Mercy collection	0	0	0	0	124	-	-	-	-
OTB	17	0	0	0	0	-	-	-	-
OVCARE	72	192	0	0	0	-	-	-	-
ProteoGenex	0	0	87	42	0	-	-	-	-
UKOPS	-	-	-	-	-	67	85	111	138

*No further ethnicity data available.

OTB, Ontario Tumour Bank; OVCARE, ovarian cancer research program; UKOPS, UK ovarian cancer population study.

The 42 inflammatory condition and 87 nonovarian cancer plasma samples were collected in either K_2EDTA or K_3EDTA blood collection tubes and were purchased from ProteoGenex, who sourced them from Moscow, Russia, in accordance with their ethics policy (*https://www.proteogenex.com/about-us/ethics-policy*, last accessed January 22, 2024). Clinical annotation of the samples was also provided by ProteoGenex.

Algorithm Development

The real-time quantitative PCR C_T results from the samples in the training study for each of the three biomarker combinations in the OC Test panel were used to develop a robust algorithm for the specific and sensitive detection of HGSC, as outlined in the study overview in Figure 2. Plasma samples from the 89 women diagnosed with HGSC and from the 192 women with benign ovarian conditions were selected to minimize bias as a function of sample source or collection protocol. Women with benign ovarian conditions were used in lieu of healthy controls in the algorithm development to optimize algorithm specificity. Given the estimated sixfold higher prevalence of benign adnexal masses relative to ovarian malignancy,[14,15] an algorithm must deliver high specificity in this context to increase the probability of positive clinical impact.

Three modeling techniques were built in R version 4.0.5 and evaluated: Random Forest,[46] Elastic Net,[47] and XGBoost.[48] For both Random Forest version 4.6 to 14 and XGboost version 1.0.6.1, standard parameters were used to run the model. For the Elastic Net approach, the cv.glmnet package version 4.1 to 4 was used to assess different values for the α parameters that determine the trade-off between L1 (lasso) and L2 (ridge) regularization.

Each model was rerun 200 times, each time using a random sampling of 70% of the samples as a training set, running a full model using all features, and ranking them by feature importance. For Random Forest, this was done by using the reduction of the mean Gini index; for Elastic Net, the sum of the β values across all possible λ values; and for XGBoost, the absolute magnitude of linear coefficients. On the basis of this approach, an algorithm was trained on this subset and applied to the 30% of the test set that had been left out. The area under the curve as well as the sensitivity at 98% specificity for both benign versus early-stage HGSC as well as all HGSC samples was recorded within each iteration and used as a performance metric to select the final model and features. The test algorithm was set to return a test score between zero and one for each sample tested. The final algorithm was selected for the OC Test based on maximizing its area under the curve and discrimination of

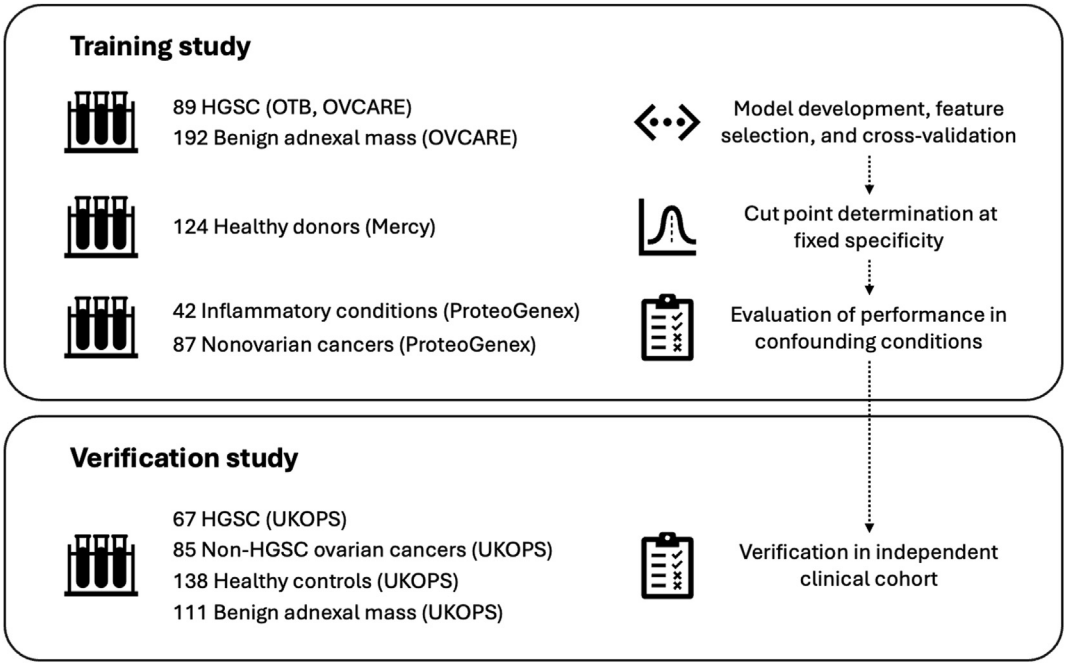

Figure 2 Overview of the design for the training and verification studies. Training study: The training study was composed of five cohorts of plasma samples sourced from academic and commercial biobanks and prospective collection. High-grade serous carcinoma (HGSC) and benign mass cohorts were used to develop a machine learning model to discriminate cancer from benign disease. A cutoff was determined in a separate cohort of healthy donors at a fixed specificity of 98.5%. Accuracy of this model was then assessed in cohorts of confounding inflammatory conditions and nonovarian cancers. Verification study: Performance of the model was assessed in samples from the UK Ovarian Cancer Population Study (UKOPS) as a forward evaluation in an independent cohort. OTB, Ontario Tumour Bank; OVCARE, Ovarian Cancer Research Program.

early-stage OC from benign gynecologic tumors while using the smallest number of different biomarker combinations, and uses an Elastic Net logistic model. The final coefficients are the average of the 200 coefficients from the model training used to optimize the algorithm. To evaluate performance of the final model for HGSC in the training study, the authors re-applied the model (algorithm and features) in a leave-one-out approach to generate predictions for each sample.

Establishment of the Clinical Cutoff Value for the OC Test Score

The test algorithm was then used to evaluate and score the 122 of 124 healthy control EDTA plasma samples in the training study that returned results for all three combinations in the OC Test and could therefore generate a test score. These samples were not used in the development of the test algorithm and were used only for the purpose of estimating the upper limit of normal in a healthy population. The cutoff was set to the 98.5th percentile OC Test score in this cohort. An initial estimate derived from calculation of a cutoff from each iteration of cross-validation was determined to be 0.259 (95% CI, 0.048−0.491). Application of the final model further refined the value to 0.243. On the basis of these results, the cutoff for the test score was set at 0.250, and the algorithm and cutoff (collectively the classifier) were then locked for a forward independent application in the verification study.

Verification Study Samples

The samples used in the verification study were from the UK Ovarian Cancer Population Study (UKOPS) biobank, which recruited 4823 women between 2006 and 2010 and included matched EDTA plasma and serum samples from healthy postmenopausal women and women with benign ovarian masses and OC.[49] The healthy UKOPS controls were recruited from women attending annual screening appointments in the multimodal screening arm of UKCTOCS. Ethical approval for the UKOPS biobank was obtained from the Joint University College London/University College London Hospital Committees on the Ethics of Human Research (Committee A) MREC number 05/Q0505/ Q11 58 and site-specific approval from the local regional ethics committees.[49] The OC cases and benign samples were recruited from UK gynecological oncology clinics (1200 in total). Copies of the surgery and histology reports were forwarded to the UKOPS research team. All were independently reviewed by a consultant gynecological oncologist who confirmed the diagnosis, stage, grade, and histotype of borderline and invasive epithelial OC, as well as the diagnosis of benign cases. The OC samples for the verification study were all from women with OC who had donated samples before starting treatment.

Blood samples were collected in red-top serum tubes (catalog number 367820; Becton Dickinson) and K_3EDTA plasma tubes (catalog number 455036; Greiner Bio-One, Stonehouse, UK). After blood draw, the serum tubes were

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allowed to stand at room temperature for 60 minutes, transferred to wet ice, and centrifuged and stored within 3 hours. The K_3EDTA plasma tubes were placed immediately on wet ice and processed within 6 hours of collection. Both plasma and serum were stored at −80°C at the centers. The blood samples were couriered on dry ice to the University College London central laboratory at prearranged intervals. All samples were then thawed and aliquoted into 500 μL straws that were then stored in liquid nitrogen tanks. The tanks were stored at Health Technology Assessment−approved commercial cryofacilities, initially at Fisher Bioservices (Bishop's Stortford, UK) and then at BioDock (Nottingham, UK). Hence, all samples had undergone one freeze thaw. Serum CA125 values were measured in the central laboratory using an electrochemiluminescence sandwich immunoassay on an Elecsys 2010 (Roche Diagnostics, Burgess Hill, UK) using two monoclonal antibodies (OC125 and M11; Fujirebio Diagnostics AB, Göteborg, Sweden).

Sample aliquots were shipped frozen on dry ice from the UKOPS biorepository to Mercy BioAnalytics and were then stored at −80°C. All samples were de-identified before shipment to Mercy BioAnalytics. Analysis of the OC Test was performed blinded to clinical status. After unblinding the clinical annotation for each sample, the performance of the OC Test classifier score was compared with results from CA125 ELISA testing.

In addition to the 401 serum samples, the sample set included matched K_3EDTA plasma samples for 20 of the healthy controls and 20 of the HGSC cases to enable evaluation of the relative performance of the OC Test and CA125 ELISA in these two sample matrices. Full sample annotation for the verification study samples can be found in Supplemental Table S5.

Statistical Analysis

Algorithm development techniques are described in detail above. Performance in the cross-validation is described as mean sensitivity across 200 iterations with empirical CIs. Specificity in healthy controls and sensitivity for detection of HGSC (overall and by stage) for the OC Test and for serum CA125 were calculated in the verification set and reported with proportional CIs (Wilson). In addition, in the verification set, sensitivity for detection of borderline and invasive epithelial non-HGSC was calculated. OC Test performance relative to the CA125 ELISA was evaluated in three groups of confounding conditions, benign adnexal masses, nonovarian cancers, and inflammatory conditions, all of which have been reported in the literature to generate elevated CA125 values in some women.[41] These groups were each evaluated separately in both the training and the verification studies.

In an exploratory analysis, the OC Test score for the OC cases in the verification study was compared with their corresponding 10-year survival data (145 cases from the UKOPS). Survival data were censored by UKOPS investigators on February 28, 2016, and were provided for this analysis after assignment of the OC Test score.

Statistical analysis was performed in R version 4.0.5 and JMP Pro 17.1.0 (JMP Statistical Discovery, Cary, NC). Data analysis of analytical studies followed Clinical Laboratory Standards Institute guidelines.[44,45,50,51]

The results published here are in whole or part based on data generated by The Cancer Genome Atlas Research Network (*https://www.cancer.gov/ccg/research/genome-sequencing/tcga*, last accessed January 17, 2024). The RNA-sequencing data (Genotype-Tissue Expression Analysis V8) used for the analyses described in this article were obtained from the Genotype-Tissue Expression Portal (*https://www.gtexportal.org/home*, last accessed April 8, 2021).

Results

Analytical Validation

Test Linearity

Before running the training study, the linearity of the C_T values for each biomarker combination and the linearity of the final assay score were assessed. The test score was linear over the 4-log range of 19,500 to 320 million EVs per well, significantly above the algorithm's cutoff between healthy and OC and did not exhibit a high-dose hook effect (Figure 3 and Supplemental Table S3). [F3]

OC Test Reproducibility

To establish interday reproducibility, the five-level EV controls were run on 16 separate days of the training study, and the overall test reproducibility of the OC Test score was calculated from these data. Higher CVs (12.8%) were observed for the unspiked plasma control, representing healthy background, with lower CVs observed (<2%) for the EV-spiked controls. This result was subsequently confirmed during the 10 days of the verification study (Supplemental Tables S1-S3).

Serum and Plasma Matrix Equivalency

The initial equivalency study on 45 donor-matched serum and EDTA plasma samples showed strong correlation in performance between sample types [y (serum) = $0.9732x$ (EDTA plasma) − 0.0088, $R^2 = 0.983$, $N = 45$]. A similar equivalency study on 38 matched serum and EDTA plasma samples selected from within the verification study also exhibited strong correlation [y (serum) = $0.9105x$ (EDTA plasma) + 0.0741, $R^2 = 0.930$, $N = 38$] (Supplemental Figure S1 and Supplemental Table S3).

For the CA125 ELISA assay, the healthy control EDTA plasma ($N = 124$) and serum samples ($N = 138$) from the training and verification studies (Tables 1 and 2) were used to determine the upper limit of normal at 98% specificity for each sample matrix independently.[51] The upper limit of

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normal for EDTA plasma was determined to be 19.6 U/mL, and the upper limit of normal for serum was determined to be 15.5 U/mL (Supplemental Tables S4 and S5).

Training and Verification Study Results

The demographics for the women in the training and verification studies are summarized in Tables 1 and 2. More detailed sample annotation and all test results for both studies can be found in Supplemental Tables S4 and S5. To verify that the CA125 ELISA values obtained during the training and verification studies using biobanked samples that had been stored frozen at −80°C for up to 15 years were still representative of their original CA125 values, the authors compared the in-house assay results with the previously reported CA125 values. The CA125 values from the R&D Systems CA125 ELISA run at Mercy BioAnalytics and the OVCARE assay from the same donors at the time of original sample collection was compared and showed good correlation of test results [y (Mercy CA125) = 0.8289x (OVCARE CA125) + 0.9653, R^2 = 0.8025, N = 72] (Supplemental Figure S2A). Similarly, the comparison of the results from the R&D Systems CA125 ELISA run at Mercy BioAnalytics and the Roche Elecsys CA125 II assay run at UCL from the same UKOPS donors at the time of original sample collection also showed good correlation of test results [y (Mercy CA125) = 0.8717x (UCL CA125) + 1.4963, R^2 = 0.959, N = 318] (Supplemental Figure S2B). On this basis, the samples were deemed to have been properly stored and representative of the original CA125 test results.

Because of limited sample volume, samples that did not return a CA125 ELISA or OC Test result on the first run were excluded from the downstream data analysis. In the training study cross-validation, the OC Test exhibited mean all-stage HGSC sensitivity of 85.9% (95% CI, 72.4%–96.3%) and mean early-stage HGSC sensitivity of 79.4% (95% CI, 60.0%–94.1%). The authors applied a leave-one-out approach to generate predictions for each sample in the training set to compare pairwise performance between the OC Test and CA125. In this application, the OC Test result was available for 87 of the 89 women with HGSC and for 122 of the 124 healthy controls (Figure 4, A and C). The overall OC test sensitivity for HGSC detection was 93.1% (81/87; 95% CI, 85.8%–96.8%). Stage information was not available in two women. In the remaining 85 women, the OC Test detected 82.4% (14/17) of stage I, 91.2% (31/34) of stage II, and 100% (34/34) of stage III HGSC cases. In comparison, the CA125 ELISA assay had a specificity in healthy controls of 97.5% (119/122; 95% CI, 93.0%–99.5%) and a sensitivity of 92.0% (80/87; 95% CI, 84.3%–96.0%) for HGSC. Serum CA125 detected 76.5% (13/17) stage I, 91.2% (31/34) stage II, and 100% (34/34) stage III of the 85 HGSC cases with stage information.

Two stage II cases were missed only by the OC Test, one stage I case and two stage II cases were missed only by CA125, and three stage I cases and one stage II case were missed by both tests. Within the 10 HGSC cases that were known to be *BRCA1* or *BRCA2* germline mutation positive, the OC Test detected 90% (9/10) of the cases, missing one small (1.5-cm) tubal stage IA cancer in a 65-year–old *BRCA1*-positive woman. This case was also missed by CA125.

Performance of the OC Test and classifier was then evaluated in the independent, case-control verification study set. In 390 of the 401 women, it was possible to obtain both an OC Test score and a CA125 ELISA value, including serum samples from 132 healthy controls and 66 women with HGSC (Figure 4, B and D). Using the algorithm and cutoff established in the training cohort (the classifier), the multibiomarker OC Test showed a sensitivity for detection of HGSC of 97.0% (64/66; 95% CI, 89.8%–99.2%), a

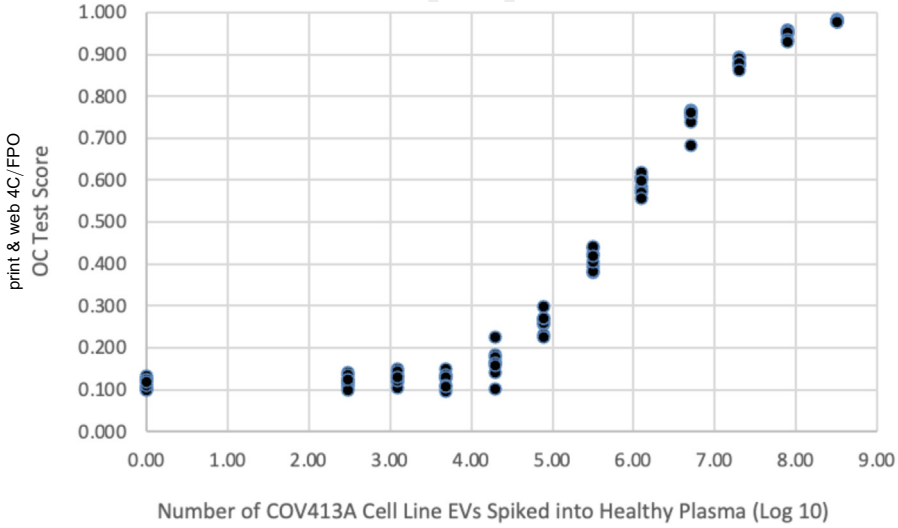

Figure 3 Linearity of the OC Test. Plasma was spiked with extracellular vesicles (EVs) isolated from the COV413A ovarian cancer cell line to cover the full range of OC Test scores.

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specificity in healthy controls of 97.0% (128/132; 95% CI, 92.4%–99.6%), and an area under the curve of 0.97 (95% CI, 0.93–0.99). By comparison, CA125 had a sensitivity for detection of HGSC of 87.9% (58/66; 95% CI, 77.5%–94.6%), a specificity in healthy controls of 95.5% (126/132; 95% CI, 90.3%–98.3%), and an area under the curve of 0.90 (95% CI, 0.86–0.93). The difference in sensitivity between the OC Test and CA125 was statistically significant (McNemar test $P = 0.0143$). The OC Test sensitivity for stage I/II HGSC was 89.5% (17/19; 95% CI, 66.9%–99.2%) compared with a CA125 sensitivity of 63.2% (12/19; 95% CI, 38.6%–83.7%). The only HGSC cases missed by the OC Test were two stage I cases, which were also missed by CA125.

The OC Test detected 73.5% (61/83; 95% CI, 62.7%–82.6%) of the borderline and non-HGSC cases compared with CA125, which detected 85.5% (71/83; 95% CI, 76.1%–92.3%) of the borderline and non-HGSC cases (Figure 4 and Table 3). The OC histotypes missed most [T3] often by the OC Test were borderline serous and low-grade

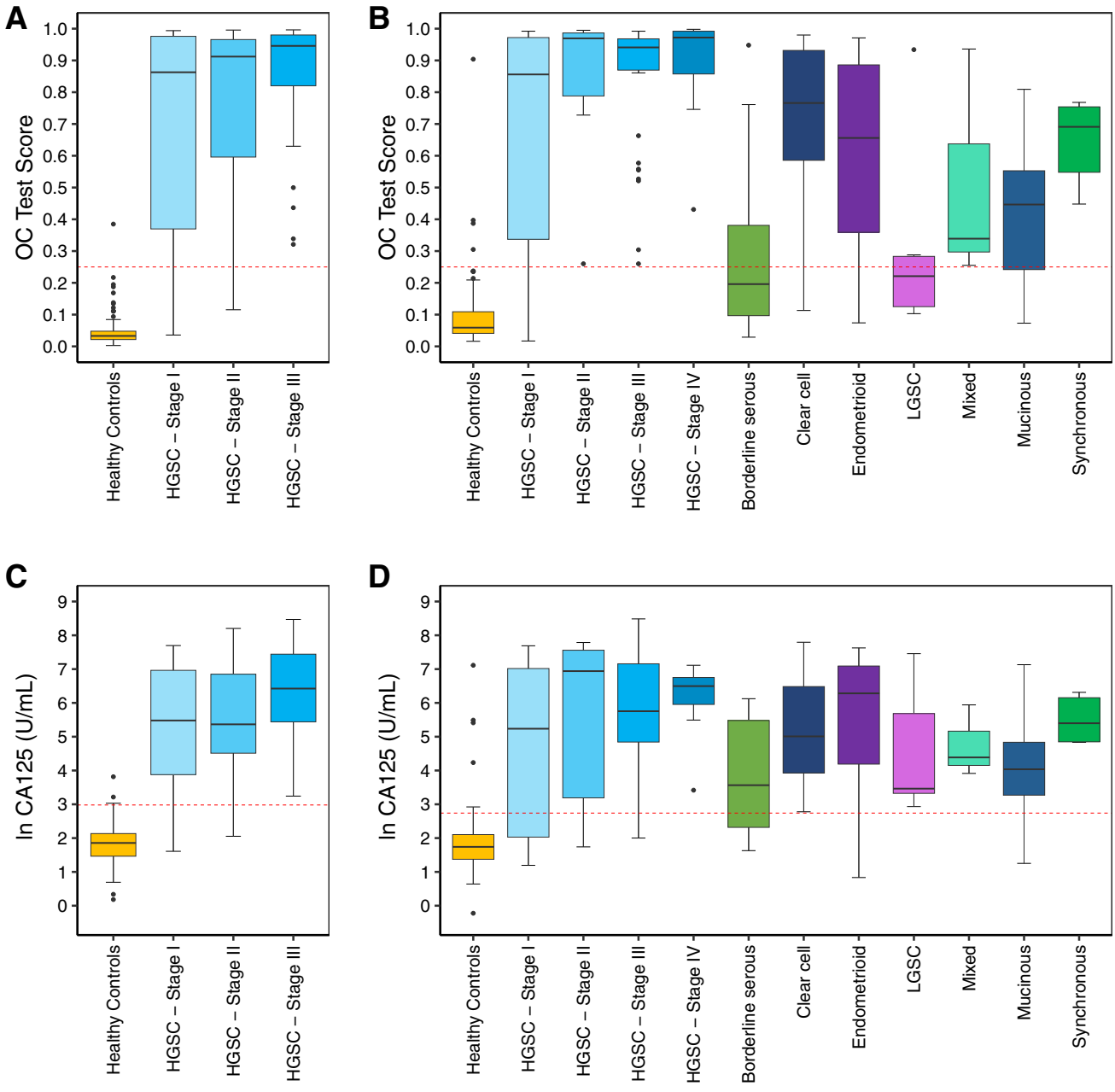

Figure 4 OC Test and CA125 enzyme-linked immunosorbent assay performance in healthy controls and in ovarian cancers. **A** and **C:** Training study EDTA plasma samples are shown. **B** and **D:** The verification study serum samples are shown. The cutoff set between healthy controls and OC cases is shown as a **red dotted line** in each graph. HGSC, high-grade serous carcinoma; LGSC, low-grade serous carcinoma.

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Table 3 Comparison of the Sensitivity of the OC Test and CA125 by Ovarian Cancer Histotype and Stage in the Verification Study

Ovarian cancer histotype	Total sample, *N*	OC Test positive, *N*	OC Test sensitivity, %	CA125 positive, *N*	CA125 sensitivity, %
HGSC (all)	66	64	97.0	57	86.4
HGSC (stage I)	13	11	84.6	8	61.5
HGSC (stage II)	6	6	100.0	4	66.7
HGSC (stage III)	40	40	100.0	39	97.5
HGSC (stage IV)	7	7	100.0	6	85.7
Borderline serous (all)	**20**	**8**	**40.0**	**13**	**65.0**
Borderline serous (no stage reported)	7	2	28.6	4	57.1
Borderline serous (unable to stage)	2	1	50.0	2	100.0
Borderline serous (stage I)	10	5	50.0	6	60.0
Borderline serous (stage III)	1	0	0.0	1	100.0
Clear cell (all)	**20**	**19**	**95.0**	**19**	**95.0**
Clear cell (stage I)	14	13	92.9	13	92.9
Clear cell (stage II)	3	3	100.0	3	100.0
Clear cell (stage III)	2	2	100.0	2	100.0
Clear cell (stage IV)	1	1	100.0	1	100.0
Endometrioid (all)	**15**	**13**	**86.7**	**13**	**86.7**
Endometrioid (stage I)	12	10	84.6	10	84.6
Endometrioid (stage II)	1	1	100.0	1	100.0
Endometrioid (stage III)	2	2	100.0	2	100.0
LGSC (all)	**6**	**3**	**50.0**	**6**	**100.0**
LGSC (stage I)	2	1	50.0	2	100.0
LGSC (stage II)	2	1	50.0	2	100.0
LGSC (stage III)	2	1	50.0	2	100.0
Mixed (all stage I)	**3**	**3**	**100.0**	**3**	**100.0**
Mucinous (all)	**14**	**10**	**71.4**	**12**	**85.7**
Mucinous (stage I)	12	9	75.0	10	83.3
Mucinous (stage III)	2	1	50.0	2	100.0
Synchronous (all)	**5**	**5**	**100.0**	**5**	**100.0**
Synchronous (stage I)*	3	3	100.0	3	100.0
Synchronous (stage II)†	1	1	100.0	1	100.0
Synchronous (stage III)‡	1	1	100.0	1	100.0
Overall performance	**149**	**125**	**83.9**	**129**	**86.6**

In this study, the OC Test had a specificity in healthy controls of 97.0%, and CA125 had a specificity in healthy controls of 95.5%.

*Endometrioid adenocarcinoma, synchronous tumors of ovary (stage IC) and endometrium (stage IIB); endometrioid adenocarcinoma; synchronous tumors of ovary (stage I, grade 2) and endometrium (stage IB, grade 1); clear cell adenocarcinoma; synchronous tumors of ovary (stage IC grade 3) and endometrium (stage IB, grade 2).

†Endometrioid adenocarcinoma; synchronous tumors of ovary (stage II, grade 2) and endometrium (stage IA, grade 2).

‡Endometrioid adenocarcinoma; synchronous tumors of ovary (stage IIIC, grade 2) and endometrium (stage I, grade 2).

HGSC, high-grade serous carcinoma; LGSC, low-grade serous carcinoma.

serous OC. Overall, the OC Test detected 83.9% (125/149; 95% CI, 77.0%–89.4%) of all the OC cases compared with CA125, which detected 86.6% (129/149; 95% CI, 80.0%–91.6%) of all the OC cases (Figure 4 and Table 3).

Effect of Confounding Conditions on Test Specificity

Improvement in test performance relative to CA125 was seen with three groups of confounding conditions, benign adnexal masses, nonovarian cancers, and inflammatory conditions.

Specificity in Subjects with Benign Ovarian Tumors

The false-positive rate for the 188 of 192 samples collected from women with benign adnexal masses in the training study that returned both an OC Test score and a CA125 ELISA result was significantly lower using the OC Test (14/188, 7%; 95% CI, 5%–12.2%) compared with using plasma CA125 (103/188, 55%; 95% CI, 48%–62%) (Figure 5, A and C). Similarly, the false-positive rate for the 109 of 111 benign samples in the verification study with test results available from both assays was lower for the OC Test (16/109, 15%; 95% CI, 9%–23%) compared with serum CA125 (38/109, 35%; 95% CI, 27%–44%) (Figure 5, B and D).

Specificity in Subjects with Nonovarian Cancers and Inflammatory Conditions

The OC Test had fewer false-positive results for nonovarian (off-target) cancers (14/83, 17%; 95% CI, 10%–26%) when

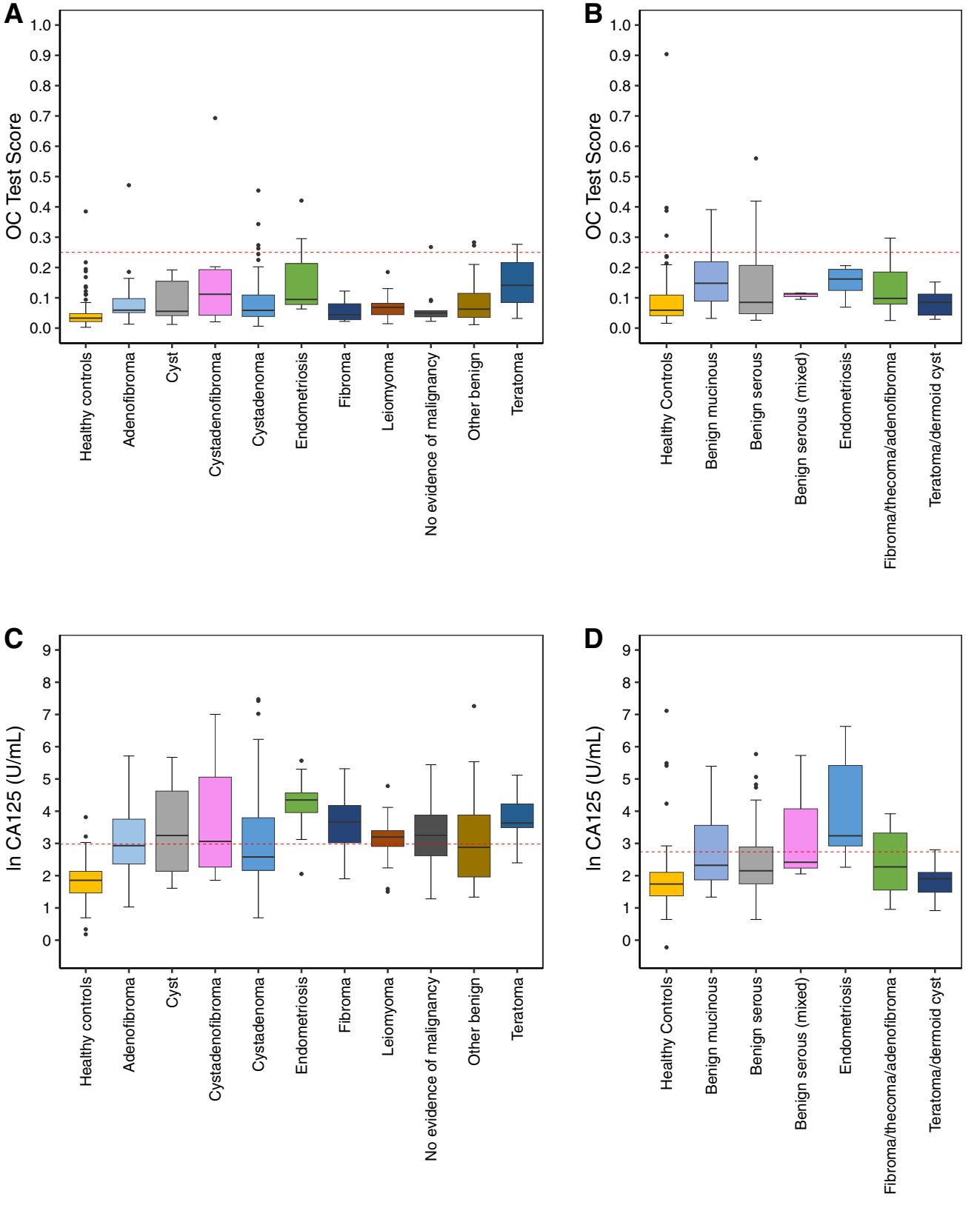

Figure 5 OC Test and CA125 enzyme-linked immunosorbent assay performance in benign ovarian conditions. **A** and **C:** Training study EDTA plasma samples are shown. **B** and **D:** The verification study serum samples are shown. The cutoff set between healthy controls and OC cases is shown as a **red dotted line** in each graph.

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Table 4 Comparison of the Performance of the OC Test and CA125 in Nonovarian Cancers and Inflammatory Conditions

Variable	Ovarian cancer test			CA125		
	Detected, *N*	Not detected, *N*	False-positive rate, %	Detected, *N*	Not detected, *N*	False-positive rate, %
Nonovarian cancers	14	69	16.8	23	60	27.7
Bladder (urothelial carcinoma)	0	14	0	2	12	14.3
Breast (infiltrating ductal carcinoma)	1	14	6.7	2	13	13.3
Colorectal (adenocarcinoma)	0	12	0	2	10	16.7
Lung (adenocarcinoma)	5	8	38.5	5	8	35.7
Pancreatic (adenocarcinoma)	2	13	13.3	4	11	26.7
Uterine (endometrial adenocarcinoma)	6	8	42.9	8	6	57.1
Inflammatory conditions	**1**	**41**	**2.4**	**7**	**35**	**16.7**
Crohn disease	0	7	0	3	4	42.9
Diabetes, type 2	1	6	14.3	3	4	42.9
Endometriosis	0	7	0	0	7	0
Acute pancreatitis	0	7	0	0	7	0
Rheumatoid arthritis	0	7	0	1	6	14.3
Ulcerative colitis	0	7	0	0	7	0

compared with CA125 (23/85, 27%; 95% CI, 19%–37%) (Table 4 and Figure 6). The frequency of false positives with the OC Test due to nonovarian cancers in an asymptomatic screening population is expected to be <0.04% based on the population incidence of these cancers (Table 5).

The OC Test also significantly outperformed CA125 in samples from women with the inflammatory conditions listed in Table 4 and Figure 6, with a much lower false-positive rate observed for these conditions (1/42, 2%; 95% CI, 0%–12%) compared with the false-positive rate with the CA125 assay (7/42, 17%; 95% CI, 8%–31%).

Correlation of the OC Test Score with Overall Survival

In the 145 cases from the verification study where survival data were available, the OC Test score was compared with their corresponding 10-year survival data. Survival by histotype is shown in Table 6 and the distribution of the OC Test scores across survival outcomes is shown in Figure 7. Overall, 51.7% of the OC cases were alive at the time of data censoring, with survival decreasing as a function of cancer stage at diagnosis. As expected, survival varied by histotype and stage at diagnosis, with stages II to IV HGSC, mixed, and mucinous OCs having the poorest 10-year survival. By contrast, stage 1 HGSC, low grade serous, borderline serous, and endometrioid OCs had the best overall survival. The distribution of the OC Test scores across survival outcomes (Figure 7) showed a trend toward higher test scores in those who died than in those who survived (median score of 0.687 for those still alive compared with 0.912 for those who died); however, the OC Test score alone is not an accurate predictor of survival outcome.

Discussion

This study confirms that targeting tumor-associated EVs, coupled with the rational design of OC-specific biomarker combinations, enables sensitive and specific detection of early-stage HGSC, where concentrations of OC-associated EVs from serum or plasma are likely to be low. This OC Test was specifically designed to detect single EVs displaying up to three colocalized, OC-associated biomarkers, all with a strong biologic basis for inclusion.

Detection of early-stage clinically diagnosed HGSC is an important first step in the development of an OC test to be used for OC screening with the ultimate goal to decrease disease-specific mortality. Despite being developed primarily for detection of HGSC, the OC Test also picked up invasive clear cell, endometrioid, and mucinous OC histotypes (Table 3 and Figure 4).

The lower rate of false-positive calls from most benign conditions with the OC Test, compared with CA125 (Figure 5), is a particularly valuable attribute of the OC Test, as benign adnexal masses are six times more common than OCs.[15] It is a crucial requirement in the OC screening setting where benign masses and other more prevalent cancers often yield false-positive results, leading to harms due to patient anxiety, complications from additional diagnostic testing and surgical interventions, and increased costs to the health care system. Endometrial carcinomas and late-stage lung adenocarcinomas generated the highest level of false positives with the OC Test (Figure 6). Endometrial carcinomas are likely to be detected during abdominal imaging that would follow a positive OC Test result. False-positive results due to the presence of late-stage lung adenocarcinoma occurred with both the OC Test and CA125. Therefore, this possibility would need to be considered for any woman with a positive result with either test, but negative abdominal imaging results. In the UKCTOCS trial, a chest computed tomography scan was in fact part of the protocol in such scenarios.[10]

To date, neither efforts to achieve similar performance by the addition of more serum biomarkers, such as HE4, CA72-4, or MMP-7, to CA125, nor the use of circulating tumor

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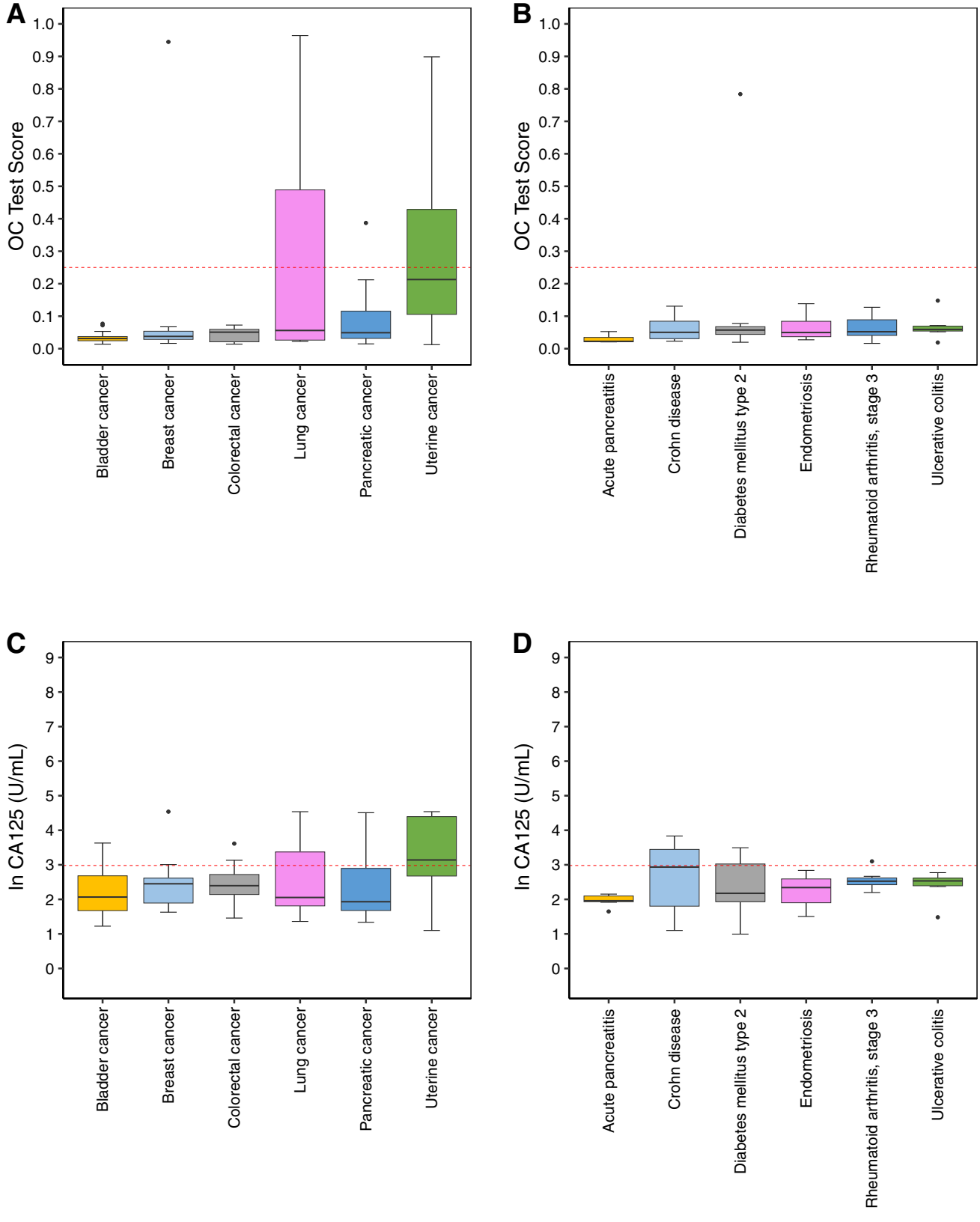

Figure 6 OC Test and CA125 enzyme-linked immunosorbent assay performance in nonovarian cancers and inflammatory conditions. **A** and **C:** Nonovarian (off-target cancer) samples are shown. **B** and **D:** The inflammatory condition samples are shown. The cutoff set between healthy controls and OC cases is shown as a **red dotted line** in each graph.

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Table 5 Estimated Rates of False Positives due to Nonovarian Cancers if the OC Test or CA125 Was to Be Used for Screening an Average Risk Population

Cancer	Seer 5-year female incidence rate per 100,000 (2000 to 2017)*	% Positivity of the OC Test	Estimated number of false-positive OC Tests per 100,000 subjects tested	% Positivity of CA125	Estimated number of false-positive CA125 tests per 100,000 subjects tested
Bladder	8.2	0	0.0	14.3	1.1726
Breast	127.3	6.7	8.529	13.3	16.9309
Colorectal	34.0	0	0.000	16.7	5.678
Lung	47.1	38.5	18.134	35.7	16.8147
Pancreatic	11.5	13.3	1.530	26.7	3.0705
Uterine	26.8	42.9	11.497	57.1	15.3028
Total estimated false-positive rate (per 100,000)			**39.689**		**58.970**

*Data available (*https://seer.cancer.gov/statistics/preliminary-estimates/preliminary.html*, last accessed January 17, 2024).

DNA as an alternate analyte, have exceeded the performance of CA125.[52–54] There have been multiple smaller studies supporting the feasibility of using a variety of biomarkers found on or in circulating EVs for the detection of ovarian cancer.[55–61] However, none of these small studies included an evaluation of benign ovarian tumors to assess their potential to generate false-positive results. In contrast, the assay development efforts for the OC Test were purposefully focused on identifying biomarker combinations that would reduce all sources of false positives compared with CA125, while still maintaining high sensitivity for detection of OC. The study reported here, using large and independent training and verification sets from centers in the United States, Canada, Russia, and the United Kingdom, demonstrates that this novel OC Test can detect HGSC at an early stage and has reduced false positives compared with CA125.

These performance characteristics are important for both high- and average-risk women. In 2019, the US Preventive Services Task Force recommended against OC screening in asymptomatic women without hereditary cancer risk factors.[20] However, they noted that women with *BRCA1* and *BRCA2* germline mutations, Lynch syndrome, or a family history of OC are at increased risk for OC. They acknowledged that screening using a combination of CA125 and TVUS was the most common way to manage OC detection in hereditary-risk women who have not yet had risk-reducing salpingo-oophorectomy surgery, even if these screening modalities do not have the sensitivity and specificity to catalyze endorsement in the US guidelines.[20] In contrast, multimodal screening using ROCA and TVUS every 4 months has been recently recommended in the latest guidelines from NICE in the United Kingdom (*https://www.nice.org.uk/guidance/indevelopment/gid-ng10225*, last accessed September 16, 2024) for OC screening in high-risk women who have not yet had risk-reducing salpingo-oophorectomy surgery,[22] setting the test performance expectation for other high-risk screening approaches.

There are limitations in the design of the training and verification studies for the OC Test. First, all OC samples were prospectively collected and banked at the time of diagnosis and were not from an asymptomatic screening population. This was done intentionally, to establish test performance in symptomatic subjects before moving on to an evaluation of test performance in a screening cohort. Potential sources of bias in the training study include the

Table 6 Ten-Year Survival Status in the Verification Study Women by Ovarian Cancer Histotype

Histotype	Cases, *N*	Alive at time of censorship, *N* (%)	Deceased at time of censorship, *N* (%)
Borderline serous	20 (1 with unknown survival status)	16 (84.2)	3 (15.8)
Clear cell	20 (1 with unknown survival status)	12 (63.2)	7 (36.8)
Endometrioid	16	12 (75.0)	4 (25.0)
High-grade serous (all stages)	67 (3 with unknown survival status)	21 (32.8)	43 (67.2)
Stage 1	*13 (2 with unknown survival status)*	*8 (72.7)*	*3 (27.3)*
Stage 2	*7*	*3 (42.9)*	*4 (57.1)*
Stage 3	*40 (1 with unknown survival status)*	*8 (20.5)*	*31 (79.5)*
Stage 4	*7*	*2 (28.6)*	*5 (71.4)*
Low-grade serous	6	6 (100.0)	0 (0)
Mixed	3	1 (33.3)	2 (66.7)
Mucinous	15 (2 with unknown survival status)	5 (38.5)	8 (61.5)
Synchronous	5	3 (60.0)	2 (40.0)
Overall	**152 (*7 with unknown survival status*)**	**75 (51.7)**	**70 (48.3)**

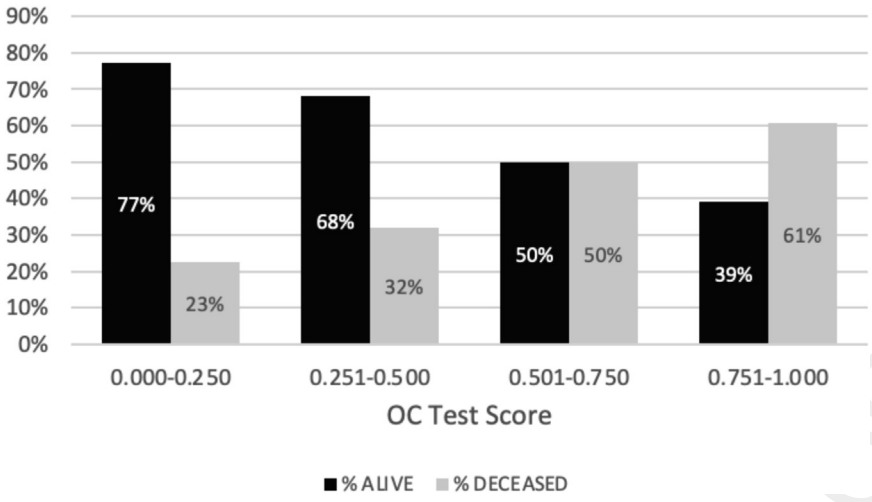

Figure 7 Correlation of the OC Test score with 10-year survival. Distribution of OC Test scores by survival status across all ovarian cancer cases in the verification study.

acquisition of samples from multiple sources, and the variability in duration of frozen storage from the time of sample collection. Concerns about long-term frozen storage were addressed by measuring CA125 in the same frozen sample aliquots that were used to produce results with the OC Test and comparing them with the CA125 values originally reported at the time of sample collection. Strong correlation across the CA125 test range was used as a surrogate for long-term sample stability. The concern over use of multiple sample sources in the training study was eliminated in the verification study (UKOPS), as these samples were all drawn from a single high-provenance collection.

There are novel targeted therapies available today that were not on the market during the time period when previous screening studies were conducted.[62–64] Moreover, several therapies have since demonstrated effectiveness in women with genetic risk factors, and expanded genetic testing enables broader application of these therapeutic options. *BRCA2*-mutated cancers have better responses than *BRCA* wild-type or *BRCA1*-mutated cases to platinum-based chemotherapy (cisplatin, carboplatin, and oxaliplatin).[65] Poly (ADP-ribose) polymerase inhibitors are known for their effectiveness against tumors with homologous repair defects, such as *BRCA*-mutated and Lynch syndrome–related tumors.[62] It is intriguing to speculate whether a randomized clinical trial assessing OC screening conducted in the context of currently available therapies would demonstrate a mortality benefit. The association of the OC Test score with overall survival, even in a group of women who at the time the study was conducted did not have these therapeutic options, is a promising finding that suggests the test is detecting aggressive cancers and warrants further evaluation.

The sensitivity of the OC Test for detection of OC in symptomatic women and the lower rate of false positives from confounding conditions supports further investigation of this novel approach as a potential blood-based OC screening test in asymptomatic women. Assessment of performance in prediagnostic samples from asymptomatic women in a general population screening trial (UKCTOCS) is in progress. Future studies will investigate its potential clinical application in high-risk populations.

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Author Contributions

D.G., L.T.B., A.D.C., E.S.W.-D., S.J.S., U.M., S.A., and A.G.-M. conceptualized the study; J.N.M., A.J., D.H., U.M., S.A., and A.G.-M. performed sample collection, biobanking, and clinical annotation;; S.B., J.G., U.M., A.G.-M., and S.A. performed methods; D.G., L.T.B., A.D.C., E.S.W.-D., D.P.S., I.O.Z., C.R.S., D.M.B., B.F.H., M.S.K., L.T.C., and P.A.D. performed study execution; K.C., D.G., K.M.B., E.S.W.-D., B.J.M., and T.B.H. performed investigation and data analysis; K.C., T.B.H., and E.S.W.-D. performed visualization; A.J., J.N.M., and D.H. acquired

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Disclosure Statement

L.T.B., A.D.C., D.P.S., S.B., I.O.Z., D.M.B., M.S.K., L.T.C., B.J.M., T.B.H., T.G., and D.M. are current employees of Mercy BioAnalytics Inc. E.S.W.-D. is a retired Mercy BioAnalytics employee and is currently a paid consultant of Mercy BioAnalytics Inc. She was a full-time employee when this work was performed. K.M.B., P.A.D., J.G., D.G., B.F.H., and C.R.S. are former employees of Mercy BioAnalytics Inc., who were active employees at the time this work was performed. S.J.S. and K.C. are paid consultants for Mercy BioAnalytics Inc. A.J., J.N.M., and D.H. are employees of University of British Columbia and provided the patient samples used for the training study. A.G.-M., S.A., and U.M. are employees of University College London and provided the patient samples used for the verification study. They also report research collaborations with Cambridge University, QIMR Berghofer Medical Research Institute, Intelligent Lab on Fiber, RNA Guardian, Micronoma, Imperial College London, University of Innsbruck, and Dana Farber USA in the area of early detection of cancer. U.M. had stock ownership (2011 to 2021) awarded by University College London in Abcodia, which held the license for the Risk of Ovarian Cancer Algorithm. She has received grant funding from the Medical Research Council, Cancer Research UK, the National Institute for Health Research UK, the Eve Appeal, and the Australian National Health and Medical Research Council. She is also a member of Tina's Wish Scientific Advisory Board (United States) and the Research Advisory Panel, Yorkshire Cancer Research (United Kingdom). L.T.B. and D.P.S. are inventors on US patent number 11,085,089 B2, Systems, Compositions and Methods for Target Entity Detection (issued August 10, 2021). L.T.B., D.P.S., E.S.W.-D., D.G., K.M.B., and A.D.C. are inventors on US patent application 63/417309, Composition and Methods for Detection of Ovarian Cancer (filed October 18, 2022). U.M. holds patent number EP10178345.4 for Breast Cancer Diagnostics.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2024.09.001*.

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Supplemental Figure S1 Comparison of the performance of the OC Test with serum and EDTA plasma. **A:** Data based on paired samples sourced from multiple vendors. **B:** Data based on paired samples from within the verification cohort. Both cohorts contain approximately equal numbers of healthy controls and high-grade serous carcinoma cases.

Supplemental Figure S2 Comparison of the serum CA125 assay results by different test methods and sample types. **A:** R&D Systems CA125 enzyme-linked immunosorbent assay (ELISA) run by Mercy BioAnalytics concurrently with the OC Test and the Ovarian Cancer Research Program (OVCARE) assay from the same EDTA plasma donors at the time of original sample collection. **B:** R&D Systems CA125 ELISA run by Mercy BioAnalytics concurrently with the OC Test and the Roche Elecsys CA125 II assay run at UCL from the same serum donors at the time of original sample collection.