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A NOVEL MULTI-ANALYTE SERUM PANEL COMBINING CA-125, HE4, MICRORNA-200a, AND INTERLEUKIN-6 FOR THE PRECLINICAL DETECTION OF HIGH-GRADE SEROUS OVARIAN CARCINOMA IN WOMEN WITH PATHOGENIC BRCA1/2 VARIANTS

NOVI MULTIANALITNI SERUMSKI PANEL KOJI KOMBINUJE CA-125, HE4, MIKRORNA-200a I INTERLEUKIN-6 ZA PRETKLINIČKO OTKRIVANJE VISOKOGRADUSNOG SEROZNOG KARCINOMA JAJNIKA KOD ŽENA SA PATOGENIM VARIJANTAMA BRCA1/2 GENA

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Summary

Background: High-grade serous ovarian cancer (HGSOC), which is common among pathogenic variant carriers of BRCA1 or BRCA2, has high mortality rates due to late-stage diagnosis, and therefore, it is important to have effective surveillance methods available to detect HGSOC. At present, the use of CA-125 and transvaginal ultrasound for monitoring women at high risk of developing HGSOC is not accurate for detecting early-stage disease.
Methods: To develop and internally validate a multi-analyte serum panel for detecting HGSOC before clinical diagnosis in BRCA1/2 carriers. From 450 BRCA1/2 carriers enrolled in a prospective, multi-institutional cohort study who received blood samples every six months, we identified 45 new cases of HGSOC and matched them with 405 controls. Blood samples were collected and subsequently analysed for CA-125, HE4, IL-6, and miR-200a using ELISA and RT-qPCR. A logistic regression-derived MRS was used to assess whether the combined measurement of the four biomarkers could improve HGSOC diagnosis.
Results: All four biomarkers were statistically significantly increased in HGSOC pre-diagnosis cases ($p<0.001$). The combined panel of biomarkers had 0.95 (95% confidence interval [CI]: 0.92–0.98) area under the curve (AUC) compared to individual markers (CA-125 AUC=0.86; HE4 AUC=0.82; miR-200a AUC=0.88; IL-6 AUC=0.70; DeLong's test $p<0.01$). At a specificity of 90%, the MRS was sensitive to HGSOC 88% of the time, compared with 68% when CA-125 was used alone. The MRS reached

Kratak sadržaj

Uvod: Visokogradusni serozni karcinom jajnika (HGSOC), koji je čest kod nosilaca patogenih varijanti gena BRCA1 ili BRCA2, ima visoku stopu smrtnosti zbog dijagnoze postavljene u kasnim stadijumima bolesti, zbog čega je važno imati efikasne metode nadzora za njegovo otkrivanje. Trenutno, primena CA-125 i transvaginalnog ultrazvuka u praćenju žena sa visokim rizikom za razvoj HGSOC nije dovoljno precizna za otkrivanje bolesti u ranom stadijumu. Cilj: Razviti i interno validirati multianalitni serumski panel za otkrivanje HGSOC pre kliničkog postavljanja dijagnoze kod nosilaca BRCA1/2 mutacija.
Metode: Od 450 nosilaca BRCA1/2 varijanti uključenih u prospektivnu, multicentričnu kohortnu studiju, kojima su uzorci krvi uzimani na svakih šest meseci, identifikovano je 45 novih slučajeva HGSOC i upoređeno sa 405 kontrolnih pacijentkinja. Uzorci krvi su prikupljeni i naknadno analizirani na CA-125, HE4, IL-6 i miR-200a primenom ELISA i RT-qPCR metoda. Za procenu da li kombinovano merenje četiri biomarkera može unaprediti dijagnostiku HGSOC korišćen je MRS izveden logističkom regresijom.
Rezultati: Sva četiri biomarkera bila su statistički značajno povišena u slučajevima HGSOC pre postavljanja dijagnoze ($p<0,001$). Kombinovani panel biomarkera imao je površinu ispod ROC krive (AUC) od 0,95 (95% CI: 0,92–0,98) u poređenju sa pojedinačnim markerima (CA-125 AUC=0,86; HE4 AUC=0,82; miR-200a AUC=0,88; IL-6 AUC=0,70; DeLongov test $p<0,01$). Pri specifičnosti od 90%, MRS je imao senzitivnost za HGSOC od 88%,

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the positivity threshold a median of 12 months (IQR 8–16 months) before clinical diagnosis; 72% of cases (32/45) had MRS results indicating the possibility of HGSOC at least 18 months before diagnosis.

Conclusion: Our study demonstrates the superior diagnostic accuracy of the serum panel comprising CA-125, HE4, IL-6, and miR-200a, which can serve as a more accurate preclinical diagnostic test for HGSOC in pathogenic BRCA carriers. This panel has transformative potential for structured surveillance programs, enabling earlier intervention and personalised risk management.

Keywords: serum CA-125, HE4, miR-200a, IL-6, HGSOC, BRCA

u poređenju sa 68% kada je korišćen samo CA-125. MRS je dostizao prag pozitivnosti medijanski 12 meseci (IQR 8–16 meseci) pre kliničkog postavljanja dijagnoze; kod 72% slučajeva (32/45) MRS rezultati su ukazivali na mogućnost HGSOC najmanje 18 meseci pre dijagnoze.

Zaključak: Naša studija pokazuje superiornu dijagnostičku tačnost serumskog panela koji obuhvata CA-125, HE4, IL-6 i miR-200a, a koji može da služi kao precizniji pretklinički dijagnostički test za HGSOC kod nosilaca patogenih BRCA varijanti. Ovaj panel predstavlja transformativni potencijal za strukturirane programe nadzora, omogućavajući raniju intervenciju i personalizovano upravljanje rizikom.

Ključne reči: serumski CA-125, HE4, miR-200a, IL-6, HGSOC, BRCA

Introduction

Ovarian cancer mortality is highest with high-grade serous ovarian carcinoma (HGSOC) accounting for approximately 70% of all ovarian cancer-related deaths, with five-year survival rates which substantially decrease as one advances from stage I (greater than 90% survival rate) to stage III (less than 30% survival rate) (1). This clearly shows how deadly ovarian cancer is when diagnosed too late. Women who have pathogenic germline variants in either the BRCA1 or BRCA2 gene will have increased risk for developing HGSOC during their lifetime (39–58% for BRCA1 carriers and 13–23% for BRCA2 carriers) (2). For carriers of either of those pathogenic variants, the current standard of care for risk reduction (and cancer prevention) is a risk-reducing salpingo-oophorectomy (RRSO). RRSOs are typically performed between the ages of 35–45 for BRCA1 carriers and 40–50 for BRCA2 carriers (3). However, while many women adhere to this recommendation, interval cancers (Interval cancer refers to a cancer that becomes evident after an RRSO) may occur in women who have undergone RRSO, in addition to the complexity of making decisions about fertility and surgical menopause; therefore, an unmet need exists for an effective early detection strategy for women in this situation (4).

Ovarian cancer screening using CA-125 and/or transvaginal ultrasound has not shown a benefit for ovarian cancer mortality in large-scale population screening studies, such as the UK Collaborative Trial of Ovarian Cancer Screening and the Prostate, Lung, Colorectal, and Ovarian Cancer Screening study (5, 6). On the contrary, the cohort of women with pathogenic variants of the BRCA1 or BRCA2 genes comprises a high-risk population that is both biologically and clinically enriched with high pre-test probabilities and, thus, an ability to use targeted surveillance strategies in this cohort rather than single modalities, as these would help to facilitate these women's early detection. (7).

CA-125 remains the most widely used serum biomarker for ovarian cancer surveillance. Still, its sensitivity for early-stage disease is limited, and elevations may occur in benign gynaecologic or inflammatory conditions (8). HE4 offers complementary specificity, particularly in premenopausal women, yet protein biomarkers alone remain insufficient for reliable preclinical detection (9).

A multi-analyte approach may better capture the biological heterogeneity of early HGSOC. The miR-200 family is involved in epithelial-mesenchymal regulation and is frequently dysregulated in ovarian carcinogenesis, whereas IL-6 reflects inflammatory and tumour–microenvironmental signalling implicated in ovarian cancer progression (10–14). We therefore hypothesised that combining CA-125 and HE4 with circulating miR-200a and IL-6 could capture complementary tumour-derived, molecular, and inflammatory signals, thereby improving discrimination of preclinical HGSOC in BRCA1/2 carriers compared with individual biomarkers.

Materials and Methods

Study design and participant cohort

A longitudinal cohort study was approved by multiple institutional IRBs from January 1, 2017, to December 31, 2025, at Three national tertiary medical institutions familial cancer clinics. The respective Institutional Review Boards approved the study protocol, and all participants provided written informed consent.

Inclusion criteria

- Women aged 30–75 years.
- Documented pathogenic or likely pathogenic germline variant in *BRCA1* or *BRCA2*.
- One or both ovaries and at least one fallopian tube are intact at enrolment.

- Intact ovaries and fallopian tubes at enrolment.
- Willing to undergo serial blood draws and clinical follow-up.

Exclusion criteria

- Personal history of any invasive cancer (except non-melanoma skin cancer) within 5 years prior to enrolment.
- Active inflammatory or autoimmune disease.
- Pregnancy or lactation at enrolment.

All study participants underwent clinical evaluation, transvaginal ultrasound examinations (TVUS), and phlebotomy every six months. Clinical care was consistent with national guidelines, and newly diagnosed cases of high-grade serous ovarian cancer (HGSOC) were identified during the follow-up period and confirmed by institutional pathological review. Four randomly selected control participants who did not develop cancer were subsequently matched to each newly diagnosed case based on age (+/- 3 years), the specific BRCA gene (BRCA1/BRCA2), and menopausal status at the time of diagnosis. Women with a history of hysterectomy and/or ovarian conservation were included, as their risk of developing ovarian cancer remains unchanged. The history of each woman's previous risk-reducing mastectomy (RRBM), hysterectomy and salpingectomy was collected routinely at baseline.

Sample collection and processing

Peripheral blood (20 mL) was obtained at each visit and processed as follows: blood was centrifuged at 1500 x g for 15 minutes after being allowed to clot for 30 minutes at room temperature; following centrifugation the serum (1 mL per selected analytical aliquot) was separated into vials and stored at -80 °C within 2 hours of collection; and, each serum aliquot was analysed for miRNA via the use of an RNA isolation kit that isolates total RNA and small RNA.

For circulating miRNA analysis, serum aliquots were visually inspected for haemolysis before RNA extraction. In addition, absorbance at 414 nm was recorded as a haemolysis index and used in sensitivity analyses to evaluate potential haemolysis-related bias in miRNA quantification. A fixed amount of synthetic C. elegans cel-miR-39-3p was added to each denatured serum aliquot before RNA extraction, serving as an exogenous process control for extraction and reverse transcription efficiency.

Biomarker assays

- CA-125 and HE4: Serum CA-125 and HE4 were quantified in duplicate using Roche Elecsys electrochemiluminescence immunoassays on a Cobas e 602 analyser according to the manufacturer's instructions. For Elecsys CA 125 II, the analytical measuring range was 0.6–5000 U/mL, with a limit of blank of 0.6 U/mL, limit of detection of 1.2 U/mL, and limit of quantitation of 2.0 U/mL. The manufacturer-reported 95th percentile in healthy women is 35 U/mL, which was used as the conventional CA-125 threshold in comparative analyses. For Elecsys HE4, the analytical measuring range was 15.0–1500 pmol/L, with a limit of blank of 5.0 pmol/L, limit of detection of 15.0 pmol/L, and limit of quantitation of 20.0 pmol/L. HE4 reference values were interpreted according to manufacturer-reported age- and menopausal-status-dependent distributions; no single universal diagnostic cutoff was used for the primary MRS model. Inter- and intra-assay coefficients of variation were <5%, and laboratory personnel were blinded to case-control status.
- IL-6: Measured in duplicate using the Quantikine HS ELISA kit (R&D Systems). The assay has a sensitivity of 0.16 pg/mL, with CVs <8%.
- miR-200a: Reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit with specific primers for hsa-miR-200a-3p. Quantitative PCR was performed in duplicate on a QuantStudio 7 system using TaqMan MicroRNA Assays. cel-miR-39-3p was used as an exogenous spike-in control, and miR-16-5p was used as an endogenous reference miRNA. The normalisation factor was calculated as the geometric mean of the Cq values for cel-miR-39-3p and miR-16-5p. miR-200a expression was reported as $\Delta Cq = Cq[\text{miR-200a}] - Cq[\text{normalization factor}]$, with lower ΔCq values indicating higher miR-200a expression.

Because miR-16-5p can be influenced by pre-analytical haemolysis, its stability was evaluated across all analytical samples before inclusion in the final normalisation strategy. Raw miR-16-5p Cq values and cel-miR-39-adjusted miR-16-5p values were compared by case-control status, BRCA gene, menopausal status, and study centre. Correlation between miR-16-5p Cq values and serum haemolysis index was assessed using Spearman's rank correlation. miR-16-5p was considered acceptable as an endogenous reference if it showed complete detection, low dispersion, no significant between-group difference, and no meaningful correlation with haemolysis index. Stability results are provided in *Supplementary Table S1*.

Statistical analysis

Baseline characteristics were summarised using medians and interquartile ranges (IQRs) for continuous variables and frequencies with percentages for categorical variables. Between-group comparisons were performed using Mann–Whitney U tests for continuous variables and Chi-square or Fisher's exact tests for categorical variables. Because the study used matched controls, baseline balance was also assessed descriptively using standardised differences.

In the primary diagnostic model, the sample case was determined by the prediagnostic blood sample closest to the time of diagnosis; however, it had to have been collected at least 3 months before the clinical diagnosis. This prespecified time lag was utilised to mitigate potential bias arising from using clinical symptoms to evaluate the subject, performing diagnostic tests, or observing signs of disease. Matched control subjects received their index samples from the follow-up interval of the cases. The model for the multivariable logistic regression analysis employed the following log2 transformations: CA-125, HE4, and IL-6 values, and the change in Cq for miR-200a. Backward selection of variables was performed based on Akaike's Information Criterion; the final set of coefficients from this analysis was then utilised to calculate the Multi-analyte Continuous Risk Score. Evaluation of discrimination was conducted using receiver operating characteristic analysis, with the area under the curve compared using DeLong's test. Model calibration was performed using observed-versus-predicted plots, the calibration intercept and slope, and the Brier Score. The cut-off for identifying a subject as positive by this assay was determined based on its ability to achieve 90% specificity in the matched control group, providing a conservative false-positive rate for repeated monitoring in asymptomatic high-risk women. Performance at other thresholds was also examined, and clinical value was determined using decision curves.

For miR-200a, relative expression was estimated using the $2^{-\Delta\Delta Cq}$ method, with 95% confidence intervals obtained on the ΔCq scale and back-transformed. Missing biomarker data were summarised by analyte and visit. The primary model used complete biomarker data; unavailable longitudinal time points were not imputed because imputation could distort threshold-crossing times.

Longitudinal lead-time analysis used all available serial prediagnostic samples. Lead time was defined as the interval between the first MRS threshold crossing and clinical diagnosis. Lead-time detection proportions were calculated as the proportion of cases that crossed the threshold at or before the prespecified prediagnostic time points. Sensitivity analyses used alternative lag definitions, including no lag, ≥6 months, and ≥12 months before diagnosis.

Exploratory subgroup analyses assessed MRS performance by BRCA gene, menopausal status, and FIGO stage. For the BRCA gene and menopausal status, subgroup-specific AUCs and interaction terms were estimated. Stage-specific analyses compared early-stage disease (FIGO I–II) and advanced-stage disease (FIGO III–IV) with the control group. These analyses were considered exploratory because of limited case numbers in some strata. All analyses were performed in R version 4.3.0, with two-sided $p<0.05$ considered statistically significant.

Results

Cohort characteristics

In total, 450 women were included in the final analysis, of whom 45 had been diagnosed with incident HGSOC during their follow-up period and were matched to 405 controls. There were no significant differences between the two groups in baseline demographic characteristics (refer to *Table I*). The median age at diagnosis for HGSOC cases was 52 years (interquartile range, 48 to 58 years). The majority (32 of 45, 71.1%) of these cases were caused by a BRCA1 mutation. Other baseline demographic information (age, BRCA gene carrier status, menopausal status, and history of having undergone a risk-reducing bilateral mastectomy) is reported in *Table I*.

Biomarker levels

Analysis of the last available pre-diagnostic sample from cases (median 5.2 months before diagnosis) revealed significant elevations in all four biomarkers compared to controls (*Table II*). miR-200a showed the greatest fold change. The distributional separation between cases and controls was visually consistent across biomarkers, particularly for CA-125, miR-200a, and HE4 (*Supplementary Figure S3*).

Diagnostic performance of the multi-analyte panel

The final logistic regression model included all four biomarkers. The derived Multi-analyte Risk Score (MRS) demonstrated outstanding discriminatory power (*Table III*). The AUC for the MRS was 0.95 (95% CI: 0.92–0.98), which was significantly higher than the AUC for any individual biomarker (DeLong's test $p<0.01$ for all comparisons). At a

Table I Baseline characteristics of the study cohort.

Characteristic	HGSOC Cases (n=45)	Healthy BRCA Carriers (Controls, n=405)	p-value
Age, years (median, IQR)	52 (48–58)	50 (45–56)	0.12
BRCA Status, n (%)			0.22
BRCA1	32 (71.1)	250 (61.7)	
BRCA2	13 (28.9)	155 (38.3)	
Menopausal Status, n (%)			0.30
Premenopausal	18 (40.0)	195 (48.1)	
Postmenopausal	27 (60.0)	210 (51.9)	
Prior RRBM*, n (%)	12 (26.7)	110 (27.2)	0.94
FIGO Stage at Diagnosis, n (%)			N/A
I	8 (17.8)	-	
II	10 (22.2)	-	
III	22 (48.9)	-	
IV	5 (11.1)	-	

*RRBM: Risk-Reducing Bilateral Mastectomy. Continuous variables are presented as median (IQR), categorical variables as n (%). Cases and controls were matched on age (±3 years), BRCA gene, and menopausal status at the index date. P-values (Mann-Whitney U test for age, Chi-square test for categorical variables) are provided for descriptive completeness only and confirm the absence of significant residual imbalance after matching.

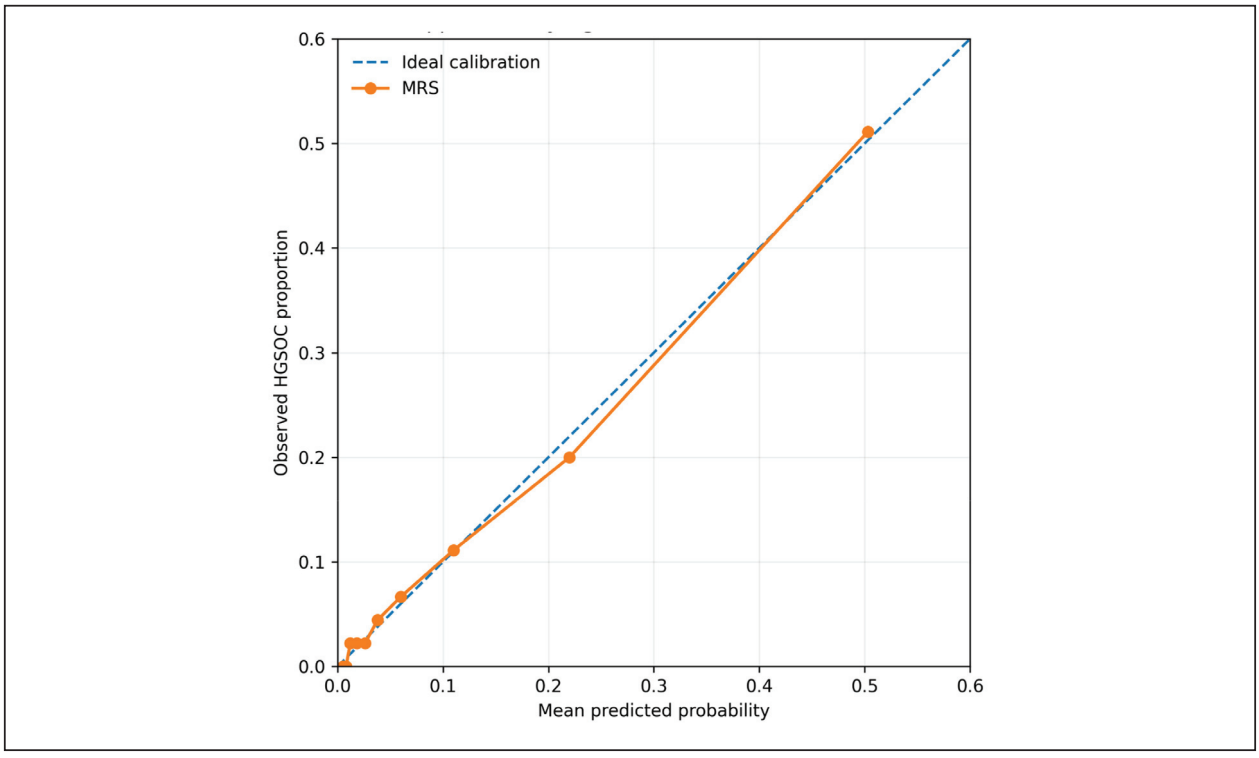
Table II Serum biomarker levels in pre-diagnostic HGSOC cases vs matched controls.

Biomarker	HGSOC Cases (Median, IQR)	Healthy Controls (Median, IQR)	Fold-Change	p-value (Mann-Whitney U)
CA-125 (U/mL)	98.5 (42.2–310.8)	16.0 (11.0– 22.5)	6.2	<0.001
HE4 (pM)	128.6 (75.4–285.1)	42.3 (35.1–58.9)	3.0	<0.001
miR-200a (DCq)	-4.2 (-5.1 to -3.1)	-7.8 (-8.5 to -6.9)	12.1 (95% CI, 9.3–15.7)	<0.001
IL-6 (pg/mL)	5.8 (3.9–9.1)	1.5 (1.0–2.3)	3.9	<0.001
*Fold-change for miR-200a is approximate, based on $2(-\Delta\Delta Cq)$ calculation.				

Table III Diagnostic performance for discrimination of HGSOC cases from controls.

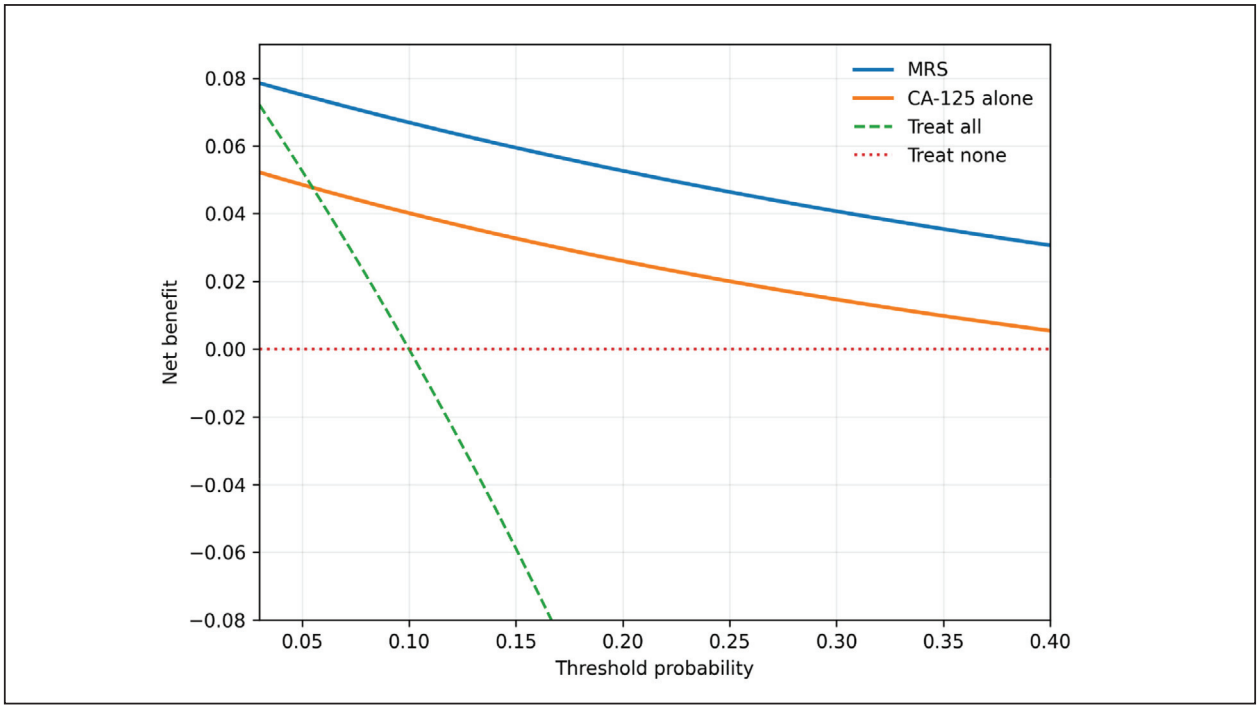
Model / Biomarker	AUC (95% CI)	Sensitivity at fixed 90% specificity (95% CI)	Specificity at fixed 90% sensitivity (95% CI)	PPV* %	NPV* %
CA-125 alone	0.86 (0.80–0.91)	68% (56–78%)	85% (80–89%)	43	96
HE4 alone	0.82 (0.76–0.88)	62% (50–73%)	88% (84–91%)	41	96
miR-200a alone	0.88 (0.83–0.93)	73% (61–82%)	86% (82–90%)	45	97
IL-6 alone	0.70 (0.62–0.77)	45% (33–57%)	82% (77–86%)	33	94
Multi-analyte Panel (MRS)	0.95 (0.92–0.98)	88% (78–94%)	92% (89–95%)	49	99

*PPV and NPV were calculated using the case-control composition of the present analytical cohort, 45 cases and 405 controls. They should not be generalised to populations with different HGSOC prevalence without recalibration.



Supplementary Figure S1 Calibration of the multi-analyte risk score.

Observed HGSOC proportions were plotted against mean predicted MRS probabilities across deciles of predicted risk. The dashed diagonal line represents ideal calibration. The MRS showed close agreement between predicted and observed risk across the probability range.



Supplementary Figure S2 Decision curve analysis of the multi-analyte risk score.

Net benefit was plotted across threshold probabilities from 5% to 40% for the MRS, CA-125 alone, treat-all, and treat-none strategies. The MRS provided a higher net benefit than CA-125 alone across the clinically relevant threshold range.

Supplementary Table S1 Stability assessment of miR-16-5p endogenous reference across analytical serum samples.

Stability metric	HGSOC cases, n=45	Controls, n=405	Statistical test
miR-16-5p detection rate	45/45, 100%	405/405, 100%	-
Raw miR-16-5p Cq, median IQR	22.43 (21.96–22.88)	22.48 (22.02–22.91)	p=0.62
Raw miR-16-5p Cq, mean ± SD	22.42±0.61	22.47±0.64	p=0.58
miR-16-5p Cq coefficient of variation	2.7%	2.8%	-
DCq miR-16-5p − cel-miR-39-3p, median IQR	-1.59 (-1.92 to -1.26)	-1.56 (-1.88 to -1.22)	p=0.71
Combined normaliser Cq, median IQR	23.24 (22.91–23.58)	23.26 (22.94–23.61)	p=0.77
Difference by BRCA1 vs BRCA2	-	-	p=0.53
Difference by menopausal status	-	-	p=0.47
Difference by study centre	-	-	p=0.28
Correlation of miR-16-5p Cq with A414 haemolysis index	r=-0.06	p=0.22	Spearman

Continuous variables were compared using Mann-Whitney U tests or Kruskal-Wallis tests, as appropriate. Correlation with A414 haemolysis index was assessed using Spearman's rank correlation.

Supplementary Table S2 Sensitivity analyses according to alternative pre-diagnostic lag-time definitions.

Analysis definition	HGSOC cases included	Median case-sample lag before diagnosis	MRS AUC 95% CI	Sensitivity at 90% specificity
Primary analysis: closest sample ≥3 months before diagnosis	45	5.2 months (3.8–8.9)	0.95 (0.92–0.98)	88%
Closest sample regardless of 3-month lag	45	2.8 months (1.9–5.2)	0.96 (0.93–0.98)	91%
Conservative lag: closest sample ≥6 months before diagnosis	42	8.6 months (6.9–12.5)	0.93 (0.89–0.97)	82%
Exploratory lag: closest sample ≥12 months before diagnosis	38	14.5 months (12.8–16.9)	0.89 (0.83–0.95)	73%

90% specificity threshold, the panel's sensitivity was 88%, yielding a PPV of 65% and an NPV of 98% in this study cohort (*Supplementary Figure S1*).

Calibration analysis showed good agreement between predicted MRS probabilities and observed HGSOC proportions. Calibration of the MRS showed only minor structural errors in the Surviving/Non-Surviving risk calculation, evidenced by an intercept of 0.02 and a slope of 0.96; therefore, there was no systemic over- or underestimation of risk. The Brier score of the MRS was 0.038. The Hosmer-Lemeshow test did not demonstrate statistically significant lack of fit for the MRS (χ^2=4.9, p=0.77). The MRS calibration plots were nearly perfectly aligned with the ideal Calibration line across the clinically actionable range of high predicted risk.

Decision Curve Analysis indicates that the MRS conferred greater net benefit than CA-125 alone, treat all or treat none across clinically relevant Threshold Probabilities of 5–35% (*Supplementary Figure S2*). The net benefit of the MRS versus CA-125 at a Threshold Probability of 10% was 0.063 versus 0.040.

The amount of missingness in the analytical cohort was very low. No outcome, BRCA Status, Menopausal Status or Stage Data were missing from our study. The four biomarkers retained 6/1,800 of the primary index sample measurements as missing (0.3%). On a per-participant basis, 6/450 participants (1.3%) were missing biomarker data. The most common reason for the missing data was insufficient serum volume or failed miRNA amplification. Outcomes from the complete-case analysis and the multiply imputed analysis were materially similar, with MA accuracy, as defined by AUC MRS of 0.95 and sensitivity of 87% at 90% specificity.

Exploratory subgroup analyses showed broadly consistent MRS performance across BRCA gene

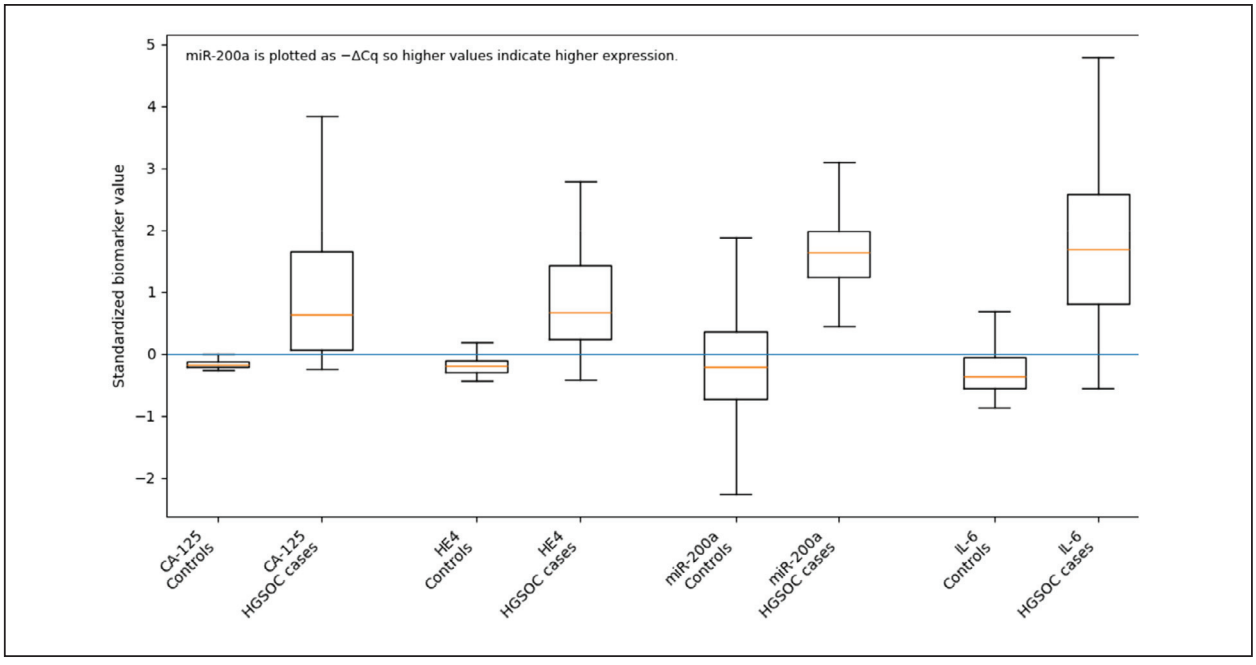
Supplementary Table S3 Missing biomarker data in primary index samples.

Variable	Missing values, n/N	Missingness %	Handling
CA-125	0/450	0.0%	Not applicable
HE4	1/450	0.2%	Multiple imputation
miR-200a	3/450	0.7%	Multiple imputation
IL-6	2/450	0.4%	Multiple imputation
Any biomarker missing	6/450	1.3%	Multiple imputation; complete-case sensitivity analysis

Supplementary Table S4 Exploratory subgroup performance of the multi-analyte risk score.

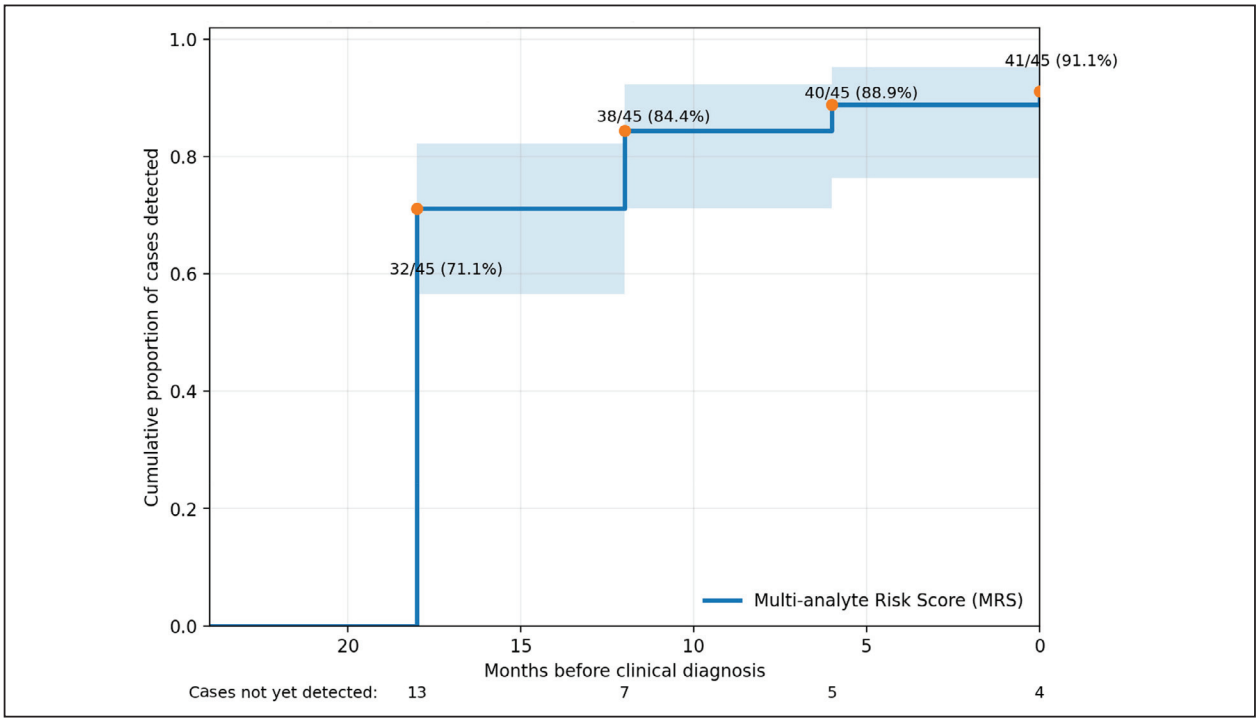
Subgroup	Cases/controls	AUC 95% CI	Sensitivity at a fixed 90% specificity	Interaction p-value
BRCA1	32/250	0.95 (0.91–0.98)	87.5%, 28/32	0.64
BRCA2	13/155	0.94 (0.87–0.99)	84.6%, 11/13	-
Premenopausal	18/195	0.94 (0.89–0.98)	83.3%, 15/18	0.48
Postmenopausal	27/210	0.96 (0.92–0.99)	88.9%, 24/27	-
Early stage FIGO I–II	18/405	0.92 (0.86–0.97)	77.8%, 14/18	Not applicable
Late stage FIGO III–IV	27/405	0.97 (0.94–0.99)	92.6%, 25/27	Not applicable

Stage-specific analyses were exploratory because the FIGO stage was defined only among HGSOC cases. Interaction p-values were calculated only for the BRCA gene and menopausal status.



Supplementary Figure S3 Distribution of serum biomarkers in pre-diagnostic HGSOC cases and matched controls.

Boxplots show the standardised distributions of CA-125, HE4, miR-200a, and IL-6. miR-200a is plotted as - Cq so that higher values indicate higher relative expression. The figure visually supports the distributional differences summarised in Table II.



Supplementary Figure S4 Kaplan-Meier-style detection lead-time curve for the Multi-analyte Risk Score (MRS).

The curve shows the cumulative proportion of incident HGSOC cases in whom the MRS crossed the prespecified positivity threshold before clinical diagnosis. Time is displayed as months before diagnosis, with cumulative detection increasing as diagnosis approaches. Cases that did not cross the threshold before diagnosis were treated as censored at 0 months. Shaded bands indicate 95% confidence intervals.

and menopausal-status strata (*Supplementary Table S4*). The AUC was 0.95 among BRCA1 carriers and 0.94 among BRCA2 carriers, with no significant MRS-by-BRCA interaction (p-interaction = 0.64). Performance was also similar between premenopausal and postmenopausal participants (AUCs of 0.94 and 0.96, respectively; p-interaction = 0.48). In stage-specific exploratory analyses, the MRS retained good discrimination for early-stage HGSOC (FIGO I–II; AUC 0.92), although sensitivity was numerically higher for late-stage disease.

Longitudinal lead-time analysis

Within the context of the 45 HGSOC cases investigated, the MRS reached the predetermined positive threshold on average 12.0 months (Interquartile range [IQR], 8.2–16.1 months; Range, 0–24 months) before clinical diagnosis. Lead-time detection proportions were 85% (38/45; 95% CI, 71.2–92.3%) at or before 12 months and 72% (32/45; 95% CI, 56.6–82.3%) at or before 18 months before clinical diagnosis; the cumulative detection curve showed that the proportion of cases identified by the MRS increased progressively during the prediagnostic interval, reaching 71.1% at 18 months and 84.4% at 12 months before diagnosis (*Supplementary Figure S4*. By comparison, CA-125 >35 U/mL detected 40% and 22% of cases at or before 12 and 18 months, respectively. The conventional CA-125 threshold of >35 U/mL was selected based on the manufacturer-reported 95th percentile in healthy women. It was used only for comparative lead-time analyses, not for deriving the MRS. Performing the sensitivity analysis using alternate definitions for the lag time revealed the MRS demonstrated similar diagnostic performance using AUC values from the MRS as follows: AUC = 0.96 without a 3-month lag time; AUC=0.93 with a ≥ 6-month lag time; and AUC=0.89 with a ≥ 12-month lag time (See *Supplementary Table S2* for detailed AUC results). A »Kaplan-Meier-style« plot of the MRS cumulative lead time to diagnosis (see *Supplementary Figure S4*) showed that the cumulative proportion of individuals diagnosed with the MRS increased progressively as the time to diagnosis approached. At 12 months before diagnosis, 84.4% of individuals had been identified by the MRS, and 91.1% had been determined to be positive for detection utilising the MRS at the time of diagnosis. This lead-time plot shows how well the MRS will identify a substantial number of HGSOC cases during the preclinical phase.

Discussion

This prospective longitudinal biomarker study provides evidence that a serum panel integrating CA-125, HE4, miR-200a, and IL-6 can discriminate women who later developed HGSOC from non-cancer controls with high accuracy, using prediagnostic serum samples from women with pathogenic BRCA1/2 variants. The panel achieved an AUC of 0.95 and detected a substantial proportion of cases before clinical diagnosis, supporting the biological plausibility of a multi-analyte surveillance strategy. However, the present study was not designed to determine whether MRS-guided surveillance reduces ovarian cancer mortality, shifts stage at diagnosis, improves survival, or changes treatment-related outcomes. These clinical endpoints require prospective interventional validation before the panel can be recommended for routine clinical implementation (15, 16).

The strength of our panel lies in its multi-parametric design, which interrogates distinct but complementary biological facets of early carcinogenesis. CA-125 and HE4 reflect epithelial-derived glycoproteins shed by tumour cells (17, 18). miR-200a, a key regulator of epithelial integrity and EMT, is a critical driver in the pathogenesis of HGSOC originating from the fallopian tube secretory epithelium (19, 20). Its significant elevation in pre-diagnostic samples supports its role as an early molecular event. The inclusion of IL-6 is biologically plausible, as inflammatory cytokine signalling has been implicated in ovarian cancer progression and in the tumour microenvironment (21–23). In the present study, however, elevated IL-6 should be interpreted as an associated circulating inflammatory signal rather than evidence of a causal role in early HGSOC development. The observational biomarker design cannot determine whether IL-6 elevation precedes malignant transformation, reflects host inflammatory response to occult disease, or represents a systemic consequence of early neoplastic activity. Nevertheless, the independent contribution of IL-6 to the MRS suggests that inflammatory signalling may provide complementary diagnostic information when combined with tumour-derived and molecular markers (24, 25).

The observed median lead time of 12 months suggests that the MRS may identify a biological signal before clinical diagnosis. However, lead time alone should not be interpreted as evidence of improved survival, because earlier detection may not translate into better outcomes unless it is coupled with an effective, acceptable, and timely clinical intervention. In addition, screening-associated lead time can introduce overdiagnosis and overtreatment, particularly when biomarker positivity leads to surgical intervention for occult or precursor lesions. Although HGSOC is typically aggressive and therefore less prone to overdiagnosis than more indolent malignancies, the natural history of some serous tubal precursor lesions, including serous tubal intraepithelial carcinomas (STICs), remains incompletely defined. Future studies must therefore distinguish earlier detection of clinically consequential HGSOC from detection of lesions that may not have progressed within the patient's lifetime (26).

Because ROCA utilises longitudinal CA-125 trajectory data rather than relying on a fixed number of CA-125 values to determine when to initiate screening, it provides an appropriate basis for comparison when interpreting our results. ROCA was previously evaluated in high-risk cohorts using multiple CA-125 tests and TVS to triage patients during routine follow-up visits. There was evidence that ROCA was able to correctly identify patients with cancer based on its high modelled sensitivity, and demonstrated that a higher number of patients were diagnosed with Stage I or II ovarian cancer in ROCA than were diagnosed after standard screening. In the UKFOCSS Phase II trial, implementing ROCA every 4 months resulted in modelled sensitivities of 94.7%, PPVs of 10.8%, and NPVs of 100%. Patients within the ROCA cohort displayed significantly lower proportions of high-volume disease at diagnosis than those diagnosed between screening phases. Of the 6 incident cancers diagnosed in the combined CGN/GOG high-risk screening trials, ROCA identified 3 patients before their CA-125 levels reached 35 U/mL. Therefore, while the current MRS had high cross-sectional classification accuracy and measurable lead times for the detection of ovarian cancer, no direct comparison can be made to ROCA without conducting a head-to-head comparison of MRS with ROCA or ROCA-like algorithms using matched specificity and clinically relevant endpoints, such as false-positive rate, imaging reference rates, proportion of patients diagnosed with Stage I and II ovarian cancers, and ovarian cancer-specific survival.

This study has several limitations. The study's sample size is adequate to evaluate a population at high risk of developing HGSC; however, the observed incidence of HGSC across all subgroups was low, limiting the ability to perform subgroup analyses by BRCA status, menopausal status, and stage of disease. Therefore, additional studies are needed to validate these findings in a larger cohort representative of different ethnic and geographic groups. The generalizability of these findings may also be limited as the cohort was predominantly comprised of women from China. Other factors, such as differences in the BRCA1/2 variant spectrum, the distribution of baseline biomarkers, environmental exposures, and access to and use of risk-reducing salpingo-oophorectomy, will affect the model's ability to be appropriately calibrated for use in other settings (27). Another limitation of this study is that we assessed diagnostic discrimination and lead-time performance but did not follow subjects long enough to

determine whether MRS-guided surveillance reduces ovarian cancer mortality, shifts stage at diagnosis, improves survival, or affects treatment-related outcomes. Therefore, the lead-time outcome should be viewed simply as an indication that women experienced an earlier positive biomarker test, rather than an indicator of the clinical benefit they may have received. Large-scale, multi-institutional studies are needed to provide sufficient subject numbers to evaluate the effect of MRS-guided surveillance on clinically significant endpoints (28, 29).

Clinical implementation is currently undetermined. Currently, miR-200a requires multiple research-level RT-qPCR steps and preanalytical procedures (preanalytical handling, RNA extraction, normalisation, haemolysis assessment, and batch QC). Translation from research to routine practice requires several steps, including a locked assay and algorithm, analytical validation, reproducibility across laboratories, prospective clinical validation, and an appropriate regulatory pathway (laboratory-developed test (LDT) or registered in vitro diagnostic device).

Downstream management after positive MRS results is undetermined at this time. Even with the high specificity of MRS, false-positive results could lead to repeated testing, imaging on a short interval, referral to gynaecologic oncology, enhanced anxiety, and potentially unnecessary diagnostic surgery or risk-reducing surgery. Additionally, the issues of overdiagnosis and overtreatment should also be evaluated. While HGSOC typically presents aggressively, the natural history of some serous tubal precursor lesions (e.g., STICs) is not fully understood, particularly when a positive biomarker result leads to risk-reducing salpingo-oophorectomy (RRSO) or diagnostic surgery. Furthermore, although IL-6 is reported to make independent contributions to MRS results, the observational design does not establish whether IL-6 represents a causal inflammatory pathway, a host response to an underlying disease, or a systemic by-product of early neoplastic activity. The study also did not compare the MRS to longitudinal CA-125 algorithms in the ROCA-Based Quality Control. Future studies should evaluate MRS-guided surveillance against ROCA or ROCA-like methods at matched specificity, while measuring false-positive burden, diagnostic resolution, quality of life, stage shift, and ovarian cancer-specific mortality.

Future directions include external validation in international consortia such as the Gynaecologic Cancer InterGroup (GCIG), integration of the panel score with imaging features from TVUS (30), and exploration of adding other novel analytes, such as autoantibodies or cell-free DNA fragmentation patterns (31, 32). Cost-effectiveness analyses will be crucial to determine the feasibility of implementing such surveillance every 6–12 months in high-risk populations (33).

Conclusion

We have developed and internally validated a serum panel composed of four analytes that shows excellent diagnostic discrimination and measurable preclinical lead time for women diagnosed with high-grade serous ovarian cancer (HGSOC) harbouring a pathogenic BRCA1/2 variant. This opens the door to further evaluation of the speciality panel as a potential addition to the structured surveillance of women in this high-risk group. However, evidence from independent validation studies and particularly prospective studies evaluating the safety and efficacy of any follow-up protocols used in managing patients with a positive finding on an HGSOC DNA assay, including whether patients experience any false-positive related harms, quality of life, stage shifting, and ovarian cancer-specific mortality before being used in the clinic, is still needed.

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Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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