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Evaluating the effectiveness of pre-operative diagnosis of ovarian cancer using minimally invasive liquid biopsies by combining serum human epididymis protein 4 and cell-free DNA in patients with an ovarian mass

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ABSTRACT

Objective To assess the feasibility of scalable, objective, and minimally invasive liquid biopsy-derived biomarkers such as cell-free DNA copy number profiles, human epididymis protein 4 (HE4), and cancer antigen 125 (CA125) for pre-operative risk assessment of early-stage ovarian cancer in a clinically representative and diagnostically challenging population and to compare the performance of these biomarkers with the Risk of Malignancy Index (RMI).

Methods In this case–control study, we included 100 patients with an ovarian mass clinically suspected to be early-stage ovarian cancer. Of these 100 patients, 50 were confirmed to have a malignant mass (cases) and 50 had a benign mass (controls). Using WisecondorX, an algorithm used extensively in non-invasive prenatal testing, we calculated the benign-calibrated copy number profile abnormality score. This score represents how different a sample is from benign controls based on copy number profiles. We combined this score with HE4 serum concentration to separate cases and controls.

Results Combining the benign-calibrated copy number profile abnormality score with HE4, we obtained a model with a significantly higher sensitivity (42% vs 0%; $p<0.002$) at 99% specificity as compared with the RMI that is currently employed in clinical practice. Investigating performance in subgroups, we observed especially large differences in the advanced stage and non-high-grade serous ovarian cancer groups.

Conclusion This study demonstrates that cell-free DNA can be successfully employed to perform pre-operative risk of malignancy assessment for ovarian masses; however, results warrant validation in a more extensive clinical study.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Previous research has explored minimally invasive biomarkers and cell-free DNA for ovarian cancer diagnosis, focusing on advanced-disease cases and specific subtypes.

WHAT THIS STUDY ADDS

⇒ This study examines cell-free DNA use in a diverse, clinically representative population, including early-stage ovarian cancer patients, develops the benign-calibrated copy number profile abnormality score, and creates a joint prediction model that outperforms the Risk of Malignancy Index (RMI).

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The benign-calibrated copy number profile abnormality score could be implemented in clinics for pre-operative ovarian tumor diagnosis. Additional research is needed to find complementary biomarkers.

INTRODUCTION

Pre-operative differentiation between benign ovarian masses and early-stage ovarian cancer is challenging because a minority of all ovarian masses is malignant.[1 2] However, accurate pre-operative distinction is essential for providing proper treatment and referring patients with ovarian cancer to an oncological center.[3–6] Current strategies use clinical, biochemical, and ultrasound data. Ultrasound requires experience for optimal performance.[7–10] In contrast, biomarkers offer objective evaluation on a routine basis.

Serum cancer antigen 125 (CA125) is widely used in ovarian cancer diagnosis. However, its value for ovarian cancer detection in a general hospital population of patients with an ovarian mass is limited, with a sensitivity of 72% and a specificity of 53% due to elevation in benign conditions such as endometriosis.[11–13] Serum human epididymis protein 4 (HE4) has a 25% higher specificity compared with CA125, but its sensitivity is only 65%.[13] The Risk of Malignancy Index (RMI) is also frequently used that employs both ultrasound variables and CA125.[14] However, its heavy reliance on CA125 leads to a sensitivity of 72% and a specificity of only 59% at a threshold of 200.[13]

Thus, the need for novel biomarkers for pre-operative diagnosis remains.

Blood-based liquid biopsies are emerging as a promising technology for risk assessment since they provide comprehensive snapshots of tumors and can be performed minimally invasively. Previous studies have reported encouraging results but all had limitations which prevented clinical implementation.[15–17] These limitations include the selection of patients with advanced-stage disease, who often present with higher circulating tumor DNA levels and clinically suspicious symptoms such as ascites and extensive intra-abdominal disease and, hence, are easy to identify. In the study by Cohen et al,[15] only patients with high-grade serous ovarian carcinoma were included. Not only is subtype information not pre-operatively available, but this subtype is also more accurately diagnosed by serum biomarkers and the RMI.[18]

The current study focuses on using cell-free DNA copy number profiles for malignancy classification by shallow whole-genome sequencing in suspected early-stage ovarian cancer. As ovarian cancer is a copy number-driven cancer, changes in cell-free DNA copy number profiles are expected.[19] Furthermore, shallow whole-genome sequencing is widely used in non-invasive prenatal testing, which would facilitate clinical implementation.

In non-invasive prenatal testing, the goal is to detect copy number aberrations in cell-free fetal DNA indicative of genetic disorders such as trisomy 21 in the fetal karyotype. In contrast, with the detection of malignancy, the goal is to detect circulating tumor DNA with chromosomal aberrations associated with malignancy. Incidental detection of copy number aberrations indicative of maternal malignancy has been reported in several non-invasive prenatal testing studies, raising the possibility that the same method could be utilized to detect cancer systematically.[20–22]

(Online supplemental figure S1A) illustrates the currently used RMI in general hospitals while Online supplemental figure S1B illustrates how our proposed method aims to improve clinical decision-making. We aimed to evaluate the classification performance of cell-free DNA-derived variables combined with existing biomarkers (HE4 and CA125) and to compare the performance of this approach with the performance of the RMI. Furthermore, we evaluated our classifier in a clinically representative and diagnostically challenging population consisting of patients presenting with an ovarian mass containing both early-stage ovarian cancer with a wide variety of histological malignant and benign subtypes.

To this end, we measured performance in terms of the (partial) area under the curve (AUC) of the receiver operating characteristic (ROC) curve, particularly in the high-specificity region. In cases of high specificity, the ovarian mass is predicted as malignant with a high certainty, which can be beneficial for planning and referring patients for surgery and to allow physicians to counsel patients for surgery.

METHODS

In accordance with the Journal's guidelines, we will provide our data for independent analysis by a selected team by the Editorial Team for the purposes of additional data analysis or for the reproducibility of this study in other centers if such is requested.

Patient selection

We selected patient samples from Lof et al (NL58253.031.16).[13] Each patient gave written informed consent before enrollment. The protocol was approved by the Netherlands Cancer Institute Institutional Review Board (Biobank number: CFMPB600).

In the study from Lof et al,[13] patients aged ≥18 years, who presented with an ovarian mass on ultrasound, in whom CA125 and RMI were assessed for risk stratification, and with an indication for surgery, were consecutively enrolled between April 2017 and February 2021 in nine general hospitals in the Netherlands. Exclusion criteria were: (a) suspicion of high-stage disease at first outpatient visit, such as the presence of ascites on ultrasound and palpable lymph nodes, (b) a medical history of cancer in the past 5 years, and (c) a medical history of decreased kidney function (glomerular filtration rate <60 mL/min/1.73m^2) as this influences HE4 concentration . The consecutively first 50 patients with malignant mass and the first 50 patients with benign or borderline mass, matched by age (±2 years) were selected. Patients with borderline tumors were treated as benign according to Dutch clinical guidelines, because staging surgery does not influence survival.[23] The diagnosis was based on the *WHO Classification of Female Genital Tumors* (5th Edition, Volume 4). Both patients with low and high RMI scores were included. A gynecological pathologist routinely reviewed all histological slides of ovarian cancer and the benign or borderline tumors of patients operated on in an oncological center.

RMI, CA125, and HE4 measurements

During the regular diagnostic workup, pre-operative blood was collected for obtaining cell-free DNA and for measurement of CA125 and HE4. Serum CA125 (kU/L) was measured using electrochemiluminescence immunoassay or two-site immunometric assay on the Cobas (6000 or 8000) or Abbott (Architect I-module or Alinity I). RMI (III) was calculated according to the criteria described by Jacobs et al.[14] Serum HE4 (pmol/L) was measured in plasma from Streck tubes using the electrochemiluminescence immunoassay on the Cobas 6000 analyzer.

Cell-free DNA copy number profiling

Workflow of processing and transferring the blood samples, DNA extraction, and DNA sequencing is described in the Online supplemental information.

Copy number profile abnormality score

We used WisecondorX (v1.2.5)[24] for generating copy number profiles and calculating copy number profile abnormality scores.[25] Briefly, it divides the genome in bins and for every bin it calculates a Z-score, representing the amount of aberration, by normalizing the observed read count in the bin with the mean and variance observed in a set of reference bins. Neighboring bins having similar Z-scores are joined into segments, each having a segmental Z-score as defined by Raman et al.[24] We chose 250 kbp as bin size, since it has been used previously by Huijsdens-van Amsterdam et al,[26] and after observing high noise for small bin sizes (50 kbp or lower) and almost no calls for large bin sizes (5 Mbp or higher). We derived reference bins to calculate WisecondorX's Z-score from a set of 190 healthy reference samples. (Note that, according to the WisecondorX protocol, these reference samples do not have to be resequenced when testing new samples.) The copy number

Table 1 Patient and tumor characteristics.

Characteristic	Malignant ovarian mass (cases)		Benign or borderline ovarian mass (controls)	
	(n=50)		(n=50)	
Age (years), mean (SD)	63	(10)	62	(10)
Post-menopausal, n (%)	47	(94)	42	(84)
Histological type of malignant mass, n (%)				
High-grade serous ovarian carcinoma	21	(42)		
Low-grade serous ovarian carcinoma	5	(10)		
Mucinous ovarian carcinoma	4	(8)		
Endometrioid ovarian carcinoma	4	(8)		
Clear cell ovarian carcinoma	5	(10)		
Other type of ovarian carcinoma[1]	3	(6)		
Non-epithelial ovarian cancer[2]	5	(10)		
Ovarian metastases[3]	3	(6)		
FIGO stage, n (%)[4]				
I	20	(40)		
II	15	(30)		
III	12	(24)		
Metastases	3	(6)		
Histological type of benign/ borderline mass, n (%)				
Borderline			5	(10)
Fibroma			4	(8)
Cystadenoma			16	(32)
Endometriotic cyst			4	(8)
Cystadenofibroma			5	(10)
Other[5]			9	(18)
Unknown			7	(14)

[1]Category 'Other' includes mixed endometrioid and clear cell adenocarcinoma (n=1) and carcinoma not otherwise specified (n=2). [2]Category 'Non epithelial' includes granulosa cell tumor (n=4) and sarcoma (n=1). [3]The primary tumor of all ovarian metastases originated from the gastrointestinal tract. [4]The patients with a non-epithelial type of ovarian cancer were not completely staged according to International Federation of Gynecology and Obstetrics (FIGO) criteria. In these cases, the stage was also based on peri-operative findings. [5]Category 'Other' includes Brenner tumor (n=2), combined endometriosis and corpus luteum (n=1), fibrothecoma (n=2), mature teratoma (n=1), ovarian torsion and hydrosalpinx (n=1), leiomyoma (n=1), and low-grade appendicular neoplasm with pseudomyxoma peritonei (n=1).

profile abnormality score was calculated by summing the absolute segment Z-score multiplied by the segment length for each segment.[25]

Benign-calibrated copy number profile abnormality score

As we want to differentiate between malignant and benign samples, we also generated a set of reference bins based on benign samples. As the set of benign samples is limited, we used a leave-one-out cross-validation procedure: that is, when calculating the copy number profile abnormality score for malignant samples we used all benign samples as reference, and when calculating the copy number profile abnormality score for a benign sample we used all benign samples but the one selected for testing as reference samples. We denoted the resulting score as the benign-calibrated copy number profile abnormality score.

Statistical analysis

To discriminate between malignant and benign ovarian masses, we either used each of the copy number profile abnormality score, RMI, or HE4, or we combined them using a logistic regression model. To evaluate the predictive power, we report the AUC (area under the receiver operating characteristic (ROC) curve), the sensitivity at 95%, the sensitivity at 99%, and the sensitivity and specificity at the optimal Youden's index as defined by the equation: J = Sensitivity + Specificity

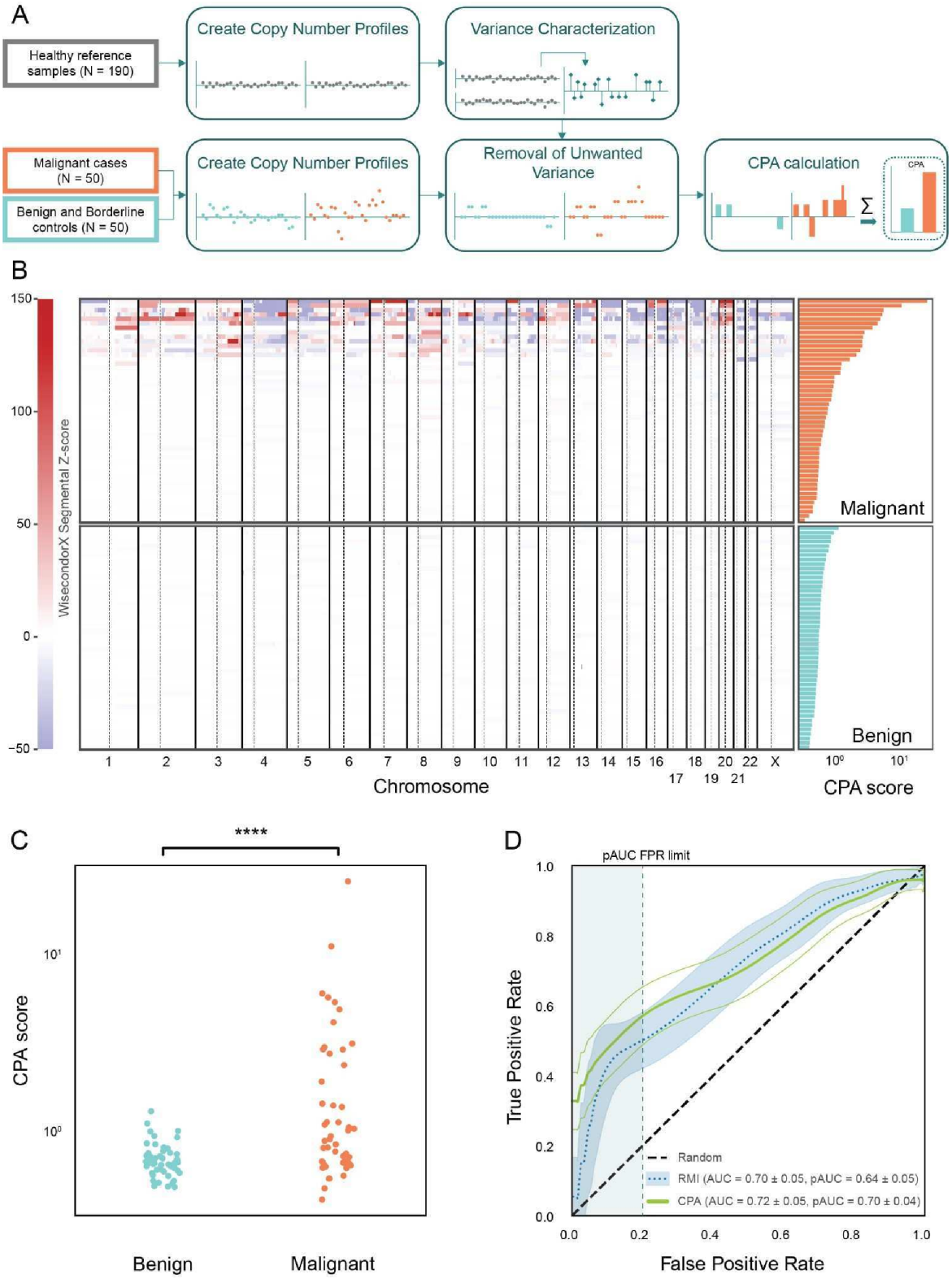

Figure 1 Copy number profile abnormality (CPA) score calculation and evaluation using WisecondorX when using healthy samples as a reference set. (A) Overview of the CPA score calculation pipeline when using healthy non-invasive prenatal testing samples as a reference set. (B) CPA profiles of the 50 patients with a malignant mass and 50 patients with benign tumors, sorted by CPA score as shown on the right. Red segments have gains, while blue segments have losses, colored by WisecondorX's segmental Z-score. (C) Strip plot of CPA score separated by malignancy. The malignant group has a significantly higher CPA score than the benign group (Mann–Whitney U test, p<0.0001). (D) Receiver operating characteristic curves for the CPA score (green) and the Risk of Malignancy Index (blue) as well as the partial area under the curve. AUC, area under the curve; CPA, copy number profile abnormality; FPR, false-positive rate; pAUC, partial area under the curve; RMI, Risk of Malignancy Index; ROC, receiver operating characteristic.

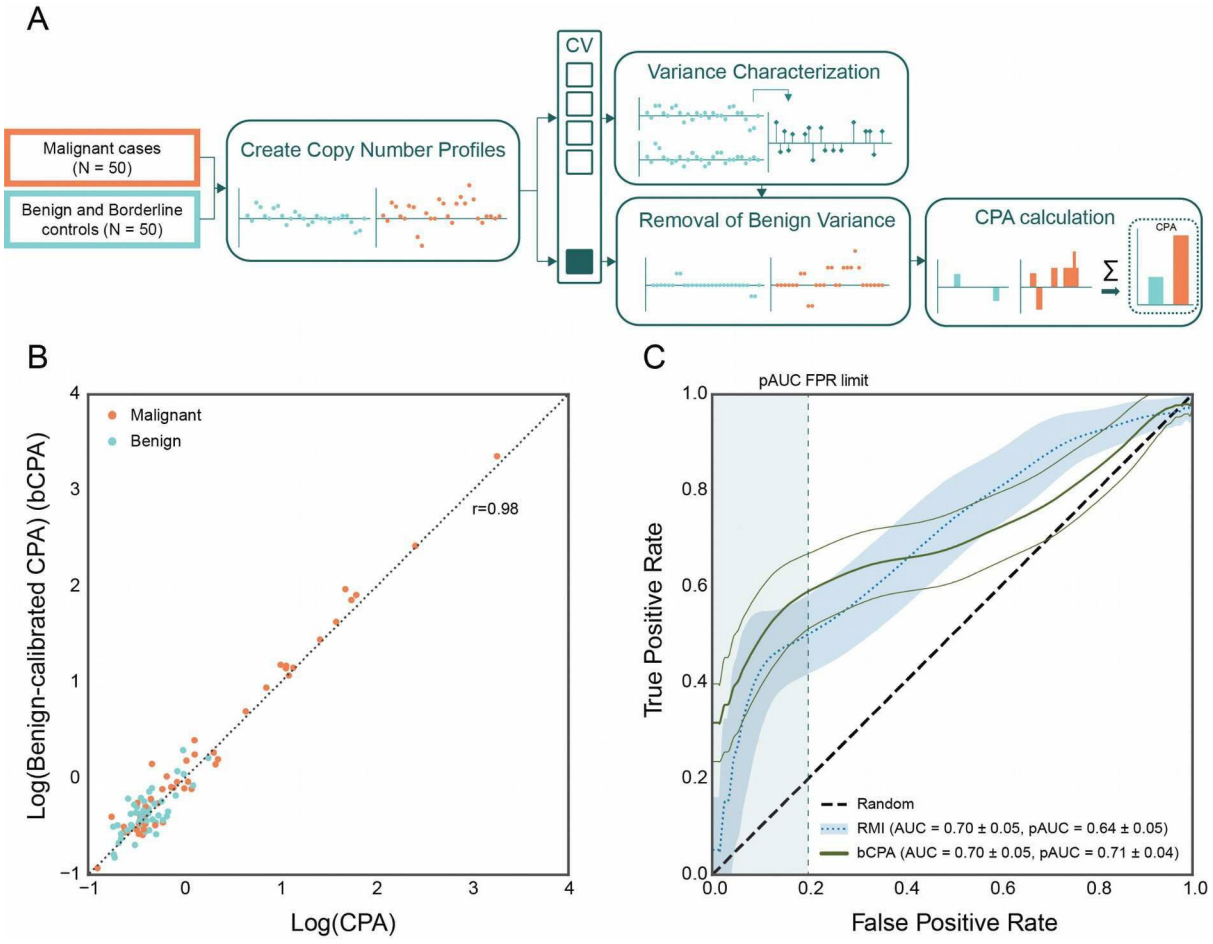

Figure 2 Benign-referenced copy number profile abnormality (bCPA) scores using benign samples as a reference set. (A) Overview of the pipeline to calculate the bCPA score that uses benign samples as a reference set when adopting WisecondorX. In a leave-one-out cross-validation setting, a reference is generated on the training set, which is used to remove unwanted variance from the test case. (B) Scatterplot showing the relationship between the CPA score (based on healthy samples) and the bCPA score (based on benign samples) with Pearson's R correlation coefficient (r=0.98). (C) Receiver operating characteristic curves for the bCPA and CPA score, with partial area under the curve (pAUC) values indicating their performances. Differences for both the AUC and pAUC are not statistically significant. AUC, area under the curve; bCPA, benign-referenced copy number profile abnormality; CPA, copy number profile abnormality; FPR, false-positive rate; pAUC, partial area under the curve; RMI, Risk of Malignancy Index; ROC, receiver operating characteristic.

– 1. To compare the performance of predictors for operating points that have a high specificity (ranging from 100% to 80%) we also report the partial AUC of the ROC curve.[27] P-values were obtained by bootstrapping 10 000 times according to the method utilized in the pROC R package.[28] A p-value≤0.05 was considered statistically significant.

RESULTS

Patient characteristics

Patient characteristics are shown in Table 1. We included 45 patients with a benign, five patients with a borderline, and 50 patients with a malignant ovarian mass, of whom 42 (84%) had epithelial ovarian cancer, 5 (10%) had non-epithelial ovarian cancer, and 3 (6%) had ovarian metastases originating from another primary cancer. Of all patients with ovarian cancer (n=47), 35 (74%) patients had International Federation of Gynecology and Obstetrics (FIGO) stage I-II and 12 (26%) were shown to have stage III disease after a surgical staging procedure.

Detection based on circulating tumor DNA

We used WisecondorX to calculate a copy number profile abnormality score (see Methods). WisecondorX uses a reference set for the removal of unwanted variance, for which we explored two options: (1) healthy samples as reference set and (2) benign samples as reference set.

Copy number profile abnormality score

Cell-free DNA copy number profiles were generated for all cases and controls with 190 non-invasive prenatal testing samples of healthy pregnant women sequenced on the same platform as a reference set. Subsequently, we calculated the copy number profile abnormality score which aggregates all copy number deviations, hence serving as a measure of 'abnormality'. This process is depicted in Figure 1A.

A Univariate Logistic Regression models

Variable	Z (p)	AUC (p)	pAUC (p)
bPA score	3.36 (0.001)	0.70 (<0.001)	0.71 (<0.001)
HE4 protein serum concentration	4.31 (<0.001)	0.76 (<0.001)	0.72 (<0.001)
CA125 protein serum concentration	2.6 (0.009)	0.64 (0.012)	0.57 (0.175)

B Multivariate Logistic Regression model

Variable	Coef (std error)	Z (p)
bCPA score	1.17 (0.50)	2.36 (0.018)
HE4 protein serum concentration	1.12 (0.36)	3.27 (0.001)
CA125 protein serum concentration	0.09 (0.28)	0.32 (0.752)

C Joint Prediction Model (JPM)

Variable	Coef (std error)	Z (p)
bCPA score	1.17 (0.50)	2.36 (0.018)
HE4 protein serum concentration	1.12 (0.36)	3.27 (0.001)

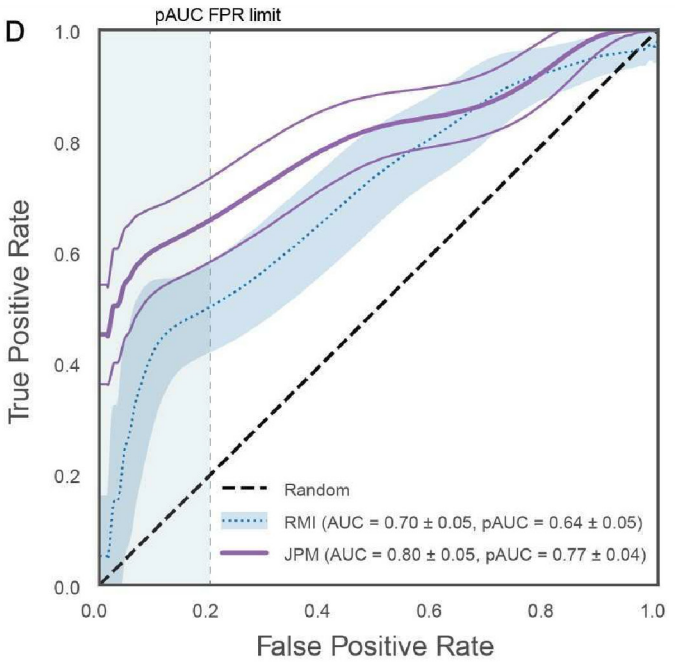

Figure 3 Joint prediction model (JPM). (A) Univariate logistic regression analyses for the benign-referenced copy number profile abnormality (bCPA) score, and human epididymis protein 4 (HE4) and cancer antigen 125 (CA125) measurements, individually. (B) Multivariate logistic regression analysis based on all three variables. By combining all three variables into a single model, CA125 does not add any significant predictive value, while the combination of bCPA score and HE4 remain significantly predictive. (C) Coefficients for the JPM. (D) Receiver operating characteristic comparison of the Risk of Malignancy Index and the JPM incorporating the bCPA score and the HE4 measurement, with area under the curve values indicating the performance of each method in classifying observations. AUC, area under the curve; bCPA, benign-referenced copy number profile abnormality; CA125, cancer antigen 125; FPR, false-positive rate; HE4, human epididymis protein 4; JPM, joint prediction model; pAUC, partial area under the curve; RMI, Risk of Malignancy Index; ROC, receiver operating characteristic.

Figure 1B shows the resulting copy number aberrations profiles. A difference in signal strength is observed between the malignant and benign tumors and behaves as expected: benign tumors resembling healthy reference samples more closely. This difference in similarity is also apparent from the copy number profile abnormality score (Figure 1B and (C): with copy number profile abnormality scores in patients with a malignant mass being significantly higher than in benign controls (Mann–Whitney U test, p<0.0001) (Figure 1C). Finally, we evaluated the classification performance of the copy number profile abnormality score and benchmarked it against the RMI evaluating their ROC curves. The partial AUCs are statistically indistinguishable (Figure 1D).

A limitation of calibrating WisecondorX on healthy reference samples is the requirement for 50 healthy reference samples to be sequenced on the same sequencer and under the same protocol as the samples to be classified, which poses a barrier to clinical implementation. Additionally, our reference set contained pregnant women, so there is potential confounding with circulating fetal DNA. Finally, while genetic aberrations are present in malignant and benign tumors, these are absent in healthy samples, making them less ideal as a reference source when wanting to distinguish between malignant and benign tumors. We therefore used the benign samples as a reference set when employing WisecondorX, accumulating into benign-calibrated copy number profile abnormality scores (see Methods).

Benign-calibrated copy number profile abnormality score

As we have a limited set of patients with a benign tumor, we use a leave-one-sample-out cross-validation scheme, as shown in Figure 2A and described in the Methods section, to calculate benign-calibrated copy number profile abnormality scores for all cases and controls. The (benign-calibrated) copy number profile abnormality scores showed a high concordance (Pearson's correlation coefficient r=0.98) (Figure 2B) and similar classification performance (Figure 2C), indicating that healthy reference samples are not necessary when computing copy number profile abnormality scores.

Detection using circulating DNA and liquid biopsy-based measurements

Next, we evaluated whether performance based on benign-calibrated copy number profile abnormality scores can be improved by integrating these scores with CA125 and HE4. We trained a logistic regression model using these three input variables. Despite all three univariate models being statistically significant (Figure 3A), this combined model showed that CA125 becomes insignificant in the presence of the other two variables (Figure 3B). The joint prediction model based on benign-calibrated copy number profile abnormality scores and HE4 significantly outperforms all univariate predictors alone (Figure 3C) (pAUC difference of 0.13; p=0.02). Especially large differences in sensitivity at high specificity between the joint

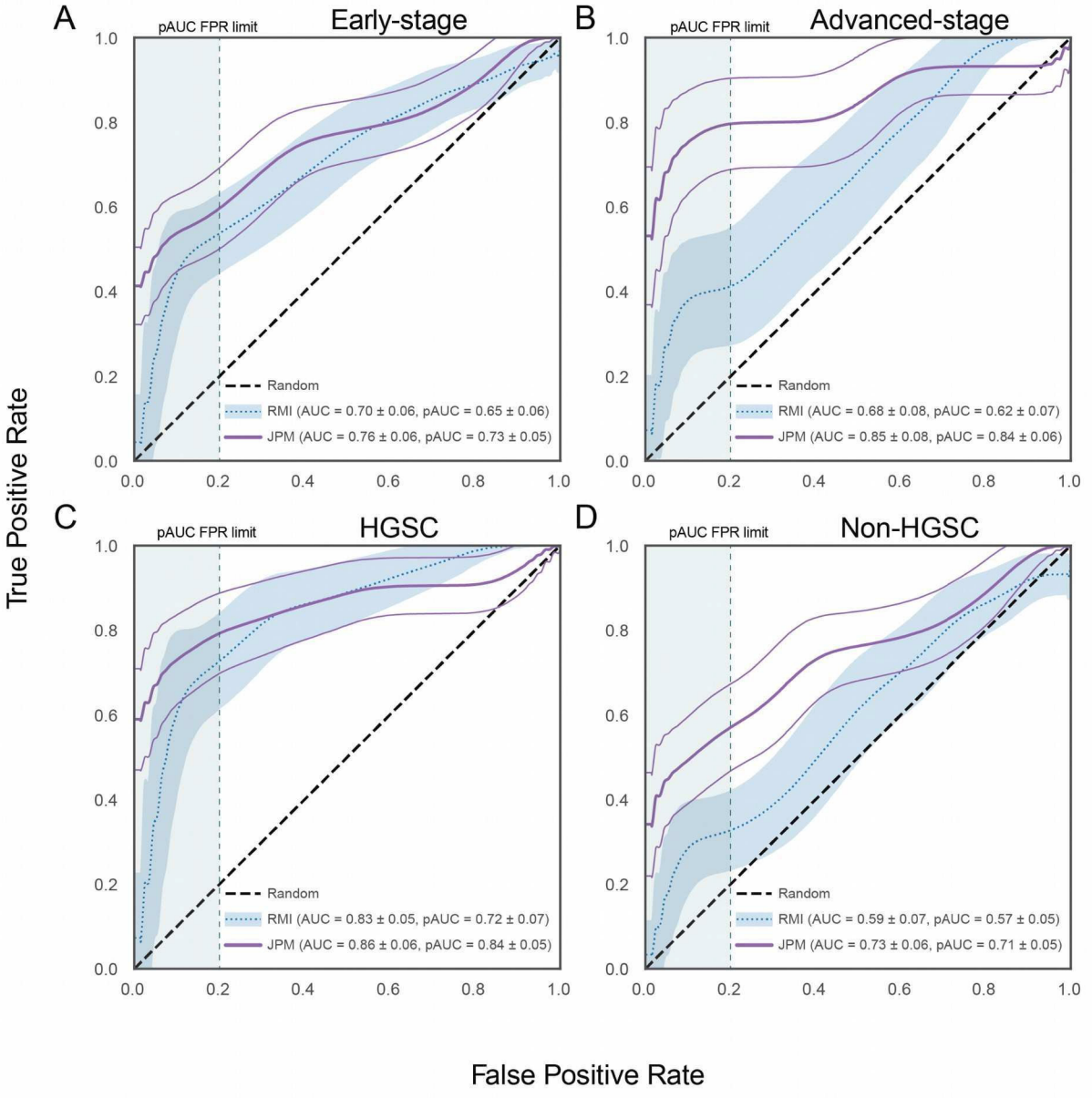

Figure 4 Comparison of classification performance of the joint prediction model (based on the benign-referenced copy number profile abnormality score and the human epididymis protein 4 measurement) to the Risk of Malignancy Index on receiver operating characteristic curves for: (A) early-stage malignant, (B) advanced-stage malignant, (C) high-grade serous ovarian cancer, and (D) all other malignant histological subtypes. AUC, area under the curve; bCPA, benign-referenced copy number profile abnormality; FPR, false-positive rate; HE4, human epididymis protein 4; HGSC, high-grade serous ovarian cancer; JPM, joint prediction model; pAUC, partial area under the curve; RMI, Risk of Malignancy Index; ROC, receiver operating characteristic.

prediction model and the RMI were observed (Online supplemental table S2) (99% sensitivity: 0.42 vs 0.00, respectively; 95% sensitivity: 0.58 vs 0.20, respectively).

We wondered how our predictor performs for different malignant subtypes. We stratified into early-stage disease, advanced-stage disease, high-grade serous ovarian carcinoma, and non-high grade serous ovarian carcinoma. Then we compared the joint prediction model to the RMI in each context (Figure 4). Number of patients per category is shown in Online supplemental table S1. The AUC, pAUC, and sensitivity for different specificities are shown in Online supplemental table S2. The sensitivity and specificity for the optimal Youden's index are shown in Online supplemental table S3. Most notably, the joint prediction model outperforms the RMI in all groups on all metrics.

For high-grade serous ovarian carcinoma patients, both the RMI and the joint prediction model perform well. However, in advanced-stage tumors and in the non-high-grade serous ovarian carcinoma subtypes, the joint prediction model significantly outperforms the RMI. In non-high-grade serous ovarian carcinoma subtypes, the RMI obtains an AUC and pAUC indistinguishable from random (for AUC and pAUC p=0.20 and p=0.10, respectively).

DISCUSSION

Summary of main results

We showed that copy number aberrations profiles derived from cell-free DNA can be used to classify malignant from benign ovarian masses. Adding serum HE4 to the benign-calibrated copy number profile abnormality score resulted in a significantly better pAUC (0.77) compared with the RMI (0.64). This joint prediction model outperforms the RMI model on all malignant subtypes. The copy number profile abnormality score derived with WisecondorX when using benign ovarian masses as a reference set achieved a similar performance as when using healthy reference samples. Thus, it suffices to train WisecondorX on benign masses, removing the need for healthy reference samples.

Results in the context of published literature

Our method's ability to outperform the RMI in all subtypes, including and especially those where the RMI performs poorly, highlights its potential clinical utility, particularly as subtype information is often unknown preo-peratively. This also emphasizes that the RMI is dependent on patient selection, which explains differences in the AUC values reported in the literature.

In our study, the RMI showed 0% sensitivity at 99% specificity because the highest RMI score was linked to a benign case, leading to no malignant detections at this threshold. Although a larger cohort might show increased RMI sensitivity, the joint prediction model still detected 42% of malignancies at the same specificity, indicating a statistically significant difference in performance.

Many international guidelines recommend performing staging surgery in borderline tumors, while in the Netherlands, borderline tumors are often treated at general hospitals, because staging surgery does not influence survival.[23] Consistent with the existing literature,[29] few copy number aberrations were detected in these cases, rendering them closer to benign masses than malignant in this context. As our prediction model relies on copy number aberrations, it might classify these borderline tumors as benign due to the absence of copy number aberrations, which could slightly lower its effectiveness if borderline tumors were considered malignant. While in terms of generalizability it is useful to analyze borderline tumors as a separate group, this cohort only contained five patients with borderline tumors, making a subgroup analysis statistically meaningless.

Strengths and weaknesses

A limitation is that we did not measure tissue material alongside the blood samples. Therefore, we could not confirm whether copy number aberrations found in liquid biopsy was in agreement with copy number aberrations found in tumor tissue. However, for clinical practice, this is less relevant as long as the pre-operative classification into benign and malignant ovarian masses has high sensitivity and specificity. Another limitation is that we could not combine our profiles with data from the ADNEX (Assessment of Different NEoplasias in the adneXa) model from the International Ovarian Tumor Analysis (IOTA) consortium, as this was not available for this cohort and is increasingly being used to assess ovarian masses.

Implications for practice and future research

Future research should focus on combining different liquid biopsy-based markers and ultrasound-based risk models to increase performance. The findings of this study need to be replicated in a larger cohort and with other ultrasound models before the Joint Prediction Model can be implemented in clinical practice. To this end, the OVI-DETECT trial (Dutch trial number: NL75690.031.20) is currently enrolling patients to evaluate the efficacy of utilizing cell-free DNA-derived multimodal data to distinguish benign from malignant ovarian masses. Finally, future research should focus on the cost-effectiveness of cell-free DNA implementation in clinical settings.

Conclusion

We conclude that a shallow whole-genome sequencing-derived benign-calibrated copy number profile abnormality measure has added benefit in detecting a malignancy in patients with an ovarian mass suspected to be early-stage ovarian cancer.

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Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by the Institutional Review Broad of the Netherlands Cancer Institute Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. WisecondorX-based copy number profiles dataset used for predictor training.

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