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TUMOR MARKERS AND SIGNATURES

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# Proteomic signature for detection of high-grade ovarian cancer in germline *BRCA* mutation carriers

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## Abstract

No current screening methods for high-grade ovarian cancer (HGOC) guarantee effective early detection for high-risk women such as germline *BRCA* mutation carriers. Therefore, the standard-of-care remains risk-reducing salpingo-oophorectomy (RRSO) around age 40. Proximal liquid biopsy is a promising source of biomarkers, but sensitivity has not yet qualified for clinical implementation. We aimed to develop a proteomic assay based on proximal liquid biopsy, as a decision support tool for monitoring high-risk population. Ninety Israeli *BRCA1* or *BRCA2* mutation carriers were included in the training set (17 HGOC patients and 73 asymptomatic women),

**Abbreviations:** AUC, area under the curve; HGOC, high-grade ovarian cancer; HPLC, high-pressure liquid-chromatography; LFQ, label-free quantification; MS, mass spectrometry; NPV, negative predictive value; PCA, principal component analysis; PPV, positive predictive value; ROC, receiver operating characteristic; RRSO, risk-reducing salpingo-oophorectomy; STIC, serous tubal intraepithelial carcinoma; UtL, utero-tubal lavage.

Tamar Geiger and Keren Levanon contributed equally to our study.

  

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(BEDOCA trial; ClinicalTrials.gov Identifier: NCT03150121). The proteome of the microvesicle fraction of the samples was profiled by mass spectrometry and a classifier was developed using logistic regression. An independent cohort of 98 *BRCA* mutation carriers was used for validation. Safety information was collected for all women who opted for uterine lavage in a clinic setting. We present a 7-protein diagnostic signature, with AUC >0.97 and a negative predictive value (NPV) of 100% for detecting HGOC. The AUC of the biomarker in the independent validation set was >0.94 and the NPV >99%. The sampling procedure was clinically acceptable, with favorable pain scores and safety. We conclude that the acquisition of Müllerian tract proximal liquid biopsies in women at high-risk for HGOC and the application of the *BRCA*-specific diagnostic assay demonstrates high sensitivity, specificity, technical feasibility and safety. Similar classifier for an average-risk population is warranted.

**KEYWORDS**
*BRCA*, early detection biomarker, ovarian cancer, utero-tubal lavage

**What's new?**

There is no diagnostic test for early stage high-grade ovarian cancer (HGOC), so women at high risk are recommended to have their ovaries and fallopian tubes surgically removed. However, the surgery comes with some unwanted side effects. Here, the authors present a 7-protein signature for detecting HGOC in a proximal liquid biopsy from the uterine cavity. The protein assay distinguished between patients and controls with high sensitivity, and the sampling method was clinically acceptable with respect to pain and safety. This approach could help assist women carrying BRCA mutations and their physicians determine the appropriate timing for prophylactic surgery.

## 1 | BACKGROUND

High-grade ovarian cancer (HGOC) remains the most lethal gynecologic malignancy, since over 75% of ovarian cancer patients are diagnosed with advanced-stage disease, which results in a 5 year survival of ~40%.[1] Currently, there is no reliable diagnostic test for early stage HGOC. Periodic surveillance with pelvic ultrasound and serum CA-125 has not shown a survival benefit in the general population or in women at high risk.[2-4] Therefore, current recommendation for women at an increased risk for HGOC, such as *BRCA1* and *BRCA2* mutation carriers, is to undergo risk-reducing salpingo-oophorectomy (RRSO) at age 35 to 45 after completing their reproduction plans.[5] Although RRSO has been proven to reduce mortality significantly,[5,6] early surgical menopause and its related health consequences are a significant cause of patient reluctance or procrastination. The incidence of HGOC in *BRCA1* and *BRCA2* carriers before age 50 is relatively low (17% and 1.2%, respectively[7]), yet as long as no risk stratification assay exists, the recommendation of RRSO cannot be individualized. Any decision-support tool which may safely delay RRSO in at least a subset of women at risk, and by that improve their quality of life and decrease long-term menopause-associated morbidity, is an important unmet clinical need.

Over the past two decades the origin of most HGOC has been proven to be the epithelium of the distal fallopian tube.[8,9] Serous tubal intraepithelial carcinomas (STICs) are the precancerous lesions that tend to shed tumor cells into the tubal lumen and the pelvis[10-12] and eventually become invasive and spread to the peritoneum and the regional lymph nodes.[13] Mutational evolutionary analyses identify a window of 6 years between the development of *TP53* mutated precursor and the initiation of HGOC.[14] This knowledge holds an opportunity to utilize proximal liquid biopsies to retrieve tubal cells and their secreted products as a source for early-stage biomarkers. This may potentially lead to the detection at a curable stage and reduce HGOC-specific mortality. Transcriptomic analyses of normal *BRCA*-mutant compared to *BRCA*-WT fallopian tube epithelium showed significant differences and differential response to stimuli.[15-17] Furthermore, the transcriptional program of premenopausal *BRCA*-mutant fallopian tube epithelium shares high similarity with HGOC.[18] These data raise the hypothesis that *BRCA* carriers require a specific diagnostic biomarker, which is different from that of an average-risk population.

Liquid biopsy is an approach that allows diagnosis of cancer without an invasive procedure. Proximal liquid biopsies are sampled directly from the body cavity in which a tumor arises, increasing the likelihood of detecting early-stage or even precancerous lesions. Since the entire gynecological tract is a communicating lumen, several research groups attempted to detect *TP53* mutated ctDNA from exfoliated cells of HGOC and STIC lesions using Papanicolau cytology smear or uterine lavage samples.[19-22] Maritschnegg et al published their experience of performing utero-tubal lavage with a new

proprietary catheter in 22 healthy patients.[23] Although overall promising, this technique required local cervical anesthesia and cervical dilatation in a large fraction of the cases. Similarly, we reported the utilization of utero-tubal lavage liquid biopsies for the development of a 9-features proteomic signature for diagnosis of HGOC.[24] The sensitivity and specificity of this diagnostic test are 70% and 76%, respectively, better than previously reported and with promising predictive power for early-stage lesions. However, this signature has unsatisfactory specificity in a cohort of *BRCA* carriers, who are the most urgent target population for this assay.

In this work we describe the performance of a proteomic signature for identification of HGOC in *BRCA* carriers, using proximal liquid biopsy. We also show that the UtL is a feasible technique that can be widely adopted in the clinic to monitor high-risk populations.

## 2 | METHODS

### 2.1 | Study population

The Biomarkers for Early Detection of Ovarian Cancer Using Uterine Lavage (BEDOCA) trial (ClinicalTrials.gov identifier: NCT03150121) recruits the following groups of participants[1]: Clinically healthy *BRCA1/2* mutation carriers who have not yet undergone RRSO-UtL is performed either in a clinic setting or an operating room immediately before their RRSO surgery[2]; HGOC patients, regardless of their *BRCA* status, UtL performed immediately before debulking surgery[3]; Average-risk controls, UtL performed in a clinic setting or an operating room. Pregnant women, women who were trying to conceive or menstruating at the time of clinic visit or patients with a legal guardian were excluded. Pathology reports and medical records were available to us in all cases.

To develop the diagnostic classifier, we analyzed 222 UtL samples of *BRCA* carriers, in several batches according to their dates. After exclusion of samples with low protein count or missing data, the first batches, with a total of 90 samples were included in the training set (17 HGOC patients and 73 controls), and all subsequent samples were regarded as a validation set (n = 98, 7 HGOC and 91 controls), and analyzed independently in a blinded manner. The sample distribution is illustrated in Figure 1A. Additional information can be found in Tables 1 and S1.

Two additional validation sets, included 172 and 140 samples of UtL from women with *BRCA*-WT or unknown *BRCA* status, were used to test the accuracy of the classifier for use in the general population. Information about the first cohort has been previously published by Barnabas et al.[24] Both sets are detailed in Table S2.

### 2.2 | Simplified utero-tubal lavage technique

To collect the liquid biopsy, an intrauterine insemination catheter (Insemi-Cath, Cook Inc. Bloomington, Indiana) or rigid pipelle uterine sampler (Endosampler, MedGyn, Addison, Illinois), according to the physician's discretion, are inserted into the endometrial cavity through the cervical canal without local anesthesia. Ten milliliters of sterile saline was flushed into the uterine cavity and fallopian tubes and immediately retrieved. The samples were then centrifuged to eliminate cells, and supernatants were aliquoted and stored at −80°C until proteomics analysis. The procedure was performed either in a clinic setting (awake) or an operating room (under general anesthesia).

### 2.3 | Proteomics

The UtL samples were processed and analyzed as previously described in greater detail.[24] Microvesicles were precipitated by centrifugation at 20 000*g* for 60 minutes at 4°C, washed with PBS and then solubilized, and the proteins were digested overnight with Trypsin/Lys-C mix (MS grade Promega, 1:100 enzyme to protein ratio) and sequencing grade modified trypsin (Promega, 1:50 enzyme-to-protein ratio). The peptides were analyzed by liquid-chromatography using the EASY-nLC1000 HPLC (Thermo Fisher Scientific) coupled to the Q-Exactive (QE) Plus or Q-Exactive HF mass spectrometers (Thermo Fisher Scientific, Bremen, Germany). Peptides were separated on 75 μm i.d. × 50 cm long EASY-spray PepMap columns (Thermo Fisher Scientific) packed with 2 μm, C18 material with 100 Å pore size. Raw MS files were analyzed in the MaxQuant software (version 1.5.2.18) and the Andromeda search engine. Separate analyses were performed for the training cohort and the validation cohort, using the same parameters. The label-free quantification algorithm (LFQ) in MaxQuant was used for relative quantification.

### 2.4 | Statistics

Data was analyzed using SAS version 9.4 (SAS Institute, Cary, North Carolina) software. Descriptive statistics are presented in Table 1. Logistic regression modeling was performed to assess proteomic predictors of HGOC in *BRCA* carriers. Variables were identified and entered into a multivariate model based on several model selection techniques as well as manual statistical evaluation. The variables designated to remain in the model were those factors that remained statistically significant when entered together in the models and maximized the predictive power (AUC of the ROC curve) of the model such that the AUC was >0.95. Several risk scores ranging from 0 to 1 were created from the selected logistic regression models using the model coefficients. The regression model coefficients were used to derive a risk score (termed "probability index") as $P = eY/(1 + eY)$, where $Y$ is the log of the odds of having ovarian cancer, and is a linear function of the independent factors.

ROC analysis was performed to test whether the parameters can discriminate between positive and negative subjects. ROC curve and the area under the ROC curve with 95% confidence interval (CI) are presented for each of the chosen models. For each discriminator, a cut-off value achieving a high sensitivity (100%) and approximately equal sensitivity and specificity is suggested. Sensitivity, specificity, PPV and NPV are presented with 95% CIs.

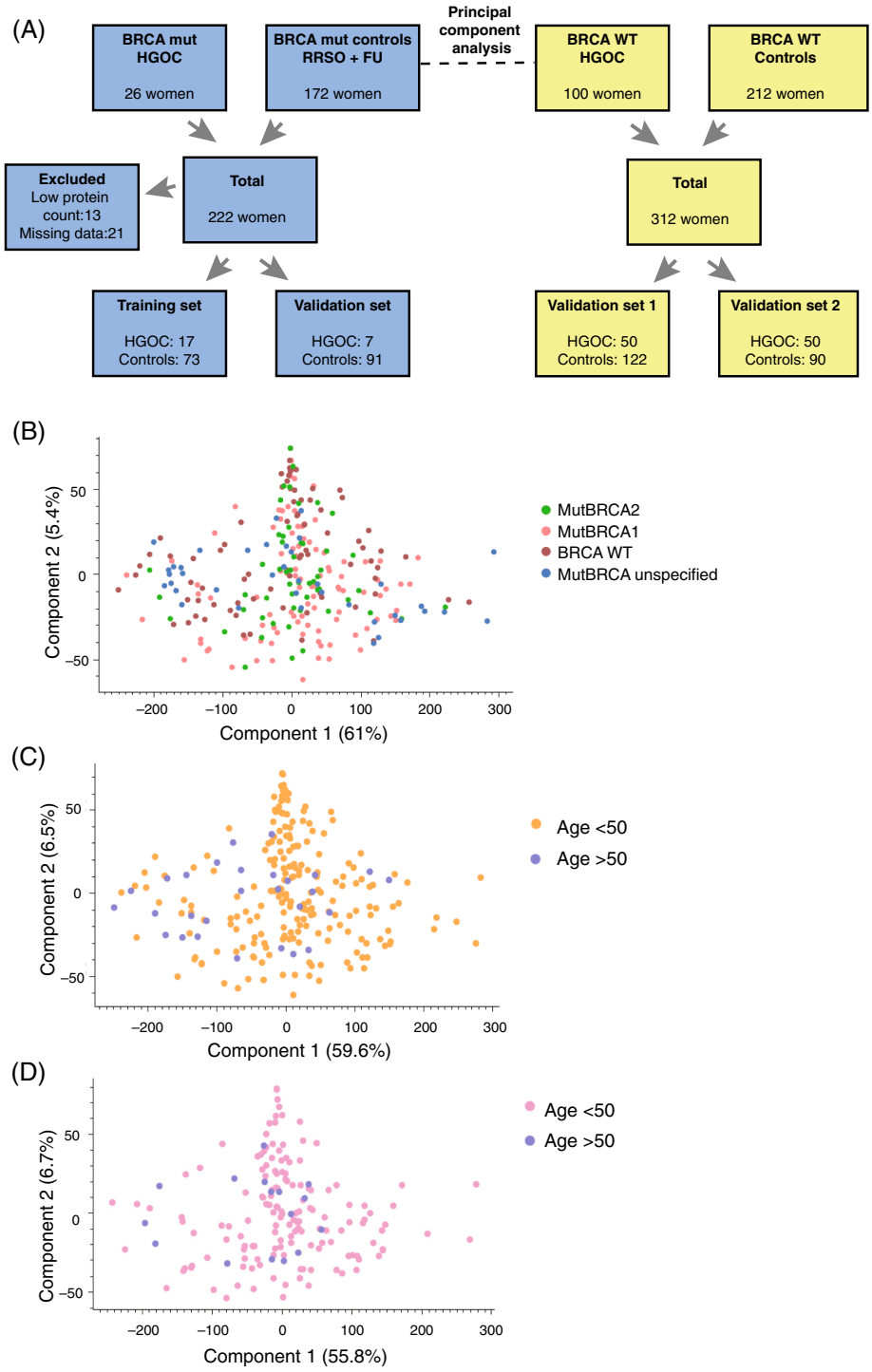


**FIGURE 1** Proteomic profiling of the microvesicle fraction of UtL liquid biopsy. (A) Illustration of the study cohorts, including *BRCA*-mutant training and validation sets and *BRCA* WT/unknown validation sets. (B-D) Principal component analysis (PCA) of all normal samples, separation according to: (B) *BRCA* mutation status mut*BRCA1*, mut*BRCA2*, mut*BRCA* unspecified and *BRCA* WT; (C) or age <50 vs ≥50. (D) PCA of *BRCA* mutant samples only, separated based on age <50 vs ≥50

## 2.5 | Immunohistochemistry

We used our previously reported tissue microarrays for evaluation of the expression of the classifier proteins in FT epithelium and HGOC tumor sections. Details of the TMAs are published.[24] All slides were simultaneously stained and scored. Primary antibodies used: (a) anti-VPS11 (HPA039020, 1:200, positive control: breast cancer); (b) anti-ATP2B4 (HPA04043, 1:1000, positive control: keratinocytes); (c) anti-CRTAC1 (HPA008175, 1:50, positive control: normal pneumocytes) from Sigma-Aldrich, St. Louis, Missouri; (d) anti-TMEM67 (13975-1-AP, 1:50, positive control: normal kidney) from ProteinTech, Rosemont, Illinois.

## 2.6 | Safety questionnaires

Participants who opted to UtL procedure in a clinic setting, completed a 6-question form assessing: (a) pre-test and (b) post-test anxiety, (c) pain at the time of the procedure and (d) shortly afterwards (up to 30 minutes), (e) subjective assessment of length of the procedure and

**TABLE 1** Summary of clinical and demographic characteristics of the study participants

| | HGOC patients (n = 26) | RRSO (n = 79) | Healthy *BRCA* carriers (n = 93) |
|---|---|---|---|
| Sampling setting | Operating room | Operating room | Clinic (n = 116) |
| | | | Operating room (n = 1) |
| Age median (range) | 55.3 (38-79) | 45.8 (37-65) | 34.7 (22-65) |
| Germline mutation | *BRCA1*: 18 (69%) | *BRCA1*: 30 (38%) | *BRCA1*: 78 (66%) |
| *BRCA1* (%); *BRCA2* (%); Other (%) | *BRCA2*: 4 (15.4%) | *BRCA2*: 23 (29%) | *BRCA2*: 35 (30%) |
| | Other: 4 (15.4%) | Other: 26 (33%) | Other: 4 (3.4%) |
| Stage—FIGO | | N/A | N/A |
| STIC (%) | 1 (4%) | | |
| 1 (%) | 5 (19.2%) | | |
| 2 (%) | 2 (7.7%) | | |
| 3-4 (%) | 18 (69%) | | |
| Prior term pregnancies | N/A | N/A | 62% |
| Number of samples contributed by participant | N/A | N/A | |
| 1 (%) | | | 93 (79.5%) |
| 2 (%) | | | 18 (15.4%) |
| 3 (%) | | | 4 (3.4%) |
| >3 (%) | | | 2 (1.7%) |
| Sample volume | | | |
| Mean (range) | 4.3 (1-10) | 3.6 (1-9) | 4.6 (1-9) |
| Excluded (%) | 2 (7%) | 21 (26.6%) | 10 (8.5%) |

(f) compliance with additional such procedures in subsequent visits. The scores were graded on a 0 to 5 scale, "0" reflecting no suffering and "5" representing immense suffering. The performing gynecologist reported the time required to complete the procedure, immediate complications and technical challenges.

## 3 | RESULTS

### 3.1 | Cohort characteristics

Figure 1A illustrates the allocation of samples to the various sets. The main clinical information is summarized in Table 1, and the individual clinical information for study participants is listed in Table S1. More detailed information about the *BRCA* carriers who underwent UtL in a clinic setting is found in Table S3.

#### 3.1.1 | High-risk cohort

From December 2016 to Sept 2021 we enrolled 172 asymptomatic women with genetically-defined high risk, including carriers of germline mutation in *BRCA1* (n = 89), *BRCA2* (n = 52) or unspecified gene (n = 31) and collected a total of 197 UtL liquid biopsies. Thirty-two samples were excluded from analysis due to missing information or low protein count. One case was diagnosed with occult malignancy (in situ carcinoma of the fallopian tube, STIC) and the sample was classified as "HGOC." Of the remaining 164 samples, 106 were collected in a clinic setting and 58 were collected during surgery. The median age of the group was 38 years (range: 22-65). Twenty-four women contributed two or more UtL liquid biopsies on consecutive visits, approximately 6 months apart.

#### 3.1.2 | *BRCA*-mutant HGOC cohort

Twenty-six HGOC patients with germline *BRCA* mutation were enrolled and contributed one UtL sample each. In most cases, the germline *BRCA* mutation was discovered after the diagnosis of HGOC. Two samples were excluded from analysis, resulting in a total of 24 HGOC samples, 6 of which are considered early stage.

All high-risk participants (except for one) were of Jewish or European descent.

#### 3.1.3 | *BRCA*-WT cohort

UtL liquid biopsies were collected during gynecological surgeries from HGOC patients and controls with WT or unknown *BRCA* mutational status. This set included two independent cohorts. "Validation set 1" includes 50 HGOC patients and 122 controls, and "validation set 2" includes 50 HGOC patients and 90 controls.

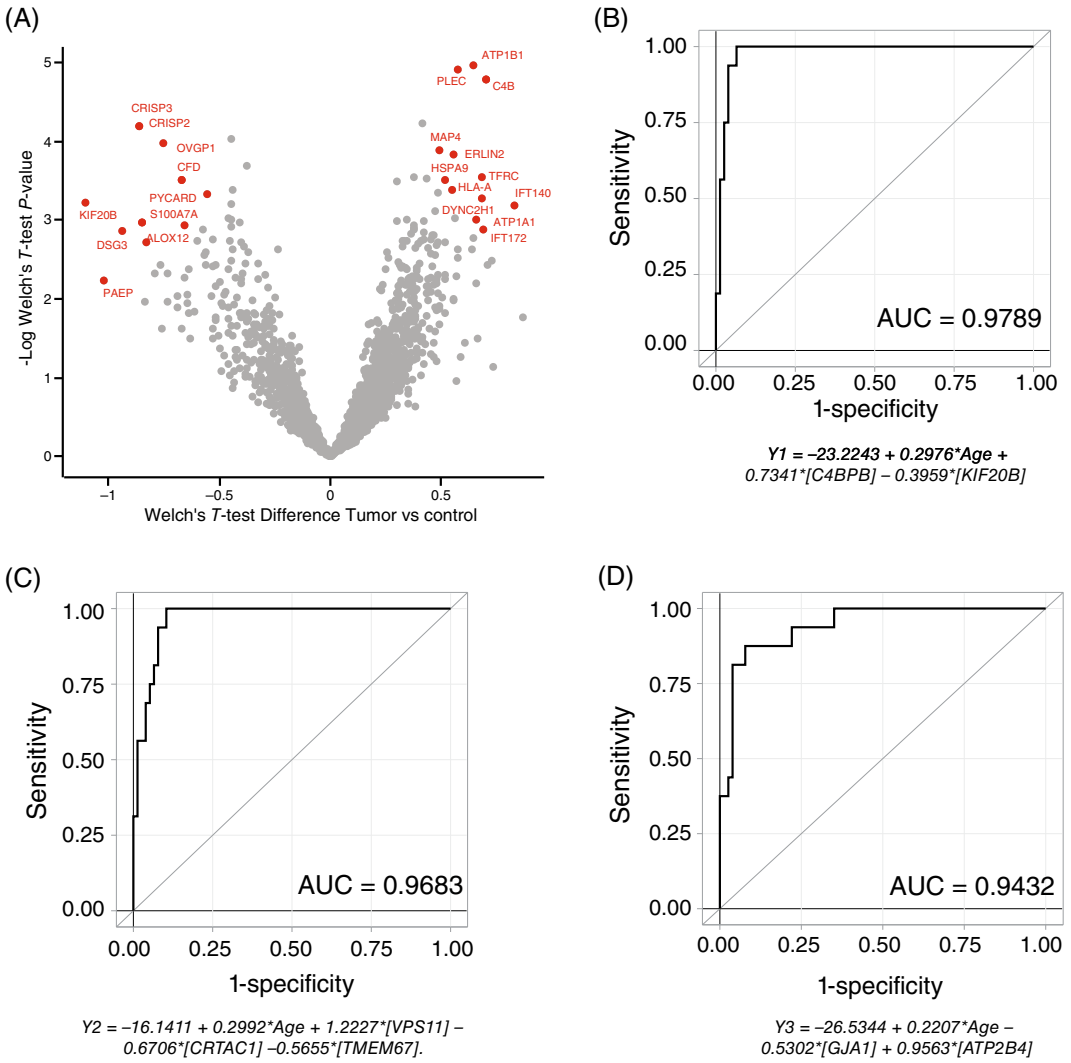


**FIGURE 2** Proteomic biomarkers for diagnosis the HGOC in *BRCA*-mutant population. (A) Volcano plot shows the differentially expressed proteins between the patient and control samples, with FDR 0.05 and s0 = 0.5. Statistically significant proteins are highlighted in red. (B-D) ROC curves of three discriminant models: (B) Model 1, AUC = 0.9789 (95% Wald CI: 0.9533-1.000); (C) Model 2, AUC = 0.9683 (95% Wald CI: 0.9379-0.9988); (D) Model 3, AUC = 0.9432 (95% Wald CI: 0.8890-0.9974)

Clinical and proteomic information of this cohort is presented in Table S2.

## 3.2 | Proteomic analysis of UtL liquid biopsy of women at high risk

To ascertain the similarity of UtL proteomic profile of *BRCA1* and *BRCA2* carriers, we compared the proteomes of 193 UtL samples of healthy *BRCA* carriers (mean age 38.0) with the proteomes of 76 UtL of healthy *BRCA*-WT controls (mean age 40.4). Figure 1B is a principal component analysis (PCA) indicating that there is no obvious difference between the UtL proteomic content of *BRCA1/BRCA2* germline mutation carriers and age-matched healthy WT controls. There are also no differences in the overall proteomic profile according to menopausal status (women ≥50 were considered as menopausal and women below 50 as premenopausal) in the entire group of healthy participants (Figure 1C), or in the subset of *BRCA* carriers (Figure 1D). Overall, these data show that the proteomes of UtL liquid biopsies of *BRCA* carriers are similar across genetic background (*BRCA1* and *BRCA2* mutations), age and hormonal status. While PCA cannot disclose fine details of differential expression between *BRCA* mutated and WT tissues, it provides support to the rationale of developing a single assay for detection of HGOC in all *BRCA* carriers.

## 3.3 | Development of BRCA-specific classifiers

Next, we compared the proteomes of all UtL liquid biopsies from *BRCA*-mutant HGOC patients and healthy controls. Combined MS

TABLE 2 Sensitivity, specificity, PPV and NPV of the validation set of Models 1 to 3, both optimum and 100% sensitivity operating points

| | Model 1 | | Model 2—100% sensitivity | | Model 2—optimum | | Model 3—100% sensitivity | | Model 3—optimum | |
|---|---|---|---|---|---|---|---|---|---|---|
| | % (n/N) | 95% CI | % (n/N) | 95% CI | % (n/N) | 95% CI | % (n/N) | 95% CI | % (n/N) | 95% CI |
| Overall accuracy | 84.00% (84/100) | [75.58%-89.90%] | 66.67% (4/6) | [30.00%-90.32%] | 66.67% (4/6) | [30.00%-90.32%] | 87.63% (85/97) | [79.61%-92.78%] | 94.85% (92/97) | [88.50%-97.78%] |
| Sensitivity | 71.43% (5/7) | [35.89%-91.78%] | 50.00% (2/4) | [15.00%-85.00%] | 50.00% (2/4) | [15.00%-85.00%] | 66.67% (4/6) | [30.00%-90.32%] | 50.00% (3/6) | [18.76%-81.24%] |
| Specificity | 84.95% (79/93) | [76.30%-90.82%] | 100.00% (2/2) | [34.24%-100.00%] | 100.00% (2/2) | [34.24%-100.00%] | 89.01% (81/91) | [80.94%-93.92%] | 97.80% (89/91) | [92.34%-99.40%] |
| PPV | 26.32% (5/19) | [11.81%-48.79%] | 100.00% (2/2) | [34.24%-100.00%] | 100.00% (2/2) | [34.24%-100.00%] | 28.57% (4/14) | [11.72%-54.65%] | 60.00% (3/5) | [23.07%-88.24%] |
| NPV | 97.53% (79/81) | [91.44%-99.32%] | 50.00% (2/4) | [15.00%-85.00%] | 50.00% (2/4) | [15.00%-85.00%] | 97.59% (81/83) | [91.63%-99.34%] | 96.74% (89/92) | [90.85%-98.88%] |

*Note*: Model 2 risk score cannot be calculated for most of the validation set, since not all the proteins were detected.

analysis identified a total of 8800 proteins, and an average of 4200 proteins per sample. A volcano scatter plot (Figure 2A) indicates the significantly different proteins (FDR < 0.1) and highlights the significant ones. Several of those are mentioned below as features in the *BRCA*-specific diagnostic classifier.

The distribution of UtL samples into "training" and "validation" sets is shown in Figure 1A. Analyses of the training and validation sets, including data normalizations and imputation of missing values, were performed independently. The training set included 110 samples, out of which 90, including 17 HGOC samples and 73 control, with high protein identification rates and complete clinical data were used to develop the diagnostic models. We applied logistic regression tools to define the best models in terms of the AUC and the shape of the ROC curve. Models 1, 2 and 3 are linear functions of the model parameter estimates (see Tables S4-S6, S7-S9 and S10-S12, respectively). Using ROC curves (Figure 2B-D) we calculated cut-off scores for two operating points (100% sensitivity and mathematical optimum) for each model. The models include an age parameter, expressed in years and the normalized intensity (LFQ) of the following seven proteins: C4BPB, KIF20B, VPS11, CRTAC1, TMEM67, GJA1 and ATP2B4.

*Model 1*: $Y1 = -23.2243 + 0.2976 \times \text{age} + 0.7341 \times \text{protein [C4BPB]} - 0.3959 \times \text{protein [KIF20B]}$.

Score model 1 = exp(Y1)/(1 + exp(Y1)). Model 1 cutoff score for 100% sensitivity is 0.191 and is equal to the optimum cutoff. AUC of the ROC curve is 0.9789.

*Model 2*: $Y2 = -16.1411 + 0.2992 \times \text{age} + 1.2227 \times \text{protein [VPS11]} - 0.6706 \times \text{protein [CRTAC1]} - 0.5655 \times \text{protein [TMEM67]}$.

Score model 2 = exp(Y2)/(1 + exp(Y2)). Model 2 cutoff for 100% sensitivity is 0.228, and for the optimum is 0.323. AUC is 0.9683.

*Model 3*: $Y3 = -26.5344 + 0.2207 \times \text{age} - 0.5302 \times \text{protein [GJA1]} + 0.9563 \times \text{protein [ATP2B4]}$.

Score model 3 = exp(Y3)/(1 + exp(Y3)). Model 3 cutoff for 100% sensitivity is 0.034, and for the optimum is 0.309. AUC is 0.9432.

We tested the correlation of each of the proteins with age, and found that the correlation is lower than 0.27, although statistically significant in three out of seven proteins (Table S13). This implies that age is not a confounder of the expression of the selected proteins, in the relevant age range. Overall, the sensitivity and negative predictive value (NPV) of each of the three models were 100% which justifies further validation.

## 3.4 | Validation of the classifiers

An independent validation set composed of 112 samples, of which 14 were excluded, resulting in 7 HGOC UtL samples (all of them were

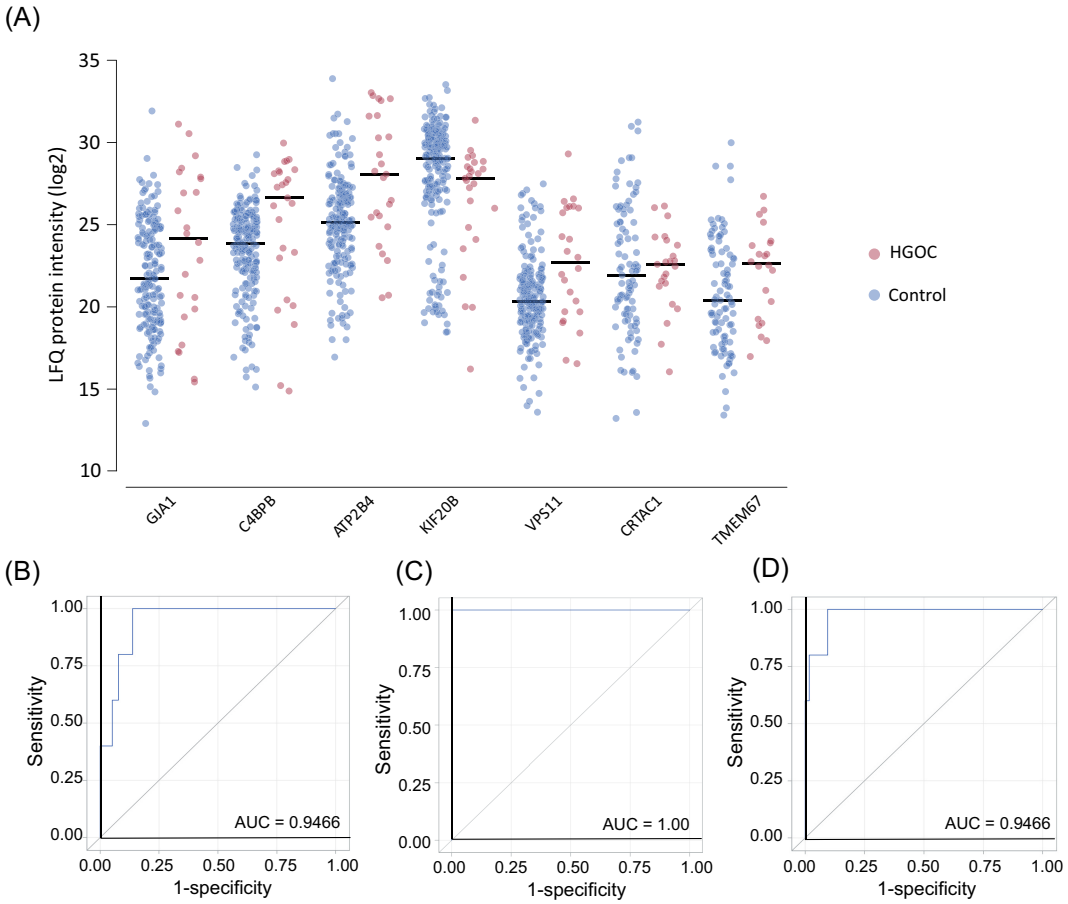


**FIGURE 3** Validation of the 7-protein classifier for *BRCA*-mutant population. (A) Differential expression of each of the seven proteins in HGOC patients vs controls (*BRCA*-mutated). Horizontal line indicates mean value. (B-D) ROC curves of three discriminant models: (B) Model 1, AUC = 0.8172 (95% Wald CI: 0.5670-1.000); (C) Model 2, AUC = 0.6250 (95% Wald CI: 0.0957-1.000); (D) Model 3, AUC = 0.9158 (95% Wald CI: 0.8187-1.000)

*BRCA1* mutant) and 91 control samples, was similarly processed, analyzed and normalized. Risk scores of the three models were calculated. The results of sensitivity, specificity, PPV and NPV are seen in Table 2. Intensities of the seven proteins in all *BRCA*-mutated UtL of HGOC patients and controls are plotted in Figure 3A). ROC curves of the independent validation sets are in Figure 3B-D, with AUC >0.94 for all models.

The levels of the proteins GJA1, C4BPB, ATP2B4, VPS11 and TMEM67 are significantly higher in UtL liquid biopsies from *BRCA*-mutant HGOC patients compared to controls. The level of KIF20B was lower in HGOC patients' samples compared to controls. We explored the TCGA HGOC database using cBioPortal[25,26] for *BRCA1* and *BRCA2* mutated samples (n = 133 across 4 studies), and confirmed that altered expression of the seven proteins does not correlate with *BRCA* mutant status (Figure S1). Next, to further investigate the expression of the seven proteins that compose the classifier we performed immunohistochemical staining of sections of tissue microarrays (TMAs) representing the following clinical-histological groups: (a) HGOC tumors; (b) fimbriae of asymptomatic *BRCA* mutation carriers, removed during RRSO; (c) grossly normal fimbriae of women with HGOC, *BRCA* WT. These TMAs were previously described in detail.[24] We used commercially available monoclonal antibodies targeting the proteins: VPS11, ATP2B4, TMEM67 and CRTAC1. Attempts to obtain reliable immunohistochemical staining for the three other proteins failed. Representative images are seen in Figure S2. For these four proteins we detected differential expression between FT epithelium of *BRCA*-WT and *BRCA* mutant women. The intensity of the staining of VPS11, ATP2B4 and TMEM in HGOC tumors was comparable to that seen in normal FT of *BRCA* mutant women.

Twenty-four healthy *BRCA* carriers were followed longitudinally the high-risk clinic at Sheba Medical Center, Israel, with UtL performed every 6 months, up to five times (Table S1, the samples are labeled with a letter a-d following their serial number). All the liquid biopsies from these women were scored as "normal," suggesting biological reproducibility which is essential for clinical implementation. Furthermore, several UtL samples were run twice in different batches, ~9 months apart, and on different MS systems to determine the technical reproducibility of the proteomic assay and the stability of the UtL samples after suboptimal storage conditions. Representative correlation plots are in Figure S3. Overall, these results suggest degree of reproducibility that is consistent with real-world limitations.

The intensity values of the seven proteins that construct the classifiers were compared between 163 UtL liquid biopsies of HGOC patients and 433 control samples (including ones that were previously excluded),

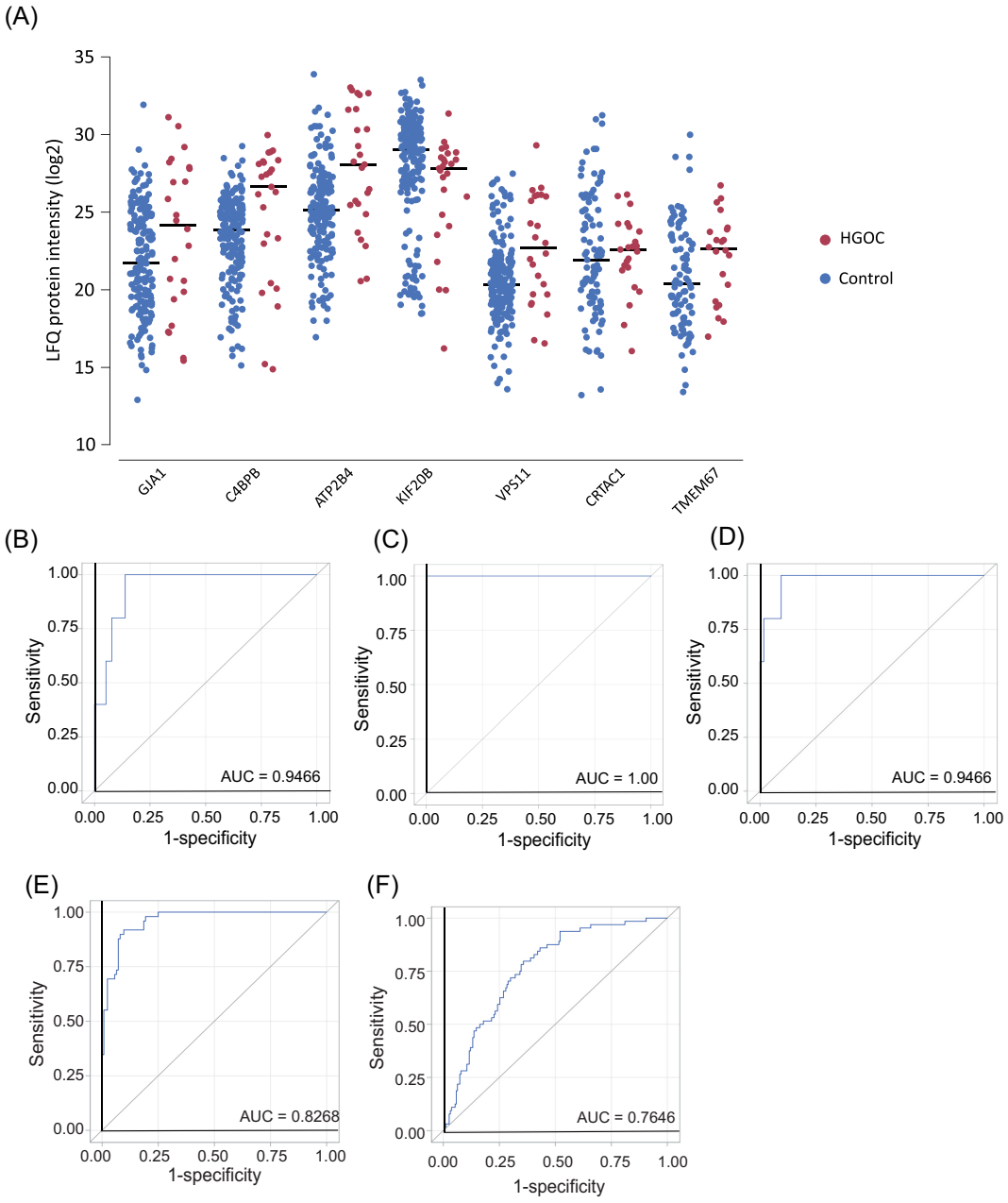


**FIGURE 4** Performance of the 7-protein classifier in *BRCA*-WT or *BRCA*-unknown population. (A) Differential expression of each of the seven proteins in HGOC patients vs controls. Horizontal line indicates mean value. (B-F) ROC curves of three discriminant models: (B&C) Model 1—set 1 and 2, respectively; (D) Model 2—set 1; (E,F) Model 3—set 1 and 2, respectively. 95% Wald CIs are indicated by each AUC

regardless of their *BRCA* status (Figure 4A). The intensities of all the proteins except C4BPB were not significantly different in UtL liquid biopsies from HGOC patients compared to controls, when *BRCA* status was ignored.

Next, we tested the discriminant performance of Models 1 to 3 on two sets of UtL liquid biopsies of participants who have WT *BRCA* or an unknown *BRCA* status. Of note, in the Israeli population, the chance of a HGOC patient to be a *BRCA* mutation carrier is ~30%. Eighty percent of HGOC patients had undergone basic genetic testing for common founder mutations, rather than full gene sequencing, and negative results indicate a residual risk of <8% for having a pathogenic mutation.[27] The two independent cohorts were normalized and analyzed separately, and included HGOC patients (n = 50 and 50, respectively), and healthy controls (n = 122 and 90, respectively). Table 3 shows the sensitivity, specificity, PPV and NPV of the three models in these cohorts. Risk score calculations for Model 2 are missing in set 2, since not all the proteins were detected. The ROC curves in Figure 4B-F indicate inconsistencies between the two sets, which warrant further validation. Overall, while the classifier's performance is worse for the WT population than the *BRCA* mutation carriers, it remains better than any other available diagnostic tool in a non-*BRCA* population as well. Importantly, the classifier also identified correctly one of two HGOC cases of women who were *BRCA*

**TABLE 3** Sensitivity, specificity, PPV and NPV of Models 1 to 3, both optimum and 100% sensitivity operating points, on *BRCA* WT and unknown *BRCA* status UtL samples

| | Model 1 | | Model 2—100% sensitivity | | Model 2—optimum | | Model 3—100% sensitivity | | Model 3—optimum | |
|---|---|---|---|---|---|---|---|---|---|---|
| | % (n/N) | 95% CI | % (n/N) | 95% CI | % (n/N) | 95% CI | % (n/N) | 95% CI | % (n/N) | 95% CI |
| *Set 1* | | | | | | | | | | |
| Overall accuracy | 53.49% (92/172) | [46.04%-60.78%] | 58.14% (100/172) | [50.67%-65.26%] | 61.63% (106/172) | [54.18%-68.57%] | 44.77% (77/172) | [37.53%-52.23%] | 57.56% (99/172) | [50.09%-64.70%] |
| Sensitivity | 80.00% (40/50) | [66.96%-88.76%] | 92.00% (46/50) | [81.16%-96.85%] | 88.00% (44/50) | [76.20%-94.38%] | 92.00% (46/50) | [81.16%-96.85%] | 74.00% (37/50) | [60.45%-84.13%] |
| Specificity | 42.62% (52/122) | [34.20%-51.49%] | 44.26% (54/122) | [35.76%-53.12%] | 50.82% (62/122) | [42.06%-59.53%] | 25.41% (31/122) | [18.52%-33.80%] | 50.82% (62/122) | [42.06%-59.53%] |
| PPV | 36.36% (40/110) | [27.98%-45.67%] | 40.35% (46/114) | [31.80%-49.53%] | 42.31% (44/104) | [33.25%-51.91%] | 33.58% (46/137) | [26.21%-41.84%] | 38.14% (37/97) | [29.10%-48.09%] |
| NPV | 83.87% (52/62) | [72.79%-91.00%] | 93.10% (54/58) | [83.57%-97.29%] | 91.18% (62/68) | [82.06%-95.89%] | 88.57% (31/35) | [74.05%-95.46%] | 82.67% (62/75) | [72.57%-89.58%] |
| *Set 2* | | | | | | | | | | |
| Overall accuracy | 76.38% (97/127) | [68.28%-82.92%] | 40.00% (6/15) | [19.82%-64.25%] | 46.67% (7/15) | [24.81%-69.88%] | 77.12% (91/118) | [68.76%-83.77%] | 77.97% (92/118) | [69.67%-84.50%] |
| Sensitivity | 93.33% (42/45) | [82.14%-97.71%] | 66.67% (2/3) | [20.77%-93.85%] | 66.67% (2/3) | [20.77%-93.85%] | 88.89% (40/45) | [76.50%-95.16%] | 66.67% (30/45) | [52.07%-78.64%] |
| Specificity | 67.07% (55/02) | [56.34%-76.28%] | 33.33% (4/12) | [13.81%-60.94%] | 41.67% (5/12) | [19.33%-68.05%] | 69.86% (51/73) | [58.56%-79.18%] | 84.93% (62/73) | [75.00%-91.37%] |
| PPV | 60.87% (42/69) | [49.07%-71.52%] | 20.00% (2/10) | [5.67%-50.98%] | 22.22% (2/9) | [6.32%-54.74%] | 64.52% (40/62) | [52.08%-75.26%] | 73.17% (30/41) | [58.07%-84.31%] |
| NPV | 94.83% (55/58) | [85.86%-98.23%] | 80.00% (4/5) | [37.55%-96.38%] | 83.33% (5/6) | [43.65%-96.99%] | 91.07% (51/56) | [80.74%-96.13%] | 80.52% (62/77) | [70.31%-87.82%] |

*Note*: Model 2 risk score cannot be calculated for part of the validation set, since not all the proteins were detected.

WT, but had a mutation in a mismatch repair gene compatible with diagnosis of Lynch syndrome.

### 3.5 | Feasibility and safety

To establish UtL as an acceptable office procedure for monitoring young high-risk population of women, it has to be technically feasible to all gynecologists, cheap, simple and safe. To assess these aspects, 18 gynecologists with varied level of skills and experience from three different Israeli medical centers participated in the study and acquired UtL liquid biopsies in a clinic setting. The average volume was 4.2 mL, and of the 137 liquid biopsies, 14 (10%) had a volume of <1 mL, which was arbitrarily defined as the volume used in the proteomic assay. The majority of samples with insufficient volume were taken by inexperienced residents.

We collected 137 questionnaires reporting pain and stress scores, as well as their attitude towards undergoing the same procedure in subsequent follow-up visits. The median pain score was 2, ranging from "0" representing no pain (n = 30), to "5" representing excruciating pain (n = 4). Local anesthesia or cervical dilatation was not allowed in this trial. The only factor which significantly correlated with the pain score is parity and only nulliparous women reported intense pain (score 4-5; Table S14).

Adverse events included prolonged pelvic pain (n = 2), vaginal discharge (n = 1) and vaginal bleeding starting the next day (n = 1), all of which did not require intervention and the severity of all adverse events were defined as Grade 1. One hundred and five women consented to additional sampling in the future, and none withdrew consent or expressed refusal to undergo the procedure in future visits. This real-life data suggests that the UtL technique is both highly safe and feasible.

## 4 | DISCUSSION

Efforts to develop clinically-meaningful biomarkers for early detection of HGOC have been futile for several decades and there are still no screening guidelines that reduce disease-specific mortality. Blood is clearly the most appealing resource for screening of vast populations since it can be easily obtained and handled, however, it remains debatable whether blood-based assays can truly detect HGOC early enough, before metastasis development, and increase cure rates. Proximal liquid biopsies are a fertile ground for discovery of biomarkers. We propose a minimally invasive technique to sample a liquid biopsy directly from the uterine cavity, which is continuous with the lumen of the site-of-origin of HGOC—the fallopian tube fimbria. This body fluid is more likely to disclose changes in the Müllerian tract epithelium, such as early stage malignancy, before they become evident in the systemic circulation. The proteomic approach captures molecular processes beyond the level of the tumor DNA, such as point mutations, copy number alterations and methylation markers, and may potentially disclose the perturbations in the tumor microenvironment as well. The 7-protein biomarker can discriminate between

patients and controls at high risk due to germline *BRCA* mutation, with high enough sensitivity and specificity to be considered clinically applicable. A decision-support tool to assist *BRCA* carriers and their physicians determine the individual risk for HGOC and the correct timing for safe RRSO should have a maximal sensitivity and maximal NPV, while specificity may be somewhat compromised, as long as RRSO remains the default solution for women with alarming positive or false-positive results. We analyzed longitudinal samples of healthy premenopausal *BRCA* carriers, and discovered variability in protein intensity that did not affect the prediction, but may be related to physiological changes. We currently record the phase of the menstrual cycle, and possibly will be able to define the best timing for sampling this liquid biopsy in premenopausal women. All the participants in this trial follow the recommendation for RRSO before age 40 to 45, so the chances of detecting latent HGOC while on the trial is extremely low. Moreover, the paucity of stage 1 and STIC lesions is an unsurpassable caveat that has plagued all early detection trials and challenges the ability to prove that our liquid biopsy technique and classifier genuinely detect clinically latent HGOC. Nonetheless, this innate problem is also the source of motivation for this type of research.

This proteomic signature is composed of three individual models that may be integrated to enhance the confidence and can compensate for failure to detect one or more of the proteins, as seen in our validation data. In future clinical trials we suggest that all cases in which at least one scores is suggestive of HGOC risk, the participant should be referred for subsequent testing (ie, pelvic MRI). Presumably more data can help define how to best adjust the weight of the three models.

The proteins that compose the signature were selected in an unbiased manner, using logistic regression methods. Their involvement in the carcinogenic process of HGOC has not been investigated yet, but previous studies associated most of them with other types of malignancies. KIF20B is a slow molecular motor protein, involved in cytokinesis, and cerebral cortex development.[28] It increases the proliferation of pancreatic, colon, and bladder adenocarcinoma, and has been shown to be a poor prognostic marker.[29-31] VPS11 is a vesicle mediated protein trafficking factor, required for fusion of endosomes and autophagosomes with lysosomes.[32] It has been shown to facilitate VEGFA secretion, in interaction with FOXM1,[33] and regulate several signaling factors and pathways, including Wnt, estrogen receptor α and NFκB.[34] CRTAC1 is a glycosylated extracellular matrix protein expressed in chondrogenic tissue.[35] It has been shown to be down-regulated in bladder cancer, losing its tumor-suppressive phenotype.[36] TMEM67 is required for ciliary structure, length, quantity and function.[37] It is also involved in cerebellar development. TMEM67 has been shown to be down-regulated in bladder carcinoma, resulting in poor prognosis.[38] GJA1 is a key component in the gap junction complex. GJA1 has been shown to regulate cell proliferation and its down-regulation is correlated with poor prognosis in several types of human cancer, particularly in HGOC.[39,40] Two additional proteins, C4BPB (which controls the classical pathway of complement activation) and ATP2B4 (play a role in intracellular calcium homeostasis in erythrocytes), have not been implicated in cancer biology. Interestingly, some of these proteins show a different expression trend than previously published, which suggests that the context of the tissue type may be critical to the protein function.

The benefits of our simplified UtL technique make it feasible in real-world setting: it uses a non-proprietary insemination or pipelle catheter, does not require anesthesia, special equipment or training. It can be performed by relatively unskilled gynecologist in an office setting, even in nulliparous women. Different independent ethics committees approved the UtL technique for our trial, as well as for other trials in worldwide (ClinicalTrials.gov: NCT02062697, NCT02039388, NCT02518256, NCT02387645, NCT03606486, NCT04794322, NCT04823871), based on evidence for lack of adverse prognosis of women who undergo hysteroscopy for endometrial carcinoma, despite consequent positive peritoneal cytology.[41-43] Extrapolating from the results of the present study regarding safety and stress levels and low complication rates, it is likely to be acceptable for *BRCA1/2* germline mutation carriers, as a semi-annual procedure, in cases where delay of RRSO is desired. The proteomic assay may be combined with other methodologies to further enhance the diagnostic accuracy.

## AUTHOR CONTRIBUTIONS

All authors read and approved the final article. **Keren Bahar-Shany:** Conceptualization, data curation, formal analysis, investigation, validation, project administration, writing - review & editing. **Georgina D. Barnabas:** Investigation, validation, data visualization. **Lisa Deutsch:** Data curation, formal analysis, data visualization. **Netanel Deutsch:** Data curation, formal analysis, data visualization. **Efrat Glick-Saar:** Data curation, investigation, validation. **Dan Dominissini:** Supervision. **Eitan Friedman:** Supervision. **Stav Sapoznik:** Conceptualization, resource acquisition. **Limor Helpman:** Conceptualization, resource acquisition. **Tamar Perri:** Conceptualization, **Jacob Korach:** Conceptualization, resource acquisition. **Anna Blecher:** Resource acquisition. **Guy Katz:** Resource acquisition. **Itai Yagel:** Resource acquisition. **Orgad Rosenblatt:** Resource acquisition. **Daniel Shai:** Resource acquisition. **Benny Brandt:** Resource acquisition. **Raanan Meyer:** Resource acquisition. **Aya Mohr-Sasson:** Resource acquisition. **Alexander Volodarsky-Perel:** Resource acquisition. **Itamar Zilberman:** Resource acquisition. **Shunit Armon:** Resource acquisition. **Ariella Jakobson-Setton:** Resource acquisition. **Ram Eitan:** Resource acquisition. **Yfat Kadan:** Resource acquisition. **Mario Beiner:** Resource acquisition. **Dana Josephy:** Resource acquisition. **Malka Brodsky:** Resource acquisition. **Liat Anafi:** Formal analysis. **Yossef Molchanov:** Formal analysis. **Tamar Geiger:** Conceptualization, formal analysis, visualization, writing - review & editing, supervision, funding acquisition. **Keren Levanon:** Conceptualization, data curation, formal analysis, writing - original draft, supervision, funding acquisition. The work reported in the article has been performed by the authors, unless clearly specified in the text.

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## CONFLICT OF INTEREST

The authors declare that they have no competing interests. United States Provisional Patent Application No. 63/216821 "Diagnostic kits and methods for early detection of ovarian cancer" is pending (inventors: Keren Bahar-Shany, Jacob Korach, Tamar Geiger and Keren Levanon).

## DATA AVAILABILITY STATEMENT

The previously published proteomics discovery dataset[24] as well as the validation dataset generated in our study are available in ProteomeXchange under identifier PXD009655 and PXD030390, respectively. Further information and other data that support the findings of our study are available from the corresponding author upon request.

## ETHICS STATEMENT

The trial was carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the Sheba Medical Center ethics committee (SMC-13-0930). All liquid biopsy contributors have signed informed consent forms. Trial registration: BEDOCA (Biomarkers for Early Detection of Ovarian Cancer Using Uterine Lavage; ClinicalTrials.gov Identifier: NCT03150121), retrospectively registered on May 12, 2017, https://clinicaltrials.gov/ct2/show/NCT03150121.

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## SUPPORTING INFORMATION

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

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