# Ember Trial: Evaluation of Multiple Protein and Molecular Biomarkers to Estimate Risk of Cancer in Gynecology Patients Presenting with a Pelvic Mass

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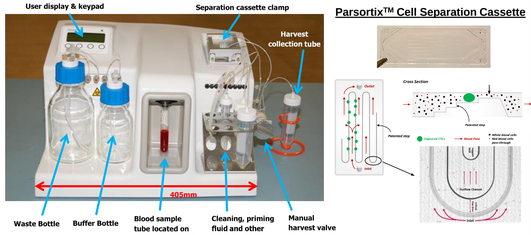
## Background

- Approximately 5-10% of women will be diagnosed with a pelvic mass during their lifetime, with 13-21% of those women subsequently being diagnosed with ovarian cancer.
- Emerging technologies for the isolation and interrogation of rare circulating cells, such as circulating tumor cells (CTCs), present an opportunity for the detection of cancer with a simple blood test, or liquid biopsy.
- The present study was designed to evaluate the potential of a liquid biopsy assay combining both serum protein biomarkers, with isolation of rare cells using the Parsortix™ Cell Separation System (Figure 1), followed by multiplexed gene expression analyses using the HyCEAD™ method and Ziplex® System (Figure 2) for detection of cancer in women with a pelvic mass prior to surgical intervention.
- This was an IRB approved prospective clinical trial (NCT ID# 02781272) conducted through the Wilmot Cancer Institute Gynecologic Oncology Division.

### Figure 1: Parsortix™ Cell Separation System



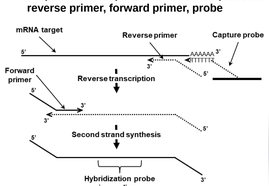

Parsortix™ Cell Separation Cassette



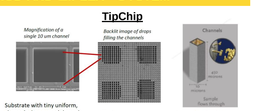
### Figure 2: HyCEAD™ Method and Ziplex® System

**HyCEAD Method**

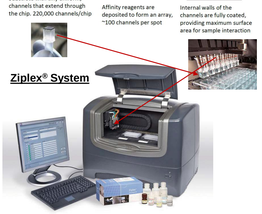
- The design of the HyCEAD™ process selects mRNA targets against genomic DNA and provides specificity comparable to qPCR.
  - Capture probe selects for polyadenylated mRNA
  - Amplification depends on three sequences: reverse primer, forward primer, probe




**TipChip**




**Ziplex® System**



## Study Design / Methods

**Diagnosis**: Women diagnosed with a pelvic mass (ovarian, uterine, retroperitoneal, etc.) who are scheduled for an imaging guided biopsy, surgical biopsy or surgical excision for evaluation of their pelvic mass.

**Imaging**: Within ~60 days prior to pelvic mass evaluation procedure, pelvic imaging studies (e.g. ultrasound, CT scan, MRI, etc.) were conducted and read to visualize the pelvic mass according to the current standard of care.

**Whole Blood (WB) Specimens**: Within 30 days prior to or on the day of the pelvic mass evaluation procedure, up to 35mL of whole blood was collected into one 5mL SST tube, which was drawn first, followed by three separate 10mL EDTA tubes.

**Pelvic Mass Evaluation**: Imaging guided biopsy, surgical biopsy or surgical excision for evaluation of the pelvic mass was performed by a qualified physician. Tissue samples were sent to the local pathology department for histological examination in accordance with the institution's standard practices. Results of the histopathological evaluation, including final diagnosis, along with histological sub-type and stage (if available) of cancer where disease was identified, were collected.

**Blood Sample Processing**: Serum obtained from blood collected into the 5mL SST tube was used to measure levels of seven commonly assayed ovarian cancer protein biomarkers using standard clinical analyzers and assays.

Blood collected into the EDTA tubes was pooled and two equal volume aliquots (7.5 – 15mL each, 12.2mL on average) were processed on the Parsortix™ System to capture and harvest rare cells. Harvested cells were pooled and lysed with RLT buffer. Lysates were split into two equal volume aliquots and stored frozen in liquid nitrogen.

Using the Qiagen® RNeasy Micro Kit, RNA was purified from one of the lysates and subsequently analyzed using a highly multiplexed assay (HyCEAD™ and Ziplex®) to evaluate the expression of 72 gene transcripts representing 52 different ovarian cancer associated genes and 8 different housekeeping genes (Table 4).

**Data Analysis**: Univariate and multivariate logistic regression analyses of the gene expression and serum protein biomarker data were performed, and ROC curves constructed and compared (Figures 3 & 4).

## Results

- A total of 200 patients were enrolled into the study, of which 183 (91.5%) were evaluable.
- The demographics, histopathological diagnoses and serum marker results for the 183 evaluable subjects are summarized in Tables 1 – 3 below.

### Table 1: Evaluable Patient Demographics

| | All Evaluable Patients | Normal/Benign | LMP/Cancer | p-value | Test |
|---|---|---|---|---|---|
| N | 183 | 104 (56.8%) | 79 (43.2%) | | |
| **Age** | | | | | |
| Mean ± SD [Median] | 56 ± 13 [56] | 53 ± 12 [53] | 60 ± 14 [60] | 0.0010 | t-test |
| Range | 19 to 91 | 20 to 82 | 19 to 91 | 0.0008 | ranksum |
| **Menopausal Status** | | | | | |
| Pre-Menopausal | 68 (37.2%) | 49 (47.1%) | 19 (24.1%) | 0.002 | Fisher's |
| Post-Menopausal | 115 (62.8%) | 55 (52.9%) | 60 (75.9%) | | Exact |
| **Race** | | | | | |
| White | 166 (90.7%) | 94 (90.4%) | 72 (91.1%) | 0.920 | Fisher's |
| Black | 13 (7.1%) | 8 (7.7%) | 5 (6.3%) | | Exact |
| Other | 4 (2.2%) | 2 (1.9%) | 2 (2.5%) | | |
| **Body Surface Area (m²)** | | | | | |
| Average ± SD [Median] | 1.86 ± 0.25 [1.84] | 1.86 ± 0.26 [1.85] | 1.85 ± 0.24 [1.82] | 0.7881 | t-test |
| Range | 1.34 to 2.64 | 1.34 to 2.64 | 1.45 to 2.41 | 0.7878 | ranksum |
| **Concurrent Conditions / Meds** | | | | | |
| Hypertension | 77 (42.1%) | 42 (40.4%) | 35 (44.3%) | 0.651 | |
| High Cholesterol | 60 (32.8%) | 32 (30.8%) | 28 (35.4%) | 0.528 | Fisher's |
| Taking Anti-Coagulants | 4 (2.2%) | 1 (1%) | 3 (3.8%) | 0.317 | Exact |
| History of Previous Cancer | 22 (12%) | 10 (9.6%) | 12 (15.2%) | 0.262 | |

### Table 2: Evaluable Patient Pathological Diagnoses

| Histopathological Diagnosis | Evaluable Subjects N (%) | |
|---|---|---|
| Normal Ovaries | 2 ( 1.1%) | 104 (56.8%) |
| Benign Condition | 102 (55.7%) | |
| Borderline/LMP | 17 ( 9.3%) | 17 ( 9.3%) |
| Granulosa Cell Ovarian Cancer | 5 ( 2.7%) | 42 (23.0%) |
| Epithelial Ovarian Cancer Stage I-II | 16 ( 8.7%) | |
| Epithelial Ovarian Cancer Stage III-IV | 21 (11.5%) | |
| GYN Metastatic Cancer | 14 ( 7.7%) | 20 (10.9%) |
| Non-GYN Metastatic Cancer | 6 ( 3.3%) | |
| TOTAL | 183 | |

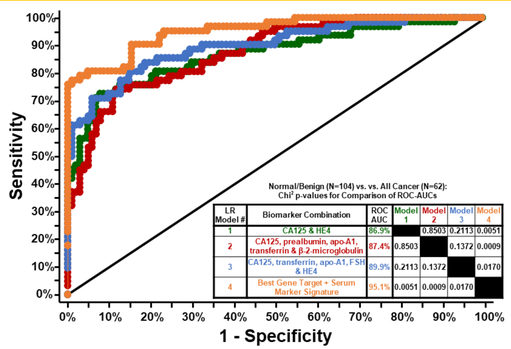
### Table 3: Evaluable Patient Serum Marker Summary

| Serum Marker or Algorithm | Mean ± Standard Deviation [Median] All Evaluable Patients | Normal/Benign | LMP/Cancer | t-test p-value |
|---|---|---|---|---|
| Prealbumin | 24 ± 8 [25] | 27 ± 7 [27] | 21 ± 8 [20] | 0.0000 |
| ApoA1 | 157 ± 33 [157] | 166 ± 31 [162] | 146 ± 33 [146] | 0.0001 |
| Transferrin | 267 ± 51 [266] | 284 ± 40 [280] | 243 ± 55 [246] | 0.0000 |
| Beta-2-microglobulin | 2.4 ± 0.9 [2.1] | 2.2 ± 0.8 [2.0] | 2.6 ± 1.0 [2.4] | 0.0013 |
| FSH | 40.5 ± 35.9 [36.8] | 41.9 ± 39.3 [29.0] | 38.7 ± 31.1 [37.4] | 0.5427 |
| CA125 | 255 ± 810 [37] | 43 ± 65 [22] | 534 ± 1177 [71] | 0.0000 |
| HE4 | 266 ± 899 [65] | 65 ± 34 [56] | 531 ± 1326 [129] | 0.0004 |
| ROMA Pred. Prob. | 32.5 ± 30.4% [17.4%] | 15.8 ± 13.5% [11.5%] | 54.5 ± 32.5% [53.1%] | 0.0000 |

### Table 4. Housekeeping and Cancer Related Genes Evaluated

| Housekeeping Genes | Cancer Related Genes | | | |
|---|---|---|---|---|
| CD45 | AFP | CXCR4 | HJURP | PLAT |
| HSP90AB1 | AGR2 | EMP2 | HUWE1 | PPIC |
| PPIA | CA125 | EN2 | INHA | PRAME |
| RPL13A | CCNE2 | EPCAM | INHBA | S100A16 |
| RPL4 | CCR2 | ERBB2 | KRT20 | SCGB2A2 |
| RPLP0 | CD274 | ERBB3 | KRT7 | SEPTIN2 |
| TBP | CDH1 | ERCC1 | LAMB1 | SERPINE2 |
| TPT1 | CDH2 | ESR1 | MAL2 | SLC6A8 |
| | CDH3 | FN1 | MSLN | TFF1 |
| | CDH5 | FOXJ1 | MUC1 | TUSC3 |
| | CEACAM5 | FXYD3 | NOTCH1 | VCAM1 |
| | CHI3L1 | GPX8 | PAX8 | VEGFA |
| | CLDN3 | HE4 | PGR | VIM |

### Figure 3: ROC Curves for Prediction of Normal/Benign (N=104) vs. Cancer (N=62)

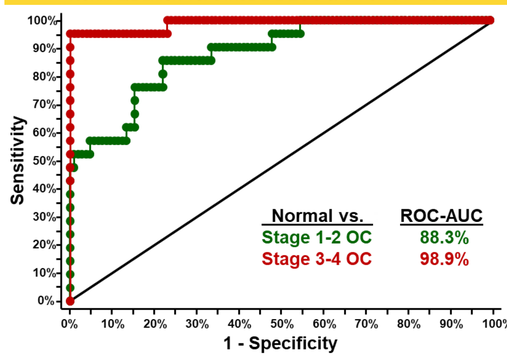



**Best Gene + Serum Marker Signature (Multivariate LR Model) Predictive Ability for Normal/Benign vs. Cancer**

| Predicted Probability Threshold | >=25% | >=50% | >=75% |
|---|---|---|---|
| Sensitivity | 90% | 81% | 74% |
| Specificity | 80% | 95% | 100% |
| Positive Predictive Value (PPV) | 73% | 91% | 100% |
| Negative Predictive Value (NPV) | 93% | 89% | 87% |
| Overall Accuracy | 84% | 90% | 90% |

- Comparison of ROC-AUCs for single and all combinations of genes and/or serum protein biomarkers to discriminate between benign (n=104) versus cancer (n=62) showed that a multivariate model combining the expression levels of 8 genes and 4 serum protein biomarkers achieved the highest AUC (95.1%).
- This combined rare cell gene expression and serum biomarker model significantly outperformed models using: HE4 & CA125 (AUC = 86.9%, p=0.005); CA125, prealbumin, apo-A1, transferrin & β-2-microglobulin (AUC = 87.4%, p<0.001); and CA125, transferrin, apo-A1, FSH & HE4 (AUC = 89.9%, p=0.017) (see Figure 3).
- Further, the combined gene expression & serum protein biomarker model achieved an AUC of 88.3% for patients with stage 1-2 EOC and 98.8% for patients with stage 3-4 EOC (Figure 4).

### Figure 4: Best Gene + Serum Marker Signature in Prediction of Normal/Benign vs. Stage 1-2 EOC & Stage 3-4 EOC




## Conclusions

- The unique rare cell gene expression profile coupled with the serum protein expression levels provides complementary insights which significantly improve the detection of cancer in women with a pelvic mass compared to the use of the current serum biomarker approach alone.
- Optimization of this combined approach is currently underway, with evaluation of additional gene targets and protein markers in further studies being planned.