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Supplementary Materials

Supplementary Methods

The study population was selected from women randomized to the intervention group in the Prostate, Lung, Colon and Ovary (PLCO) Trial, recruited at 10 screening centers in the United States. Women were eligible if they were aged 55 to 74 years and had no previous diagnosis of lung, colorectal, or ovarian cancer. Women who had bilateral oophorectomy were excluded from the ovarian cancer screening and the study. Serum samples and data were provided for 112 invasive epithelial ovarian cancers (EOCs) diagnosed and confirmed before June 30, 2006 and 706 matched control subjects (including 237 with family history of ovarian or breast cancer) sampled by PLCO investigators (PLCO EEMS 2005-009) in accordance with their design for a collaborative validation study (1). EOC was defined to include ovarian, fallopian tube and primary peritoneal cancer. Granulosa cell and low malignant potential (borderline) tumors were excluded, as were patients for which data were missing for CA125. Case ascertainment was consistent across PLCO clinical centers. The medical records of all procedures performed to evaluate an abnormal screen were obtained by study personnel and recorded on standardized reporting forms. Pathology reports from all ovarian neoplasms were abstracted by trained certified tumor registrars and were reviewed by a gynecologic oncologist. To track cancers not diagnosed through screening, study participants were sent annual study update forms to document cancer diagnoses. Pathology reports from relevant neoplasms were abstracted by trained

certified tumor registrars at the respective screening centers. Neoplasms with an International Classification of Diseases for Oncology Second Edition behavior code of 3 were considered to be malignant neoplasms (2).

For each participant the proximate serum sample was aliquoted and shipped blinded along with serum from matched control subjects for laboratory analysis. EOC patients (n = 112) provided written, informed consent, completed a baseline questionnaire, and had available pre-diagnostic serum. Non-oophorectomized control subjects were frequency matched to patients by the age at serum draw, calendar year of the blood draw, and calendar year of the ascertainment of the cancer diagnosis (patients) or cancer-free status (control subjects). For each patient, control subjects were selected without replacement from the appropriate stratum including two control subjects with a family history of breast or ovarian cancer as well as four randomly selected healthy general population control subjects. Family history control subjects were included to ensure that the results of the study are generalizable to women who are a high priority for EOC screening. Sample collection, processing, shipping, and storage of PLCO samples were standardized by PLCO investigators (1).

Laboratory analyses were performed centrally at three institutions. CA125 was measured as part of the PLCO trial, centrally at the Immunogenetics Laboratory (University of California, Los Angeles, CA) using the U.S. Food and Drug Administration-approved Centocor CA-125II RIA assay performed according to the instructions from the manufacturer (Centocor, Inc, Malvern, PA). HE4 was measured at the Clinical Laboratory Research Core of the Massachusetts's General Hospital (Partners) using an enzyme immunometric assay performed according to instructions

from the manufacturer (Fujirebio Diagnostics, Inc. Goteborg, Sweden). Samples were run in duplicate to determine both CA125 and HE4 levels. The remaining laboratory assays were performed at the Fred Hutchinson Cancer Research Center by experienced technicians who were blinded to patient and control subject status as described below. The reagents used in the assays have been reported previously (1). Matrix metalloproteinase-7 (MMP-7) and insulin-like growth factor binding protein 2 (IGFBP2) were measured by plate-based enzyme-linked immunosorbent assay (ELISA) using kits provided by R&D Systems (Minneapolis, MN) kits (Catalog # DMP700 and DY674, respectively) and performed according to the manufacturer's instructions. Each sample was tested in duplicate. The optical density was determined using a SpectraMax Microplate Reader at 450 nm (Molecular Devices, Sunnyvale, CA). Specimen-efficient single-plex Luminex bead-based ELISAs were used to measure Spondin2, SLPI, and mesothelin. Spondin2 was measured using a bead-based assay with reagents provided by diaDexus (3). Mesothelin and SLPI assays employed a new class of recombinant antibodies referred to as biobodies (4) that are site-specific biotinylated single chain variable fragments secreted by diploid yeast (5, 6) and were used in combination with polyclonal antibodies commercially available from R&D Systems to create bead-based sandwich assays (7). All incubations were carried out in 96-well Multiscreen HTS, GV filter plates (Millipore, Billerica, MA) in the dark with shaking at room temperature in phosphate buffered saline supplemented with 1% bovine serum albumen. Wells were washed 3X with 100 µL phosphate buffered saline supplemented with 0.05% Tween 20 (Fisher Scientific, Pittsburgh, PA). All plates were analyzed with the Bio-Plex Array Reader (Bio-Rad, Hercules, CA). Coefficients of variability for these assays estimated

from control samples in the PLCO set are 0.08 for HE4, SLPI and IGFBP2, 0.09 for MMP7, 0.13 for Mesothelin, and 0.18 for Spondin2 (8); the coefficient of variabiliity for the CA125 assay used in the PLCO trial is 0.04 (9).

Statistical analysis was performed at the Fred Hutchinson Cancer Research Center (Seattle, WA). Serial CA125 results were available for all years but only one proximate serum sample was provided for each PLCO participant for the measurement of other markers. Multivariable analysis was used to select the best marker for use as a confirmatory test in a second-line screen. For use in a first-line screen, a marker used to complement CA125 would need to detect cancers that CA125 misses, thereby increasing sensitivity without compromising specificity. For use in the second-line screen, a marker would need to be highly concordant with CA125 levels in patients to preserve sensitivity, and discordant with CA125 levels in control subjects to improve specificity. We therefore evaluated HE4, mesothelin, MMP7, SLPI, Spondin2 and IGFBP2 in terms of their signal in cancers that are identified or missed by increasing CA125 (interpreted using the parametric empirical Bayes longitudinal algorithm), while simultaneously adjusting for subject characteristics that may also affect marker levels (10). It is important to understand the effects of subject characteristics on marker levels to avoid misinterpretation of elevated marker levels as indicators of disease. In particular it was important to know if family history affected marker levels because if it did, the family history control subjects should not be pooled with the randomly selected control subjects in subsequent analyses. All 112 cancers and 706 control subjects for which CA125 and HE4 data were available were included because transvaginal ultrasound (TVU) data were not required for this multivariable analysis of marker levels.

To facilitate comparison across markers, all marker concentrations were re-scaled and covariable adjustment was performed using analysis of covariance on the standardized scale (11). Coefficients of this model can be interpreted as the number of standard deviations of change in a marker on the basis of one unit of change in the covariable. The rescaling does not affect statistical significance, only the interpretation of the coefficient. Increasing CA125 was measured using the parametric empirical Bayes longitudinal algorithm (12, 13), which detects a rise in CA125 by comparing a woman's current CA125 level with a regression-adjusted baseline predicted from her prior values, establishing a personal threshold for positivity for each woman on the basis of her marker history without requiring call-backs. It has the advantages of lowering the threshold for positivity in most women, thereby increasing lead time in cancers, uniformly distributing false-positive results among healthy women, and adjusting simultaneously for any stable covariables that affect marker levels. Positivity by this rule is referred to as "increasing CA125."

HE4, the best of the markers, was compared to TVU as a second-line screen. A subset of 84 cases of EOC and 516 controls (including 175 family history controls) for which TVU results as well as , CA125 and HE4 results were available was used to test hypotheses regarding inclusion of a second marker in a multimodal strategy. We compared HE4 to TVU as a second-line screen, using increasing CA125 to define positivity as the first-line screen. A positivity threshold was chosen for increasing CA125 as well as for HE4 to yield specificity of 96.9%, consistent with that of TVU based on the 516 control subjects in this analysis set. McNemar's test was used to test the

hypothesis that the number of cancers detected using HE4 as the second-line screen exceeds that using TVU as the second-line screen.

Supplementary Table 1. Characteristics reported at the time of enrollment into the Prostate, Lung, Colon and Ovary (PLCO) trial by participants with CA125 and HE4 results available *

Participant characteristics †	All patients (n=112)	All control subjects (n=706)	*P‡*	Family history control subjects (n=237)	Randomly selected control subjects (n=469)	*P§*
Age at blood draw, y (SD)	65.9 (5.3)	65.6 (5.2)	.59	65.7 (5.1)	65.6 (5.3)	.81
BMI, kg/m^2 (SD)	27.0 (6.1)	27.1 (5.2)	.93	26.9 (5.1)	27.2 (5.2)	.42
Non-white race, No. (%)	9 (8.0)	63 (8.9)	.86	20 (8.4)	43 (9.2)	.78
Family history, No. (%)	28 (25.0)	311 (44.1)	<.001	237 (100.0)	74 (15.8)	<.001
Oral contraceptive use, No. (%)	35 (31.2)	271 (38.4)	.17	79 (33.3)	192 (40.9)	.06
Nulliparous, No. (%)	9 (8.1)	46 (6.5)	.54	14 (5.9)	32 (6.8)	.75
History of endometriosis, No. (%)	10 (8.9)	28 (4.0)	.03	9 (3.8)	19 (4.1)	1.0
Current smoker, No. (%)	8 (7.1)	54 (7.6)	1.0	20 (8.4)	34 (7.2)	.65
Prior hysterectomy, No. (%)	29 (25.9)	189 (26.8)	.91	58 (24.5)	131 (27.9)	.37
Current hormone therapy with intact uterus, No. (%)	36 (32.1)	198 (28.0)	.37	60 (25.3)	138 (29.4)	.29
Current hormone therapy with prior hysterectomy, No. (%)	19 (17.0)	111 (15.7)	.78	39 (16.5)	72 (15.4)	.74

* BMI = body mass index, SD = standard deviation.

† Categorical variables included non-white race (yes or no), family history (breast or ovarian cancer, yes or no), oral contraceptive use (≥1 year, yes or no), nulliparous (yes or no), history of endometriosis (yes or no), current smoker (yes or no), prior hysterectomy (yes or no), current hormone therapy with intact uterus (yes or no), and current hormone therapy with prior hysterectomy (yes or no). The mean and standard

deviation are given for continuous variables including age at blood draw (years) and body mass index (kg/m^2).

‡ Differences in the characteristics between patients and control subjects were assessed using Student *t* test for continuous variables (age and body mass index) and by Fisher exact test for all other variables. All statistical tests were two-sided.

§ Differences in the characteristics between family history and PLCO control subjects were assessed using Student *t* test for continuous variables (age and body mass index) and by Fisher exact test for all other variables. All statistical tests were two-sided.

Supplementary Table 2. Characteristics reported at the time of enrollment by a subset of Prostate, Lung, Colon and Ovary (PLCO) trial participants with CA125, HE4 and transvaginal ultrasound imaging results available *

Participant characteristics †	**All patients (n=84)**	**All control subjects (n=516)**	***P*‡**	**Family history control subjects (n=175)**	**Randomly selected control subjects (n=341)**	***P*§**
Age at blood draw, y (SD)	65.6 (5.2)	65 (5.1)	.36	65.2 (5.0)	65 (5.2)	.60
BMI, kg/m^2 (SD)	26.9 (6.2)	27.2 (5.2)	.66	27.0 (5.1)	27.3 (5.3)	.54
Non-white race, No. (%)	7 (8.3)	48 (9.3)	1.0	14 (8.0)	34 (10.0)	.53
Family history, No. (%)	20 (23.8)	231 (44.8)	<.001	175 (100.0)	56 (16.4)	<.001
Oral contraceptive use, No. (%)	28 (33.3)	178 (34.5)	.90	51 (29.1)	127 (37.2)	.08
Nulliparous, No. (%)	5 (6.0)	35 (6.8)	1.0	9 (5.1)	26 (7.6)	.36
History of endometriosis, No. (%)	9 (10.7)	20 (3.9)	.01	6 (3.4)	14 (4.1)	.81
Current smoker, No. (%)	4 (4.8)	36 (7.0)	.64	15 (8.6)	21 (6.2)	.36
Prior hysterectomy, No. (%)	22 (26.2)	145 (28.1)	.79	46 (26.3)	99 (29.0)	.54
Current hormone therapy with intact uterus, No. (%)	27 (32.1)	145 (28.1)	.44	41 (23.4)	104 (30.5)	.10
Current hormone therapy with prior hysterectomy, No. (%)	15 (17.9)	83 (16.1)	.64	31 (17.7)	52 (15.2)	.53

* BMI = body mass index, SD = standard deviation.

† Categorical variables included non-white race (yes or no), family history (breast or ovarian cancer, yes or no), oral contraceptive use (≥1 year, yes or no), nulliparous (yes or no), history of endometriosis (yes or no), current smoker (yes or no), prior hysterectomy (yes or no), current hormone therapy with intact uterus (yes or no), and current hormone therapy with prior hysterectomy (yes or no). The mean and standard deviation is given for continuous variables including age at blood draw (years) and body mass index (kg/m^2).

‡ Differences in the characteristics between patients and control subjects were assessed by Student *t* test for continuous variables (age and body mass index) and by Fisher exact test for all other variables. All statistical tests were two-sided.

§ Differences in the characteristics between family history and PLCO control subjects were assessed by Student *t* test for continuous variables (age and body mass index) and by Fisher exact test for all other variables. All statistical tests were two-sided.

Supplemental Table 3. Time from proximate blood draw to diagnosis by screen number for proximate samples with serum marker results available from 112 patients who participated in the Prostate, Lung, Colon, and Ovarian trial

Time to diagnosis, y	Screen No. *					Total No.
	T0	T1	T2	T4	T5	
0−1	22	13	14	8	6	63
1−2	1	0	16	1	6	24
2−3	0	1	4	1	1	7
3−4	2	0	3	1	2	8
4−5	0	1	2	0	0	3
5−6	0	0	2	0	0	2
6−7	0	1	1	0	0	2
7−8	1	0	2	0	0	3
Total No.	26	16	44	11	15	112

* T0 = baseline (prevalence) screen, T1 = first incidence screen, T2 = second incidence screen, T4 = fourth incidence screen, T5 = fifth incidence screen. No values are given for the third incidence screen (T3) because serum was not available for analysis.

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