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(57) Abstract: The present invention relates to methods of diagnosing ovarian cancer comprising, detecting, in a subject, changes in serum levels of a plurality of markers associated with that disease.

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MULTIMARKER ASSAY FOR EARLY DETECTION OF OVARIAN CANCER

GRANT INFORMATION

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The subject matter of the invention was developed at least in part under National Institutes of Health Grants EDRN Associate Member Award, RO1 CA098642, RO1 CA 108990, P50 CA083639 and Avon (NIH/NCI), so that the United States Government has rights to the invention.

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PRIORITY CLAIM

This application claims priority to United States Provisional Application Serial No. 60/919,843, filed March 23, 2007, which is incorporated by reference in its entirety herein.

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1. INTRODUCTION

The present invention relates to methods of diagnosing ovarian cancer comprising, detecting, in a subject, changes in serum levels of a plurality of markers associated with that disease.

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2. BACKGROUND OF THE INVENTION

Ovarian cancer is the fourth most frequent cause of death from cancer in women in Europe and the United States (1-3). Since ovarian cancers typically cause few specific symptoms, more than 70% of patients are diagnosed when they have advanced disease and for these patients 5-year survival rates are less than 30% (1, 3). In contrast, the 25% of patients, who are diagnosed with stage I disease, have a 5-year survival of up to 90% and those with stage II up to 70% (2, 3). Therefore, early detection of ovarian cancer has great promise to improve clinical outcome.

At present, no screening techniques are recommended for early detection of ovarian cancer in the general population. CA 125, the most frequently used serum biomarker for ovarian cancer, has a sensitivity of only 50-60% for early stage disease in postmenopausal women when specificity is set at 99% (4-6). Transvaginal sonography (TVS), computerized tomography, magnetic resonance imaging, or power Doppler offer less than 90% sensitivity for early ovarian cancer and their expense precludes annual screening (7-9). Considering the low prevalence of ovarian cancer, it has been suggested that a screening strategy must achieve a minimum specificity of 99.6% and a sensitivity of >75% for early stage disease to

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avoid an unacceptable level of false-positive results, and thereby achieve a positive predictive value of 10% (10, 11). Using TVS as a second-line test, previous CA125 based screening studies indicate that a first line specificity of 98% for an annual test with a panel of serum biomarkers could assure required specificity (>99.6%) and positive predictive value (>10%), and would reduce the number of ultrasound examinations performed annually to a cost-effective level of 2% of women screened (10, 11).

Similar to CA 125, several other individual ovarian cancer-associated serum protein biomarkers lack sufficient sensitivity or specificity for detection of early stage disease (12-16). Recently, combinations of serum tumor markers have achieved greater sensitivity than individual markers, while maintaining high specificity. Two combinations, CA 125, CA 72-4, CA 15-3, and M-CSF (17), or CA 125, apolipoprotein A1, truncated form of transthyretin, and a cleavage fragment of inter-alpha-trypsin inhibitor heavy chain H4 (18), substantially improved test accuracy over CA 125 alone, with sensitivities of 70-73% at a specificity of 97-98%. A panel of 4 biomarkers, leptin, prolactin, IGF-II, and osteopontin, reportedly exhibited a sensitivity of 95% at a specificity of 95% (19). However, an independent validation set included only 24 patients with stage I/II cancer. Therefore, the need still exists to develop a diagnostic assay that detects stages I-II ovarian cancer with high sensitivity at 98% specificity in a larger population of patients with early disease.

3. SUMMARY OF THE INVENTION

The present invention relates to methods of diagnosing ovarian cancer in a subject comprising detecting changes in a set of serum markers correlating with the disease. It is based, at least in part, on several studies which used a multiplexing approach to analyze candidate serum proteins to identify a biomarker combination with the highest power to detect early stage ovarian cancer. The present invention further encompasses kits which may be used to practice said methods.

4. BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1A-B. Serum levels of ovarian cancer biomarkers in healthy controls, ovarian cancer patients and patients with benign tumors. Sera were collected from 86 patients with stage (I-II) ovarian cancer, 79 patients with benign pelvic masses and from 104 age-matched healthy women. Circulating concentrations of

markers were measured using xMAP™ technology as described in Methods. (A) Comparison of controls vs. ovarian cancer vs. benigns; (B) Comparison between different histological types of ovarian cancer, clear cell carcinoma (n=7), endometrioid adenocarcinoma (n=27), mucinous Adenocarcinoma (n=27), and serous adenocarcinoma (n=21). Horizontal lines indicate mean values. * - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$.

FIGURE 2A-D. Cumulative ROC curves using multimarker panels with XX algorithm. A. Ovarian cancer vs. controls cross validation test, 55/45 random split, 100 runs (6-marker panel), post- and pre-menopausal women; B. Evaluation of 6-markers panel in blinded validation set consisting of ovarian cancer (n=50) vs. controls (n=50), post-menopausal women; C. Classification of ovarian cancer (n=79) from benign pelvic disease (n=86) utilizing the 6-marker panel. Cross-validation test, 55/45 random split, 100 runs; D. Classification of ovarian cancer (n=86) vs. endometrial (n=120), breast (n=120) and lung (n=85) cancers utilizing the 6-marker panel. Cross-validation test, 55/45 random split, 100 runs.

FIGURE 3A-B. (A) Serum levels of ovarian cancer biomarkers in healthy controls, ovarian cancer patients and patients with benign tumors. Sera were collected from 310 healthy controls (Nor) 168 patients with stage I-II ovarian cancer (C I-II), 105 patients with stage III-IV ovarian cancer (C III-IV), and 141 patients with benign pelvic masses (Ben). Circulating concentrations of markers were measured using xMAP™ technology as described in Methods. Horizontal lines indicate mean values. * - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$. (B) Cumulative ROC curves using 4-biomarker panel with LR algorithm. Ovarian cancer vs. controls cross-validation “leave one out” estimate, postmenopausal women.

FIGURE 4A-B. Serum levels of ovarian cancer biomarkers in healthy controls, ovarian cancer patients and patients with benign tumors. Sera were collected from 136 patients with stage (I-II) ovarian cancer, 79 patients with benign pelvic masses and from 154 age- and sex-matched healthy controls. Circulating concentrations of markers were measured using xMAP technology. Measurements were performed twice. (A) Comparison of controls vs. ovarian cancer vs. benigns; (B) Comparison between different histological types of ovarian cancer. Horizontal lines indicate mean values. * above cancer group denotes statistical significance between controls and ovarian cancer patients; * above benign denotes statistical significance

between patients with benign pelvic disease and patients with ovarian cancer; *
 $p < 0.05$; ** - $0.01 < p < 0.05$; *** - $p < 0.001$.

FIGURE 5A-B. Sensitivity, accuracy, and stability of multimarker tests as a function of number of markers. For each number of markers the random split cross-validation test was run 100 times and then the upper 2.5%, upper 25%, median and corresponding lower 25% and 2.5% population boundaries were found, both for sensitivity at 98% specificity (A) and for the average percentage of correctly identified samples (B).

FIGURE 6A-F. Cumulative ROC curves for postmenopausal women using multimarker panels with ADEPT algorithm. A-C are linear representations and D-F are bar graph representations. (A,D) Ovarian vs. Healthy, test set, 55/45 random split, 100 runs (22-marker panel); (B,E) Ovarian vs. Benign, cross-validation test, 55/45 random split, 100 runs (20-marker panel); (C,F) Breast vs. Benign, crossvalidation test, 55/45 random split, 100 runs (22-marker panel).

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods of diagnosing ovarian cancer comprising, detecting, in a subject, changes in serum levels of a plurality of markers associated with that disease.

In non-limiting embodiments, the present invention provides for a method of diagnosing ovarian cancer in a human subject, comprising (i) obtaining a serum sample from a subject being evaluated for possible ovarian cancer, and (ii) identifying, in the serum sample, a change in the serum levels of at least two, or at least three, or at least four, or at least five, or at least six, or at least seven, or at least eight, or at least nine, or at least ten, or at least eleven, or at least twelve, of the following molecules relative to a healthy control subject:

PRL (prolactin);

IGFBP1 (insulin-like growth factor binding protein 1);

GH (growth hormone);

HE4 (human epididymis protein 4);

MIF (macrophage migration inhibitory factor);

IL-6 (interleukin-6);

IL-8 (interleukin-8);

IL-2R (interleukin-2 receptor)

- SMRP (soluble mesothelin-related peptides);
 MCP-1 (monocyte chemoattractant protein-1);
 MIP-1 α (macrophage inflammatory protein-1 α);
 MIP-1 β (macrophage inflammatory protein-1 β);
 5 CA-19-9 (carbohydrate antigen 19-9);
 CA-125 (cancer antigen 125);
 Cyfra 21-1 (soluble fragment of cytokeratin-19);
 CA 72-4 (cancer antigen 72-4);
 leptin;
 10 tPAI-1 (tissue plasminogen activator inhibitor-1);
 TSH (thyroid stimulating hormone);
 ACTH (adrenocorticotrophic hormone);
 CEA (carcinoembryonic antigen);
 eotaxin;
 15 MMP-2 (matrix metalloproteinase-2);
 MMP-3 (matrix metalloproteinase-3);
 sV-CAM (vascular cell adhesion molecule);
 FSH (follicle stimulating hormone);
 EGFR (epidermal growth factor receptor);
 20 ErbB2 (erythroblastic leukemia viral oncogene homolog 2);
 adiponectin;
 sI-CAM (soluble intercellular adhesion molecule);
 sE-selectin;
 MPO (myeloperoxidase);
 25 IP-10 (interferon gamma induced protein 10);
 LH (luteinizing hormone); and/or
 full length mesothelin;
 (each of the foregoing, if not soluble in its native state, is present in a form soluble in
 serum) wherein preferably, but not by way of limitation, at least one, at least two, at
 30 least two, at least three, at least four, at least five, at least six, at least seven, at least
 eight, at least nine, at least ten, at least eleven, or at least twelve, of said changes are
 changes in levels of molecules selected from the group consisting of TSH, GH,
 ACTH, eotaxin, MMP-2, MMP-3, ErbB2, adiponectin, sI-CAM, sV-CAM, sE-
 selectin, and full-length mesothelin;

wherein the presence of such changes indicates the presence of ovarian cancer in the subject. Said plurality of markers are considered a “panel”, and may, in non-limiting embodiments, be comprised in a panel of markers that consists of up to 5 markers, up to 6 markers, up to 7 markers, up to 8 markers, up to 9 markers, up to 10 markers, up to 20 markers, up to 25 markers, up to 30 markers, up to 40 markers, up to 50 markers, up to 100 markers, up to 250 markers, up to 500 markers, or up to 1000 markers.

In non-limiting embodiments, the present invention provides for a method of diagnosing ovarian cancer in a human subject, comprising (i) obtaining a serum sample from a subject being evaluated for possible ovarian cancer, and (ii) identifying, in the serum sample, at least two, or at least three, or at least four, or at least five, or at least six, or at least seven, or at least eight, or at least nine, or at least ten, or at least eleven, or at least twelve, of the following changes in serum levels relative to a healthy control subject:

- 15 an increase in PRL (prolactin);
- an increase in IGFBBP1 (insulin-like growth factor binding protein 1);
- an increase in GH (growth hormone);
- an increase in HE4 (human epididymis protein 4);
- an increase in MIF (macrophage migration inhibitory factor);
- 20 an increase in IL-6 (interleukin-6);
- an increase in IL-8 (interleukin-8);
- an increase in IL-2R (interleukin-2 receptor)
- an increase in SMRP (soluble mesothelin-related peptides);
- an increase in MCP-1 (monocyte chemoattractant protein-1);
- 25 an increase in MIP-1 α (macrophage inflammatory protein-1 α);
- an increase in MIP-1 β (macrophage inflammatory protein-1 β);
- an increase in CA-19-9 (carbohydrate antigen 19-9);
- an increase in CA-125 (cancer antigen 125);
- an increase in Cyfra 21-1 (soluble fragment of cytokeratin-19);
- 30 an increase in CA 72-4 (cancer antigen 72-4);
- an increase in leptin;
- an increase in tPAI-1 (tissue plasminogen activator inhibitor-1);
- an increase in TSH (thyroid stimulating hormone);
- an increase in ACTH (adrenocorticotrophic hormone);

- an increase in CEA (carcinoembryonic antigen);
 - a decrease in eotaxin;
 - a decrease in MMP-2 (matrix metalloproteinase-2);
 - a decrease in MMP-3 (matrix metalloproteinase-3);
 - 5 a decrease in sV-CAM (vascular cell adhesion molecule);
 - a decrease in FSH (follicle stimulating hormone);
 - a decrease in EGFR (epidermal growth factor receptor);
 - a decrease in ErbB2 (erythroblastic leukemia viral oncogene homolog 2);
 - a decrease in adiponectin;
 - 10 a decrease in sI-CAM (soluble intercellular adhesion molecule);
 - a decrease in sE-selectin;
 - a decrease in MPO (myeloperoxidase);
 - a decrease in IP-10 (interferon gamma induced protein 10);
 - a decrease in LH (luteinizing hormone); and/or
 - 15 a decrease in full length mesothelin;
- (each of the foregoing, if not soluble in its native state, is present in a form soluble in serum) wherein preferably, but not by way of limitation, at least one, at least two, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, at least eleven, or at least twelve, of said changes are
- 20 changes in levels of molecules selected from the group consisting of TSH, GH, ACTH, eotaxin, MMP-2, MMP-3, ErbB2, adiponectin, sI-CAM, sV-CAM, sE-selectin, and full-length mesothelin; wherein the presence of such changes indicates the presence of ovarian cancer in the subject. Said plurality of markers are considered a "panel", and may, in non-limiting embodiments, be comprised in a panel of markers
- 25 that consists of up to 5 markers, up to 6 markers, up to 7 markers, up to 8 markers, up to 9 markers, up to 10 markers, up to 20 markers, up to 25 markers, up to 30 markers, up to 40 markers, up to 50 markers, up to 100 markers, up to 250 markers, up to 500 markers, or up to 1000 markers.

Preferably said increase or decrease is a statistically significant

30 increase or decrease. Non-limiting specific examples of increases and decreases in concentrations of the aforelisted molecules are presented in FIGURES 1A, 3A, and 4A.

Serum levels may be measured by any method known in the art, including but not limited to multiplex analysis as provided by the technology of

Luminex Corp., Austin, TX, as well as antibody array, ELISA (enzyme-linked immunoabsorbent assay), IRMA (two-site immunoradiometric assay), Western blot, flow cytometry, etc.. Serum levels may be evaluated essentially simultaneously or sequentially. "Obtaining" the sample may mean collecting the blood sample from the patient or receiving an already-collected sample from another source, such as a blood-drawing service. A "subject being evaluated for possible ovarian cancer" is a human female, where a health care provider such as a physician desires to test the subject for ovarian cancer, either as part of routine screening (for example, but not limited to, in a postmenopausal woman or a woman having a first degree relative who has or had ovarian cancer or breast cancer) or to evaluate symptoms consistent with ovarian cancer such as one or more of abdominal bloating, pelvic or abdominal pain, loss of appetite, early satiety after eating, urgent need to urinate, and frequent urges to urinate.

In non-limiting embodiments, the method further comprises recommending a further test selected from the group consisting of a pelvic ultrasound, a laparotomy and a laparoscopy or a combination thereof. A diagnosis made according to the invention is preferably supplemented by additional tests, for example, but not limited to, pelvic ultrasound and/or laparoscopy and/or laparotomy and/or tissue biopsy (of ovarian tissue and/or a neoplasm biopsy if neoplasm is detected) and histologic evaluation.

In a specific, non-limiting embodiment, the above method identifies at least a change in the serum concentration, relative to a healthy subject, of PRL, CEA, HE4, and CA 125, wherein the presence of such changes indicates the presence of ovarian cancer in the subject.

In a specific, non-limiting embodiment, the above method identifies at least an increase in the serum concentration, relative to a healthy subject, of PRL, CEA, HE4, and CA 125 wherein the presence of such increases indicates the presence of ovarian cancer in the subject.

In a specific, non-limiting embodiment, the above method identifies at least a change in the serum concentration, relative to a healthy subject, of CA 125; HE4; CA72-4; FSH; eotaxin; IP-10, wherein the presence of such changes indicates the presence of ovarian cancer in the subject.

In a specific, non-limiting embodiment, the above method identifies at least an increase in the serum concentration, relative to a healthy subject, of CA 125,

HE4, and CA72-4, and a decrease, in the serum concentration, relative to a healthy subject, of FSH; eotaxin; and IP-10, wherein the presence of such increases and decreases indicates the presence of ovarian cancer in the subject.

In a specific, non-limiting embodiment, the above method identifies at least a change in the serum concentration, relative to a healthy subject, of CA 125, EGFR, HE4, sVCAM, wherein the presence of such changes indicates the presence of ovarian cancer in the subject.

In a specific, non-limiting embodiment, the above method identifies at least an increase in the serum concentration, relative to a healthy subject, of CA 125 and HE4, and a decrease in the serum concentration, relative to a healthy subject, of EGFR and sVCAM, wherein the presence of such increases indicates the presence of ovarian cancer in the subject.

In a specific, non-limiting embodiment, the above method identifies at least a change in the serum concentration, relative to a healthy subject, of CA125, prolactin, sV-CAM1, HE4, MMP-2, TSH, eotaxin, IGFBPI, sI-CAM-1, FSH, Cyfra-21-1, MIP-1 β , CEA, MCP-1, IL-8, MPO, CA 19-9, E-selectin, IP-IO, EGFR, ACTH, MIF, wherein the presence of such changes indicates the presence of ovarian cancer in the subject.

In further non-limiting embodiments, the present invention provides for a method of diagnosing ovarian cancer in a human subject, comprising (i) obtaining a serum sample from a subject being evaluated for possible ovarian cancer, and (ii) identifying, in the serum sample, a change in the serum levels, relative to a healthy subject, of a panel of markers comprising PRL, CEA, HE4, and CA 125, wherein the presence of such changes indicates the presence of ovarian cancer in the subject. In particular, non-limiting embodiments, said panel further comprises at least one additional marker associated with ovarian cancer, selected from the group consisting of IGFBP1 (insulin-like growth factor binding protein 1); GH (growth hormone); MIF (macrophage migration inhibitory factor); IL-6 (interleukin-6); IL-8 (interleukin-8); IL-2R (interleukin-2 receptor); SMRP (soluble mesothelin-related peptides); MCP-1 (monocyte chemoattractant protein-1); MIP-1 α (macrophage inflammatory protein-1 α); MIP-1 β (macrophage inflammatory protein-1 β); CA-19-9 (carbohydrate antigen 19-9); Cyfra 21-1 (soluble fragment of cytokeratin-19); CA 72-4 (cancer antigen 72-4); leptin; tPAI-1 (tissue plasminogen activator inhibitor-1); TSH (thyroid stimulating hormone); ACTH (adrenocorticotrophic hormone); eotaxin;

MMP-2 (matrix metalloproteinase-2); MMP-3 (matrix metalloproteinase-3); sV-CAM (vascular cell adhesion molecule); FSH (follicle stimulating hormone); EGFR (epidermal growth factor receptor); ErbB2 (erythroblastic leukemia viral oncogene homolog 2); adiponectin; sI-CAM (soluble intercellular adhesion molecule); sE-selectin; MPO (myeloperoxidase); IP-10 (interferon gamma induced protein 10); LH (leutinizing hormone); and/or full length mesothelin. In further particular, non-limiting embodiments, the panel of markers being tested has a total percentage of markers that are associated with ovarian cancer (as set forth above) of at least 50 percent, at least 75 percent, or at least 90 percent. In non-limiting embodiments, the panel of markers being tested consists of up to 5 markers, up to 6 markers, up to 7 markers, up to 8 markers, up to 9 markers, up to 10 markers, up to 20 markers, up to 25 markers, up to 30 markers, up to 40 markers, or up to 50 markers.

In further non-limiting embodiments, the present invention provides for a method of diagnosing ovarian cancer in a human subject, comprising (i) obtaining a serum sample from a subject being evaluated for possible ovarian cancer, and (ii) identifying, in the serum sample, a change in the serum levels, relative to a healthy subject, of a panel of markers comprising CA 125; HE4; CA72-4; FSH; eotaxin; IP-10, wherein the presence of such changes indicates the presence of ovarian cancer in the subject. In particular, non-limiting embodiments, said panel further comprises at least one additional marker associated with ovarian cancer, selected from the group consisting of PRL (prolactin); IGFBP1 (insulin-like growth factor binding protein 1); GH (growth hormone); MIF (macrophage migration inhibitory factor); IL-6 (interleukin-6); IL-8 (interleukin-8); IL-2R (interleukin-2 receptor); SMRP (soluble mesothelin-related peptides); MCP-1 (monocyte chemoattractant protein-1); MIP-1 α (macrophage inflammatory protein-1 α); MIP-1 β (macrophage inflammatory protein-1 β); CA-19-9 (carbohydrate antigen 19-9); Cyfra 21-1 (soluble fragment of cytokeratin-19); leptin; tPAI-1 (tissue plasminogen activator inhibitor-1); TSH (thyroid stimulating hormone); ACTH (adrenocorticotrophic hormone); CEA (carcinoembryonic antigen); MMP-2 (matrix metalloproteinase-2); MMP-3 (matrix metalloproteinase-3); sV-CAM (vascular cell adhesion molecule); EGFR (epidermal growth factor receptor); ErbB2 (erythroblastic leukemia viral oncogene homolog 2); adiponectin; sI-CAM (soluble intercellular adhesion molecule); sE-selectin; MPO (myeloperoxidase); LH (leutinizing hormone); and/or full length mesothelin. In further particular, non-limiting embodiments, the

panel of markers being tested has a total percentage of markers that are associated with ovarian cancer (as set forth above) of at least 50 percent, at least 75 percent, or at least 90 percent. In non-limiting embodiments, the panel of markers being tested consists of up to 7 markers, up to 8 markers, up to 9 markers, up to 10 markers, up to 20 markers, up to 25 markers, up to 30 markers, up to 40 markers, or up to 50 markers.

In further non-limiting embodiments, the present invention provides for a method of diagnosing ovarian cancer in a human subject, comprising (i) obtaining a serum sample from a subject being evaluated for possible ovarian cancer, and (ii) identifying, in the serum sample, a change in the serum levels, relative to a healthy subject, of a panel of markers comprising CA 125, EGFR, HE4, sVCAM, wherein the presence of such changes indicates the presence of ovarian cancer in the subject. In particular, non-limiting embodiments, said panel further comprises at least one additional marker associated with ovarian cancer, selected from the group consisting of PRL (prolactin); IGFBBP1 (insulin-like growth factor binding protein 1); GH (growth hormone); MIF (macrophage migration inhibitory factor); IL-6 (interleukin-6); IL-8 (interleukin-8); IL-2R (interleukin-2 receptor); SMRP (soluble mesothelin-related peptides); MCP-1 (monocyte chemoattractant protein-1); MIP-1 α (macrophage inflammatory protein-1 α); MIP-1 β (macrophage inflammatory protein-1 β); CA-19-9 (carbohydrate antigen 19-9); Cyfra 21-1 (soluble fragment of cytokeratin-19); CA 72-4 (cancer antigen 72-4); leptin; tPAI-1 (tissue plasminogen activator inhibitor-1); TSH (thyroid stimulating hormone); ACTH (adrenocorticotrophic hormone); CEA (carcinoembryonic antigen); eotaxin; MMP-2 (matrix metalloproteinase-2); MMP-3 (matrix metalloproteinase-3); FSH (follicle stimulating hormone); ErbB2 (erythroblastic leukemia viral oncogene homolog 2); adiponectin; sI-CAM (soluble intercellular adhesion molecule); sE-selectin; MPO (myeloperoxidase); IP-10 (interferon gamma induced protein 10); LH (luteinizing hormone); and/or full length mesothelin. In further particular, non-limiting embodiments, the panel of markers being tested has a total percentage of markers that are associated with ovarian cancer (as set forth above) of at least 50 percent, at least 75 percent, or at least 90 percent. In non-limiting embodiments, the panel of markers being tested consists of up to 5 markers, up to 6 markers, up to 7 markers, up to 8 markers, up to 9 markers, up to 10 markers, up to 20 markers, up to 25 markers, up to 30 markers, up to 40 markers, or up to 50 markers.

In further non-limiting embodiments, the present invention provides for a method of diagnosing ovarian cancer in a human subject, comprising (i) obtaining a serum sample from a subject being evaluated for possible ovarian cancer, and (ii) identifying, in the serum sample, a change in the serum levels, relative to a healthy subject, of a panel of markers comprising CA125, prolactin, sV-CAM1, HE4, MMP-2, TSH, eotaxin, IGFBPI, sI-CAM-1, FSH, Cyfra-21-1, MIP-1 β , CEA, MCP-1, IL-8, MPO, CA 19-9, E-selectin, IP-IO, EGFR, ACTH, and MIF, wherein the presence of such changes indicates the presence of ovarian cancer in the subject. In particular, non-limiting embodiments, said panel further comprises at least one additional marker associated with ovarian cancer, selected from the group consisting of: GH (growth hormone); IL-6 (interleukin-6); IL-2R (interleukin-2 receptor); SMRP (soluble mesothelin-related peptides); MIP-1 α (macrophage inflammatory protein-1 α); CA 72-4 (cancer antigen 72-4); leptin; tPAI-1 (tissue plasminogen activator inhibitor-1); MMP-3 (matrix metalloproteinase-3); ErbB2 (erythroblastic leukemia viral oncogene homolog 2); adiponectin; LH (luteinizing hormone); and/or full length mesothelin. In further particular, non-limiting embodiments, the panel of markers being tested has a total percentage of markers that are associated with ovarian cancer (as set forth above) of at least 50 percent, at least 75 percent, or at least 90 percent. In non-limiting embodiments, the panel of markers being tested consists of up to 25 markers, up to 30 markers, up to 40 markers, or up to 50 markers.

In any of the foregoing methods, identification of changes in the following markers indicates an increased likelihood of the diagnosis of stage III or stage IV ovarian cancer:

- CA 125, HE4, EGFR and PRL;
- CA125, HE4, PRL and GH;
- CA 125, HE4, MMP-3 and TSH; or
- CA 125, HE4, eotaxin, and PRL;

wherein the foregoing combinations the level of CA 125 would be increased, the level of HE4 would be increased, the level of EGFR would be decreased, the level of prolactin would be increased, the level of GH would be increased, the level of MMP-3 would be decreased, and the level of TSH would be increased relative to the level in a normal subject.

In yet another specific, non-limiting embodiment, the above method identifies changes in the combinations of molecules presented in Table VI.

In particular non-limiting embodiments, in methods of the invention, where the serum levels of multiple molecules are being determined contemporaneously, at least 50 percent or at least 75 percent or at least 90 percent of the molecules being tested are selected from the following list of ovarian cancer -

5 associated molecules: PRL (prolactin); IGFBP1 (insulin-like growth factor binding protein 1); GH (growth hormone); HE4 (human epididymis protein 4); MIF (macrophage migration inhibitory factor); IL-6 (interleukin-6); IL-8 (interleukin-8); IL-2R (interleukin-2 receptor); SMRP (soluble mesothelin-related peptides); MCP-1 (monocyte chemoattractant protein-1); MIP-1 α (macrophage inflammaotry protein-1 α);

10 MIP-1 β (macrophage inflammaotry protein-1 β); CA-19-9 (carbohydrate antigen 19-9); CA-125 (cancer antigen 125); Cyfra 21-1 (soluble fragment of cytokeratin-19); CA 72-4 (cancer antigen 72-4); leptin; tPAI-1 (tissue plasminogen activator inhibitor-1); TSH (thyroid stimulating hormone); ACTH (adrenocorticotrophic hormone); CEA (carcinoembryonic antigen); eotaxin; MMP-2 (matrix

15 metalloproteinase-2); MMP-3 (matrix metalloproteinase-3); sV-CAM (vascular cell adhesion molecule); FSH (follicle stimulating hormone); EGFR (epidermal growth factor receptor); ErbB2 (erythroblastic leukemia viral oncogene homolog 2); adiponectin; sI-CAM (soluble intercellular adhesion molecule); sE-selectin; MPO (myeloperoxidase); IP-10 (interferon gamma induced protein 10); LH (leutinizing

20 hormone); and/or full length mesothelin.

In non-limiting embodiments, the present invention provides for a kit for diagnosing ovarian cancer, set kit comprising a means for determining the serum level of a plurality of molecules, where at least 50 percent or at least 75 percent or at least 90 percent of the molecules being tested are selected from the following list of

25 ovarian cancer -associated molecules: PRL (prolactin); IGFBP1 (insulin-like growth factor binding protein 1); GH (growth hormone); HE4 (human epididymis protein 4); MIF (macrophage migration inhibitory factor); IL-6 (interleukin-6); IL-8 (interleukin-8); IL-2R (interleukin-2 receptor); SMRP (soluble mesothelin-related peptides); MCP-1 (monocyte chemoattractant protein-1); MIP-1 α (macrophage

30 inflammaotry protein-1 α); MIP-1 β (macrophage inflammaotry protein-1 β); CA-19-9 (carbohydrate antigen 19-9); CA-125 (cancer antigen 125); Cyfra 21-1 (soluble fragment of cytokeratin-19); CA 72-4 (cancer antigen 72-4); leptin; tPAI-1 (tissue plasminogen activator inhibitor-1); TSH (thyroid stimulating hormone); ACTH (adrenocorticotrophic hormone); CEA (carcinoembryonic antigen); eotaxin; MMP-2

(matrix metalloproteinase-2); MMP-3 (matrix metalloproteinase-3); sV-CAM (vascular cell adhesion molecule); FSH (follicle stimulating hormone); EGFR (epidermal growth factor receptor); ErbB2 (erythroblastic leukemia viral oncogene homolog 2); adiponectin; sI-CAM (soluble intercellular adhesion molecule); sE-selectin; MPO
5 (myeloperoxidase); IP-10 (interferon gamma induced protein 10); LH (leutinizing hormone); and/or full length mesothelin.. In specific non-limiting embodiments said means comprises, for each molecule to be tested, a capture agent selected from the group consisting of an antibody, a portion of an antibody, a single chain antibody, a non-immunoglobulin receptor for the molecule, a peptide ligand for the molecule, and
10 an oligonucleotide ligand for the molecule, where said capture agent may, in non-limiting embodiments, be bound to a solid support, which may be, but is not limited to, a bead.

6. EXAMPLE: DEVELOPMENT OF A MULTIMARKER ASSAY
FOR EARLY DETECTION OF OVARIAN CANCER - I

5 6.1 PATIENTS, MATERIALS AND METHODS

Patient populations. The patient populations are described in Table I, at the end of this section 6.

Collection and storage of blood serum. Blood processing was similar for all samples collected at the contributing centers. Samples were obtained from
10 cancer patients or patients with benign pelvic conditions prior to surgery and before administration of anesthesia. Ten ml of peripheral blood was drawn using standardized phlebotomy procedures. Blood samples were collected without anticoagulant and allowed to coagulate for up to 2 hrs at room temperature. Sera were separated by centrifugation, immediately aliquoted, frozen and stored at -80oC. No
15 more than 2 freeze-thaw cycles were allowed for any sample.

Sources of bead-based immunoassays. The xMAP™ bead-based technology (Luminex Corp., Austin, TX) permits multiplexed analysis of several analytes in one sample. Fifty-one bead-based xMAP™ immunoassays for most known or potential ovarian cancer serum biomarkers were utilized in this study.
20 Multiplexed immunoassays for cytokines were purchased from BioSource International (Camarillo, CA); Linco, assays for MMPs were from R&D Systems (Minneapolis, MN), multiplexed assays for (sICAM-1, sVCAM-1, sE-selectin, tPAI-1, MPO, MIF, sFas, sFasL), (adiponectin, leptin, active PAI-1, resistin, NGF), and (prolactin, FSH, GH, TSH, LH, ACTH), were obtained from Linco Research (St.
25 Charles, MO). Assays for (CA 125, EGFR, ErbB2, CEA, CA 15-3, CA 19-9, IGFBP-I, human kallikrein 8 (hK8) Cyfra 21-1, mesothelin) and (CA 72-4, AFP, βHCG, hK10), soluble mesothelin-related protein (SMRP) and HE4 were obtained from the Luminex Core Facility of the University of Pittsburgh Cancer Institute. Of these, antibodies for kallikreins were provided by Dr. Eleftherios Diamandis, antibodies
30 against HE4 and SMRP were a kind gift of Fujirebio Diagnostics (Malvern, PA) to Dr. Robert Bast. Chicken polyclonal antibody for human full-length mesothelin was developed by GenWay Biotech, Inc. (San Diego, CA). Overall, 7 different multiplexed panels and 2 individual bead-based assays (HE4 and SMRP) were used. The intra-assay variability of each assay was 3.5-5%. Inter-assay variability was 11-

15%. Each bead-based assay was validated in comparison with appropriate standard ELISA based on the same antibody pair and has demonstrated 89-98% correlation.

Multiplex analysis. Assays were performed according to manufacturers' protocols as previously described (21). Samples were analyzed using the Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA). For each analyte, 100 beads were analyzed and means were calculated. Analysis of experimental data was performed using five-parametric-curve fitting to the standard analyte curves.

Statistical analysis of data. The Wilcoxon rank sum test was used to determine statistical significance of differences in biomarker concentrations between patient groups. For evaluating the efficiency of standard classification methods (logistic regression, neural networks etc.), the Weka v. 3.4 software (Waikato University, NZ) was used.

15 6.2 RESULTS

Bead-based immunoassay analysis of serum concentrations of different biomarkers in ovarian cancer patients. Serum samples of postmenopausal women from three groups: early (I-II) stage ovarian cancer (n=86), benign pelvic masses (n=79), and age-matched healthy controls (n=104) (Table I) were screened using multiplexed bead-based immunoassays for following proteins: cancer antigens (CA 125, CEA, CA 15-3, AFP, CA19-9, CA 72-4); growth/angiogenic factors (VEGF, bFGF, IGFBPI, HGF, NGF, ErbB2, EGFR); apoptosis-related molecules (sFas, sFasL, Cyfra 21-1); metastasis-related molecules (MMP-2, MMP-3, tPAI-1, active PAI-1, sICAM-1, sVCAM-1, sE-selectin); cytokines (IL-6, IL-8, G-CSF, TNF- α , IP-10, MCP-1, MIP-1 α , MIP-1 β , MIF, sIL-2R, TNFRI, Eotaxin, IL-10); hormones (β HCG, FSH, LH, GH, TSH, ACTH, and prolactin); and adipokines (adiponectin, leptin, and resistin); hK8 and 10, HE4, SMRP, mesothelin, and MPO. Distributions of serum markers did not differ among the three control groups obtained from different sources indicating comparability of samples. Serum concentrations of 37 proteins demonstrated significant ($p < 0.05$ – $p < 0.001$) differences between ovarian cancer samples in comparison to healthy and benign groups (Table II and FIGURE 1A). In contrast, only 11 proteins demonstrated statistically significant differences between cancer and benign pelvic disease suggesting considerable

overlap. Serum concentrations of 12 proteins were significantly different between different histological groups of ovarian cancers (FIGURE 1B).

Statistical analysis of multimarker panels. None of the individual markers offered a high enough sensitivity at 98% specificity (Table II). To determine the optimal multimarker panel the simulation set based on 86 ovarian cancer and 104 control samples was created as described in Methods, and the projection pursuit technique (PT) was applied to determine the best set of projections as well as markers comprising these projections. The resulting panel consisted of 6 biomarkers, CA 125, HE4, Eotaxin, CA 72-4, FSH, and IL-10. Cross-validation test results are presented on FIGURE 2A. The algorithm demonstrated better performance when compared to logistic regression, decision trees, Bayesian networks and neural networks. The multimarker panel demonstrated selectivity for post-menopausal women, since the analysis performed for pre-menopausal patients resulted in a lower sensitivity of 80% at 98% specificity (FIGURE 2A). To evaluate the performance of the 6-marker panel in an independent validation set, additional samples from postmenopausal women with early stage ovarian cancer and healthy controls (50 samples in each group) were analyzed using the algorithm resulting in classification with 90% sensitivity at 98% specificity, which was significantly higher than CA 125 alone (47% sensitivity at 98% specificity) (FIGURE 2B). Based on the size of validation set, the accuracy could be defined as $\pm 8.4\%$, which for stage IA women rules out the sensitivity less than 86.6%. Furthermore, this panel was able to discriminate ovarian cancer from benign tumors with sensitivity of 75% at specificity of 99% (FIGURE 2C). The higher threshold for specificity was chosen to minimize the number of ovarian cancer cases misdiagnosed as benign.

Selectivity of the panel for ovarian cancer vs other epithelial cancers. To evaluate ovarian cancer-selectivity of the 6-marker panel, a cross-validation test (55/45 random split, 100 runs) was performed with sera from 86 patients with early stage ovarian cancer and age-matched patients with breast (n = 120), endometrial (n = 120), and lung (n = 85) cancers resulting in correct identification of 98% of ovarian cancers while misclassifying 2% of other cancers (FIGURE 2D). Thus, this marker panel appears to be highly selective for ovarian cancer.

6.3 DISCUSSION

After performing a multimarker bead-based immunoassay screening, it was observed, in agreement with published evidence, elevated serum levels of IL-6, IL-8, SMRP, IGFBP-I, MIF, HE4, IL-2R, MCP-1, MIP-1 α , MIP-1 β , CA19-9, CA 125, Cyfra 21-1, CA 72-4, leptin, prolactin, tPAI-1, and lower levels of FSH, LH, and EGFR in patients with ovarian cancer as compared with healthy women (9, 39-44). In addition, the above experiments were the first to report increased serum levels of TSH, GH, and ACTH, and lower concentrations of eotaxin, immunodetectable MMP-2,3, ErbB2, adiponectin, sI-CAM, sV-CAM, sE-selectin, and full-length mesothelin in serum of patients with ovarian cancer. Some of our findings, i.e. lower levels of sI-CAM, sV-CAM, and sE-selectin, are in disagreement with published evidence (55). This may be explained by non-representative sample sizes in the prior publications (n=28) or by the use of antibodies with different epitope recognition. Furthermore, lower concentrations of ErbB2, MMP-2,3, sICAM-1, sVCAM-1, and sE-selectin were observed in serum from patients with several other cancers, i.e. breast, endometrial, and pancreatic, compared to healthy controls.

Using an algorithm, a combination of 6 serum proteins were identified that offered 90% sensitivity at 98% specificity for early stage ovarian cancer in the independent blinded validation set. It has to be noted that serous histology was not a prevalent histology in both training and validations sets. This could be explained by the fact that for patients with early stage disease, non-serous histologies are more frequently encountered. Furthermore, the validation set had lower representation of endometrial, clear cell, serous, and mucinous histotypes as compared with the training/test sets. The fact that the multimarker assay offers high accuracy in this set indicates the likely general utility of the assay for the most common histotypes of ovarian cancer. Furthermore, 90% sensitivity at 98% specificity achieved in this study with the 6-marker panel surpasses that reported in comparable studies where fewer markers were used (70-73%) (17-18). Importantly, the most powerful biomarker panel consisted of proteins with different biological functions. The functional diversity of this multimarker panel may contribute to its power for discrimination of early stage ovarian cancer.

The resultant panel was highly selective for ovarian cancer as compared with three other cancers, i.e. breast, endometrial, and lung. These results indicate the possibility of generating different multimarker assays specific for

individual cancers. The results also demonstrate that 6-marker diagnostic panel is relatively selective for ovarian cancer in post-menopausal women.

Table I. Characteristics of Patient Population

5

Histology	Training Set		Validation Set		Premenopausal	
	N	Age	N	Age	N	Age
<u>Healthy</u>	<u>104</u>	Range 49-84 Median 57.7 Mean 54.7	<u>50</u>	Range 50-85 Median 67.5 Mean 64.8		
<u>Ovarian cancer</u>	<u>86</u>	Range 47-87	<u>50</u>	Range 50-85	<u>24</u>	Range 31-55
endometrioid	27	Median 59.0	6	Median 67.5	4	Median 47
mucinous	27	Mean 61.6	5	Mean 64.7	16	Mean 47.5
serous	21		4		1	
clear cell	7		8		3	
in adenofibroma	4		0			
Adenocarcinoma						
NOS (not otherwise specified)	0		16			
<u>Benign</u>	<u>79</u>	Range 49-87				
Adenofibroma	10	Median 66				
Brenner tumor	2	Mean 65				
Cystadenofibroma	19					
Cystadenoma	41					
Endometriosis	1					
Fibroma	2					
Ovary benign	4					

Table II. Characteristics of serum biomarkers in ovarian cancer, benign pelvic disease, and healthy groups

Biomarkers	Biomarker concentrations			Units	p-value		SN at 98% SP OC vs C, %
	Control (C) mean±SE median (range)	Ovarian cancer(OC) mean±SE median (range)	Benign(B) mean±SE median (range)		OC vs C	OC vs B	
CA 125	3.6±0.37 2.4 (0-14.3)	41.3±7.93 16.8 (0-444.3)	19.1±6.59 3.8 (0.5-434.5)	U/ml	<0.001	<0.001	55
Prolactin	41.2±14.98 7.8 (1.3-560.0)	354.4±68.11 125.7 (3.7-4593.2)	260.3±27.61 156.4 (3.8-560.0)	ng/ml	<0.001	ns	17
sI-CAM	142.5±8.32 128.7 (54.3-650.3)	99.8±4.54 88.8 (19.6-345.7)	124.3±8.03 105.0 (42.5-418.4)	pg/ml	<0.001	0.006	35
HE4	207.6±47.26 89.5 (27.4-2719.9)	1877.5±405.44 458.6 (20.0-22363.0)	549.9±154.97 127.9 (14.1-9696.4)	pg/ml	<0.001	<0.001	15
CA 72-4	0.00±0.00 0.0 (0.0-0.0)	10.1±6.60 0.0 (0.0-556.5)	5.3±4.70 0.0 (0.0-356.0)	U/ml	<0.001	0.012	30
MIF	75.1±15.52 6.7 (2.5-533.5)	451.4±62.36 242.7 (2.5-3688.5)	457.3±55.71 309.9 (2.5-2025.8)	pg/ml	<0.001	ns	12
TSH	1.8±0.31 1.3 (0.3-22.9)	3.8±0.40 2.9 (0.1-22.6)	3.4±0.34 2.6 (0.2-18.1)	ng/ml	<0.001	ns	10

IGFBPI	3.3±0.61 1.7 (0.1-28.8)	12.6±1.71 5.8 (0.8-89.1)	8.7±1.21 4.8 (0.4-62.9)	ng/ml	<0.001	ns	10
FSH	44.0±2.97 42.2 (1.8-149.3)	24.6±2.07 22.7 (0.8-88.1)	26.8±1.93 24.5 (2.8-65.1)	ng/ml	<0.001	ns	12
Mesothelin	8.6±0.87 6.6 (1.6-60.8)	4.7±0.35 3.9 (0.0-14.0)	6.2±0.58 4.9 (0.0-33.6)	ng/ml	<0.001	ns	3
sFasL	124.7±28.24 92.2 (9.6-2082.5)	107.8±29.83 64.2 (6.9-2480.8)	82.8±9.77 67.7 (9.6-766.9)	pg/ml	<0.001	ns	
Cyfra 21-1	0.4±0.15 0.0 (0.0-7.4)	4.3±0.81 2.2 (0.0-37.1)	1.4±0.49 0.0 (0.0-35.2)	ng/ml	<0.001	<0.001	7
Eotaxin	124.2±6.80 108.7 (34.8-301.8)	85.1±7.14 75.9 (4.5-532.2)	84.5±6.30 74.4 (18.7-347.8)	pg/ml	<0.001	ns	8
TNF-α	20.0±3.94 11.1 (0.0-220.8)	108.6±74.15 9.2 (0.0-6144.4)	26.9±10.05 12.1 (0.0-725.9)	pg/ml	0.040	ns	
sV-CAM	729.5±19.84 728.2 (299.3-1255.8)	480.2±17.45 479.6 (74.9-1038.7)	535.1±21.41 507.8 (193.3-987.2)	pg/ml	<0.001	ns	20
MMP-2	156.7±3.36 153.9 (84.0-277.2)	125.6±3.86 121.9 (24.7-210.1)	140.6±4.57 137.8 (51.4-283.3)	pg/ml	<0.001	0.021	22
E-Selectin	24.2±2.06 21.0 (0.0-122.2)	18.8±1.04 20.4 (0.0-63.8)	21.2±1.71 20.7 (0.0-116.7)	pg/ml	0.021	ns	2
AFP	2.5±0.56 1.6 (0.5-40.7)	1.7±0.20 1.1 (0.1-13.9)	1.4±0.11 1.1 (0.2-5.6)	ng/ml	0.003	ns	3
HGF	61.3±11.84	81.4±14.39	110.4±17.49	pg/ml	ns	0.024	

	34.6 (0.0-541.7)	27.3 (0.0-738.6)	45.9 (0.0-669.4)				
LH	25.2±1.45 24.9 (2.9-55.9)	17.5±1.46 14.7 (0.1-53.2)	19.7±1.70 17.7 (1.1-80.2)	ng/ml	<0.001	ns	10
MMP-3	8.8±0.45 8.1 (2.6-28.2)	6.6±0.43 6.1 (0.5-30.5)	7.6±0.78 6.3 (2.3-54.9)	pg/ml	<0.001	ns	7
tPAI-1	23.5±0.84 22.9 (10.5-42.2)	23.3±0.93 23.7 (18.9)	21.1±1.05 18.6 (7.6-53.5)	pg/ml	ns	0.021	1
IL-8	5.5±0.49 4.9 (0.0-36.2)	18.8±9.91 6.3 (0.0-847.3)	9.4±1.54 5.5 (0.0-87.7)	pg/ml	0.004	ns	3
IL-2R	78.9±8.74 59.4 (0.0-309.2)	207.2±40.22 121.3 (0.0- 2700.7)	146.2±23.18 106.1 (0.0- 1637.1)	pg/ml	<0.001	ns	1
MCP-1	315.8±14.55 301.3 (98.8- 647.5)	301.1±35.82 235.9 (34.3- 3038.0)	290.7±18.66 265.5 (82.5- 1326.4)	pg/ml	0.012	ns	2
MIP-1 α	42.4±12.01 0.0 (0.0-629.6)	296.0±240.02 0.0 (0.0-20415.3)	72.1±16.96 27.2 (0.0- 1047.1)	pg/ml	ns	0.045	
IL-6	23.9±13.36 7.2 (0.0-965.1)	36.5±5.82 17.5 (0.0-306.1)	39.4±7.79 16.5 (0.0-398.6)	pg/ml	<0.001	ns	4
MIP-1b	65.1±21.64 0.0 (0.0-1354.5)	346.5±199.83 38.1 (0.0- 16822.9)	154.1±39.21 43.5 (0.0- 2297.6)	pg/ml	0.005	ns	3
Leptin	15.6±1.55 12.55 (1.1-73.3)	10.3±1.18 6.5 (0.6-58.8)	12.4±1.22 9.1 (0.6-50.4)	ng/ml	<0.001	ns	
IP-10	17.7±2.43 10.7 (0.0-117.8)	13.2±1.60 8.2 (0.0-69.4)	18.6±2.44 13.7 (0.0-149.1)	pg/ml	0.024	0.009	3.5

EGFR	4.8±0.08 4.6 (3.3-6.7)	3.7±0.11 3.6 (0.9-6.7)	4.0±0.10 3.9 (2.0-6.4)	ng/ml	<0.001	ns	20
TNFR-1	1.7±0.14 1.5 (0.5-10.2)	1.3±0.13 1.3 (0.1-4.9)	1.6±0.12 1.3 (0.4-6.9)	ng/ml	0.006	ns	7
Adiponectin	28.7±1.71 26.9 (1.7-67.6)	22.7±1.52 20.6 (2.9-64.8)	24.5±2.01 19.6 (3.5-110.0)	µg/ml	<0.001	ns	
CA 19-9	0.1±0.01 0.0 (0.0-0.5)	11.8±8.77 0.2 (0.0-742.8)	19.8±13.25 0.0 (0.0-745.0)	U/ml	<0.001	<0.001	16
ACTH	9.3±1.94 4.5 (0.1-124.4)	42.8±10.26 11.8 (0.1-700.8)	28.7±5.82 11.3 (0.1-285.8)	pg/ml	<0.001	ns	12
ErbB2	9.8±0.32 9.5 (4.9-23.2)	7.9±0.44 7.4 (1.4-33.9)	7.8±0.33 7.2 (4.1-22.2)	ng/ml	<0.001	ns	15
GH	1053.1±652.98 104.0 (2.8- 46711.8)	1583.5±320.58 583.2 (18.6- 18819.5)	1814.7±393.68 722.2 (5.0- 20161.9)	pg/ml	<0.001	ns	7

OC – ovarian cancer; C- control; B- benign

SN – sensitivity; SP - specificity

7. EXAMPLE: DEVELOPMENT OF A MULTIMARKER ASSAY FOR
EARLY DETECTION OF OVARIAN CANCER- II

7.1 PATIENTS, MATERIALS AND METHODS

5 **Patient populations.** The study population was comprised of 256
healthy postmenopausal women, 268 patients with ovarian cancer in stages IA (n=68),
IB-IIIC (n=95), and III-IV (n=105), 137 patients with benign pelvic tumors, and
postmenopausal women with breast (n=210) and lung (n=74) cancers. Samples were
obtained from multiple sources (Table III) and were annotated with information
10 regarding age, cancer diagnosis, stage, histology, and grade (Table IV). The local
institutional review boards approved the appropriate protocols for use of each sample
collection.

Collection and storage of blood serum. Serum samples were
collected, processed and stored as previously described (20). Blood processing was
15 similar for all samples collected at the contributing centers.

Sources of bead-based immunoassays. The xMAP™ bead-based
technology (Luminex Corp., Austin, TX) permits multiplexed analysis of several
analytes in one sample. Eighty four bead-based xMAP™ immunoassays for most
known or potential ovarian cancer serum biomarkers utilized in this study are
20 presented in Table V. For assays obtained from the Luminex Core Facility of the
University of Pittsburgh Cancer Institute, antibodies for kallikreins were a generous
gift of Dr. Eleftherios Diamandis (University of Toronto, Toronto, CA), antibodies
against CA 125, CA 72-4, CA 15-3, soluble mesothelin-related protein (SMRP), and
HE4 were a kind gift of Fujirebio Diagnostics Inc. (Malvern, PA). Chicken polyclonal
25 antibody for human full-length mesothelin was developed by GenWay Biotech, Inc.
(San Diego, CA). The inter-assay variability of each assay was 3.5-5%. Intra-assay
variability was 7-21%. Each bead-based assay was validated in comparison with
appropriate standard ELISA based on the same antibody pair and has demonstrated
89-98% correlation (data presented on Luminex Core Facility website for in-house
30 assays; performance of purchased assays was in agreement with that claimed by a
manufacturer).

Multiplex analysis. Assays were performed according to
manufacturers' protocols as previously described (21). Samples were analyzed using
the Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA). For each
35 analyte, 100 beads were analyzed and means were calculated. Analysis of

experimental data was performed using four-parameter logistic curve fitting to the standard analyte curves.

Statistical analysis. The data were randomly split into a training and validation set with each subject having a probability of 50% to be selected for the training set. All development of statistical models for distinguishing ovarian cancer cases from controls was restricted to the training set (modelers were blinded to all data in the validation set) until one panel and one model of combining the candidate biomarkers in the panel was selected from the analyses of the training set, avoiding bias in estimate of operating characteristics due to selection of best panel from evaluation of multiple models/panels on the validation set.

The natural logarithm was applied to transform the span of concentrations to an arithmetic scale and to transform their distribution closer to a symmetric one, both of which are more appropriate for the assumptions underlying standard statistical methods. Logistic regression, using the Furnival-Wilson branch and bound algorithm, as implemented in the Statistical Analysis System (SAS version 10, Cary, NC), was used to identify an initial list of the best subsets of size 10 candidates or fewer 22.

The score statistic S was evaluated on all possible panels for each subset, and the five panels with highest S of size three or greater selected. The cross-validated "leave one out" estimate of sensitivity at 95% and 98% specificity was obtained for all resulting panels. Panels of size five did not have significant increases in sensitivity compared to panels of size four. Therefore panels of size three and four with cross-validated sensitivities above 90% were further analyzed. Bivariate scatter plots (using the R statistical software, The R Foundation for Statistical Computing, c/o Institut für Statistik und Wahrscheinlichkeitstheorie, Technische Universität Wien, Wiedner Hauptstraße 8-10/1071, 1040 Vienna, Austria) on the log-log scale of all combinations of remaining candidate biomarkers identified 3 outliers with large influence on logistic regression coefficients 23.

These outliers were removed and cross-validated estimates of sensitivity at 95% and 98% specificity re-calculated for the remaining panels of size three and four. The panels and coefficients were applied to the other groups of patients (lung cancer, breast cancer, benign disease) in the training set, and one optimal panel chosen with high cross-validated sensitivity for both early and late stage ovarian cancer. This sole panel and method of combination as estimated by logistic

regression on the combined early and late stage training cases versus healthy training controls was evaluated on the validation set to estimate, free from selection bias, the model's sensitivity at 98% specificity for early ovarian cancer, late ovarian cancer, breast and lung cancers, and benign ovarian disease. Univariate comparison of candidate biomarkers, such as between different sources of samples, or between early and late stage cases, was made with the Wilcoxon rank sum test.

7.2 RESULTS

Bead-based immunoassay analysis of biomarker serum

concentrations in ovarian cancer patients. Serum samples from postmenopausal women with early stage (I-II) ovarian cancer, benign pelvic masses, and from age-matched healthy controls (Table IV) were analyzed using multiplexed bead-based immunoassays for 84 cancer-associated biomarkers representing proteins with different biological functions (Table V). These candidate biomarkers were selected based on the published evidence documenting an association with ovarian cancer 24-26 and our preliminary unpublished observations. Distributions of serum markers did not differ among the four control groups obtained from different sources, indicating comparability of samples. Serum concentrations of 19 proteins demonstrated highly significant ($p < 0.001$) differences between ovarian cancer samples in comparison to healthy and benign groups (FIGURE 3A). Serum levels of 11 proteins differed significantly between patients with early (I-II) and late (III-IV) stage ovarian cancer (FIGURE 3A). Thirteen proteins demonstrated statistically significant ($P < 0.05$) differences between early ovarian cancer and benign pelvic disease (FIGURE 3A). No significant differences in serum concentrations of any of the biomarkers between stages I and II were found.

Statistical analysis of multimarker panels. Sensitivities of individual biomarkers for discrimination of early stage (I-II) ovarian cancer from healthy controls at a specificity of 98% varied from 48% for CA 125 to 1% for IL-2R. Training set (Table IV) was utilized to identify the best panel using the process described in Methods. This panel included 4 biomarkers, CA 125, EGFR, HE4, and sVCAM-1 and provided 92% SN (95% SN for stage IA) at 98% SP (FIGURE 3B). Several other biomarker combinations offered 1-2% lower sensitivity (Table VI).

Next, the classification results were validated in an independent blinded set (Table IV) that included 34 patients with stage IA ovarian cancer. The 4-

biomarker panel classified early stage ovarian cancers with 90% SN (85% SN for stage IA) at 98% SP, which was significantly higher than CA 125 alone. Based on the size of validation set (86 cancers), the accuracy of the sensitivity estimate could be defined as $\pm 6.6\%$, which for early stage women rules out the sensitivity less than 83%. Of 34 stage IA cases, 5 were misclassified resulting in 85% SN. The 4-biomarker set performed equally well for classification of stages III-IV cases offering 91% SN at 98% SP. Importantly, this multimarker panel correctly diagnosed 68% of 137 benign cases as non-cancers.

Selectivity of the panel for ovarian cancer vs other epithelial cancers. The panel consisting of CA 125, EGFR, HE4, and sVCAM was used to classify sera collected from postmenopausal women with breast (n=210), and lung (n=74) cancers. This panel correctly classified 92% of breast cancer cases and 54% of lung cancer cases as 'non-ovarian cancer' at specificity for ovarian cancer set at 98%. Other panels provided higher selectivity for ovarian cancer at slightly lower sensitivity (Table VI).

7.3 DISCUSSION

The goal of the present study was to identify a panel of biomarkers that would provide high sensitivity and specificity for distinguishing clinically diagnosed early stage ovarian cancer from healthy controls using a large panel of serum biomarkers chosen from groups of proteins with different biological functions representing different aspects of systemic response to a growing tumor (12, 21, 27-38). After performing a multimarker bead-based immunoassay screening, we have observed, in agreement with published evidence, elevated serum levels of IGFBP-I, MIF, HE4, IL-2R, CA19-9, CA 125, Cyfra 21-1, CA 72-4, and prolactin, and lower levels of FSH, LH, and EGFR in patients with ovarian cancer as compared with healthy women (9, 39-44). In addition, the above experiments were the first to report significant elevation of serum levels of GH, and ACTH, and lower concentrations of eotaxin, immunodetectable MMP-2,3, and sV-CAM in serum of patients with ovarian cancer.

Using a combination of logistic regression, branch and bound algorithms, cross-validation, and graphical analyses, we have identified a combination of 4 serum proteins, CA 125, EGFR, HE4, and sVCAM-1 that offered 90% sensitivity at 98% specificity for early stage ovarian cancer in the solely evaluated independent

blinded validation set. This multimarker set is comprised of 4 proteins with distinct biological functions. CA 125 is a mucin (MUC16) shed by >80% of ovarian cancers⁴⁵. HE4 is a Whey Acidic Protein with a presumptive role in natural immunity that is overexpressed in several types of cancers (46). Lower concentrations of EGFR
5 in serum of ovarian cancer patients were previously reported (13, 47). Finally, vascular endothelial cell adhesion molecule-1, VCAM-1, is involved in extravasation of circulating cancer cells, which is a key step in metastasis (reviewed in 48). We have observed lower serum sVCAM-1 concentrations in serum of patients with endometrial, pancreatic, and lung cancers (20). The mechanisms behind lower sEGFR
10 and sVCAM-1 levels in patients with ovarian and other cancers have yet to be determined.

The high sensitivity of 90% achieved with our four marker panel for distinguishing women with early stage ovarian cancer from healthy individuals at 98% specificity surpasses that previously reported (70-73%) (9, 39-44). The fact that
15 the multimarker assay offers high accuracy in a heterogeneous validation set that contained different histologies, indicating the likely general utility of the assay for the most common histotypes of ovarian cancer. Of note, in agreement with published evidence, we observed lower incidence of serous histology in early stage patients (49). The panel of biomarkers correctly classified 68% of benign lesions as non-
20 cancer. Specificity would, however, be substantially improved by the subsequent use of TVS to distinguish malignant from benign pelvic lesions in 2% of women classified at risk for ovarian cancer further reducing the number of laparotomies for benign disease (50).

The 4-molecule panel was highly selective for ovarian cancer when
25 compared to breast cancer and moderately selective when compared to lung cancer, suggesting that multimarker assays could be cancer-specific. Detection of occasional breast and lung cancers might be facilitated by incorporating mammography and spiral chest CT in the second phase of screening for those women with a negative TVS. The performance of this multimarker panel for detecting ovarian cancer in
30 asymptomatic subjects with sufficient lead time prior to clinical diagnosis remains to be further determined in longitudinal retrospective studies using the serum banks established by major ongoing prospective trials, e.g. PLCO, WHI, UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS).

Table III. Sources of serum samples

	UPCI (Pittsburgh, PA)	GOG (Columbus, OH)	MD Anderson (Houston, TX)	FCCC (Philadelphia, PA)	Duke University (Durham, NC)	University of Iowa (Iowa City, IA)
Ovarian, I-II		141	13 (Dr. Bast)	9 (Dr. Godwin)		
Ovarian, III-IV		84				21 (Dr. Lutgendorf)
Ovarian, benign		137				
Breast					210 (Dr. Marks)	
Lung	37 (Dr. Siegfried)				37 (Dr. Patz)	
Healthy	40 (Dr. Modugno) 22 (Dr. Lokshin)	65	27 (Dr. Lu)	58 (Dr. Godwin)	44 (Dr. Marks)	

Table IV. Characteristics of the Patient Population

Histology	Training Set		Validation Set	
	N	Age	N	Age
<u>Healthy</u>		Range 47-86		Range 49-85
<u>N Total</u>	<u>153</u>	Median 57	<u>103</u>	Median 62
		Mean 57.0		Mean 59.3
<u>Stages IA-II C Ovarian cancer</u>		Range 45-85		Range 47-87
<u>N Total</u>	<u>77</u>	Median 59	<u>86</u>	Median 59
Adenocarcinoma, endometrioid	28	Mean 62.4	21	Mean 61.4
Adenocarcinoma, mucinous	18		12	
Adenocarcinoma, serous	14		26	
Adenocarcinoma, clear cell	7		13	
Other	10		14	
<u>Stages III-IV Ovarian cancer</u>				Range 48-91
<u>N Total</u>			<u>105</u>	Median 66
Adenocarcinoma, endometrioid			39	Mean 65.5
Adenocarcinoma, mucinous			4	

Adenocarcinoma, serous			47	
Adenocarcinoma, clear cell			11	
Other			5	
<u>Benign</u>				
<u>N Total</u>			<u>137</u>	Range 49-87 Median 63 Mean 63.4
<u>Breast</u>				
<u>N Total</u>			<u>210</u>	Range 53-82 Median 63 Mean 64.4
<u>Lung</u>				
<u>N Total</u>			<u>74</u>	Range 47-91 Median 69 Mean 67.8

Table V. Multiplexed biomarkers

Biological groups	Proteins	Plex No.	Source
Cytokines/ Chemokines	eotaxin, TNFR I, II, IL-1R α , IL-2R, IL-6R, and DR5	1	1
	Interleukins 1b, -2, 4, 5, 6, 7, 8, 10, 12p70, 13, G-CSF, GM- CSF, IFN γ , TNF- α , IP-10, MCP-1, MIP-1 α , MIP-1 β , MIF, CD40L, fractalkine	2	2
Growth/angiogenic/ factors	EGF, VEGF, bFGF, HGF, VEGF, NGF	1	1
	EGFR, Her2/neu, IGFBPI	3	3
Anti-angiogenic factors	angiostatin, endostatin, and thrombospondin	4	3
Cancer antigens	CA 125, CA 15-3, CEA,	5	3
	CA 19-9, CA 72-4, PSA, AFP, SCC	6	3
Apoptotic proteins	Cyfra 21-1	5	3
	DR5	1	1
	sFas, sFasL	7	2
Proteases	Kallikreins 8,10	5,6	3
	MMP1-13	8	4
Adhesion molecules	sICAM, sVCAM, sE-selectin	9	2
Hormones	prolactin, TSH, LH, ACTH,	10	2

	GH	10	2
	β HCG	6	3
Adipokines	Adiponectin, leptin, resistin	11	2
Other markers	mesothelin	6	3
	HE4	12	3
	tPAI-1, active PAI-1, MPO,	13	2
	apolipoproteins (Apo) A1, AI, AII, B, CII, CIII, E	14	2

Table VI. SN, SP, and cancer selectivity of different panels after cross-validation

M#1	M#2	M#3	M#4	SN% at 98% SP		% of 'non-cancer' at 98% SP		
				Stages I-II	Stages III-IV	Benign	Lung	Breast
CA 125	HE4	EGFR	PRL	88	91	64	67	95
CA 125	HE4	MMP2	PRL	89	88	62	61	94
CA 125	HE4	PRL	GH	89	91	61	60	95
CA 125	HE4	MMP3	TSH	88	91	62	60	94
CA 125	HE4	Eotaxin	PRL	89	91	59	60	94
CA 125	HE4	PRL	ACTH	89	89	64	59	97
CA 125	HE4	MMP3	PRL	88	91	65	59	97
CA 125	HE4	VCAM-1	PRL	88	91	63	59	95

8. EXAMPLE: DEVELOPMENT OF A MULTIMARKER ASSAY
FOR EARLY DETECTION OF OVARIAN CANCER- III

8.1 PATIENTS, MATERIALS AND METHODS

5 **Patient populations.** Serum samples from postmenopausal women including 136 patients diagnosed with early (I-II) stage ovarian cancer, 79 patients with benign pelvic masses, 120 patients with breast cancer, and 154 healthy age-matched controls were tested. Serum samples from patients with ovarian cancer, women with benign pelvic disease, and some healthy women were provided by the
10 Gynecologic Oncology Group (Cleveland, OH) along with information about gynecologic diagnoses and ovarian cancer staging as well cancer histology and grade (Table VII). Additional control serum samples from healthy, age-matched women and from women with breast cancer were received from Dr. Francesmary Modugno (UPCI, Pittsburgh, P A) and from Dr. Jeffrey Marks (Duke University, Dunham, SC).

15 **Collection and storage of blood serum.** Ten ml of peripheral blood was drawn from subjects using standardized phlebotomy procedures. Handling and processing was similar for all patients. Samples were obtained from patients diagnosed with ovarian cancer or benign pelvic condition, prior to surgery, before administration of anesthesia. Blood samples were collected without anticoagulant into
20 red top vacutainers and allowed to coagulate for up to 4 hrs at room temperature. Sera were separated by centrifugation, and all specimens were immediately aliquoted, frozen and stored in a dedicated -80°C freezer. No more than 2 freeze-thaw cycles were allowed for each sample.

Sources of bead-based immunoassays. Forty-six bead-based
25 xMAPTM immunoassays for most known ovarian cancer serum biomarkers were utilized in this study. Multiplexed bead-based immunoassays for cytokines were purchased from BioSource International (Camarillo, CA); assays for MMP-2 and -3 were from R&D Systems (Minneapolis, MN), multiplexed assays for sICAM-I, sVCAM-I, sE-selectin, tPAI-I, MPO, MIF, sFas, sFasL, and prolactin, FSH, GH,
30 TSH, LH, ACTH, were obtained from Linco Research (St. Charles, MO). Assays for CA 125, EGFR, ErbB2, CEA, CA 15-3, CA 19-9, IGFBP-I, hK8 Cyfra 21-1, mesothelin and CA 72-4, AFP, pHCG, hKI0, soluble mesothelin-related protein (SMRP) and HE4 were developed in Luminex Core Facility of University of Pittsburgh Cancer Institute according to the protocol by Luminex Corp. (Austin, TX)

essentially as described earlier for CA125 (21). Antibody pairs for kallikreins were kindly provided by Dr. Diamandis, antibody pairs against HE4 and SMRP were a kind gift of Fujeribio Diagnostics (Malvem, P A). Sources of other antibody pairs are the proprietary information of the University of Pittsburgh Luminex Core Facility.

- 5 Overall, 5 different multiplexed panels and 2 individual bead-based assays (HE4 and SMRP) were used. The inter-assay variability within the replicates was in the range of 3.5 to 7%. Intra-assay variability was between 11 and 15%. Each assay was further validated in comparison with appropriate ELISA and has demonstrated 89-98% correlation. Recovery from serum was assessed and was determined to range from
10 80% to 110%.

Multiplex analysis. The multiplexed bead-based serum assays were performed in 96-well microplate format as previously described (21). All purchased assays were performed according to appropriate manufacturer's protocols. Samples were analyzed using the Bio-Plex suspension array system, (Bio-Rad Laboratories,
15 Hercules, CA). Analysis of experimental data was performed using five-parametric-curve fitting.

Statistical analysis of data. Descriptive statistics and graphical displays (i.e. dot plots) for serum concentrations of 46 biomarkers were calculated using SigmaStat (SYSTAT Software, Inc., Chjcago, Illinois) and GraphPad Prism
20 (GraphPad Software, Inc., San Diego, CA). The Wilcoxon ranks urn test was used to determine statistical sigruficance of differences in biomarker concentrations between patient groups. Logistic regression was performed as described (17). For evaluating the efficiency of standard classification methods (logistic regression, neural networks etc.) the Weka v. 3.4 software (Waikato Univ., NZ) was used.

25 **The ADEPT (ADE+PT) algorithm.** A novel approach to the multivariate two-class events classification of sparse data in a multidimensional space has been developed based on classification in multiple k - dimensional projections with subsequent combining of the classification scores obtained from these projections to form a final classifier. The discrimination within a single k-dimensional
30 projection was performed using a kernel based probability density estimator with adoptive bandwidth (ADE) by creating separate density probability estimations for both classification events and then generating the logit score reflecting the probability of a given data point to fall into one of the two event classes. The resulting score was obtained as a sum of scores over all selected projections. The optimal set of

projections was obtained by utilizing the projection pursuit technique (PT) applied to the simulation set which was comprised of series of the training subsets created by repetitive random sub-sampling of the original data set and adding the white noise to each data point as well as a scale noise to the whole training subset in form of the linear transform with random coefficients in order to reflect the scale de-synchronization between successive xMAP runs. The projections were chosen in such a way that when combined together they maximized the discrimination rate simultaneously for all training sets.

8.2 RESULTS

Bead-based immunoassay analysis of serum concentrations of different biomarkers in ovarian cancer patients. Multiplexing bead-based immunoassay was utilized to analyze 46 proteins in serum samples of postmenopausal women from three groups: early (I-II) stage ovarian cancer (n=86), benign pelvic masses (n=79), and age-matched healthy controls (n=104) (Table VII). Analyzed proteins belonged to several functional groups including cancer antigens, CA 125, CEA, CA 15-3, AFP, CAI9-9, CA 72-4; growth/angiogenic factors, VEGF, bFGF, IGFBPI, HGF; ErbB2, EGFR; apoptosis-related molecules, sFas, sFasL, Cyfra 21-1; metastasis-related molecules, MMP-2, MMP-3, tPAI-1, sICAM-1, sVCAM-1, sE-selectin; cytokines, IL-6, IL-8, G-CSF, TNF- α , IP-10, MCP-1, MIP-1 α , MIP-1 β , MIF, sIL-2R, TNFRI, Eotaxin; hormones, β HCG, FSH, LH, GH, TSH, ACTH, and prolactin; HK8 and 10, HE4, SMRP, mesothelin, and MPO. Serum concentrations of 32 proteins demonstrated significant ($p < 0.05$ - $p < 0.001$; Table VIII and FIGURE 4A) difference between ovarian cancer samples in comparison to healthy and benign groups.

Histotype-specificity of selected markers. To ascertain the general applicability of the multi marker panel for different histotypes of ovarian cancer, concentrations of these 22 markers were analyzed in four major histological groups, endometrial, serous, mucinous, and clear cell carcinomas. Ten proteins, IL-6, IP-10, AFP, MMP-2, MPO, tPAI, MIF, LH, TSH, prolactin, and ACTH, showed statistically significant differences between particular histological groups (FIGURE 4B).

Statistical analysis of multimarker panels. None of the single markers had a high enough sensitivity (1 %-55%) at 98% specificity (Table VIII). To identify a multimarker combination with the best performance for discrimination of patients with ovarian cancer from healthy women, the ADEPT algorithm was applied

as follows. Data set consisting of 86 ovarian cancer samples, 79 benign pelvic tumor samples and 104 control samples was randomly split into the training and test sets at 55/45 ratio, then the training set was used to build the classification model and the test set was used to determine the total number of classification errors as well as the specificity/sensitivity. The described procedure was repeated 20 times for each algorithm and the average error rate and the cumulative specificity/sensitivity dependency were calculated. Next, the simulation set consisting of 200 subsets of 50 ovarian and 50 cancer data points was created as described in Methods, and the projection pursuit approach was applied to determine the best combination of projections as well as set of markers comprising these projections. This set was used in a new series of 55/45 cross validation tests that were repeated 100 times for each subset of projections corresponding to the increasing number of markers. The results for sensitivity at 98% specificity, the total number of correctly identified samples as well as variation of these parameters among the 200 subsets are presented in FIGURE 5A, B. As shown in FIGURE 5A, B, the efficiency of classification reaches its near maximum at about 22 biomarkers. FIGURE 5A,B also shows that using the large number of markers not only improves the average classification rate, but substantially (by 30%) reduces the variation of correctly identified samples and dramatically (2-3 times) reduces the variation of sensitivity at high (~98%) values of specificity in compare with using small «8) number of biomarkers. The resulting panel consisted of 22 markers: CA 125, prolactin, sVCAM-1, HE4, MMP-2, TSH, Eotaxin, IGFBPI, sICAM-1, FSH, Cyfra 21-1, MIP-1b, CEA, MCP-1, IL-8, MPO, CA 19-9, E-Selectin, IP-10, EGFR, ACTH, and MIF (listed in order of importance). This panel offered an average sensitivity of 96% at a specificity of 98% in a test set after 10-fold cross-validation as demonstrate by ROC curve presented in FIGURE 6A,D.

To evaluate the performance of the 22-marker panel in an independent validation set, additional samples from 50 ovarian cancer cases and 50 controls were analyzed using xMAP technology for these 22 biomarkers. The data were blinded and subjected to analysis by ADEPT algorithm resulting in a correct classification of 95% of ovarian cancer cases with 90% sensitivity at 98% specificity.

Classification of ovarian cancer vs. benign pelvic disease.

Classification of benign vs. ovarian cancer group was performed using 86 cancer patients and 79 benign patients as described above for discrimination of ovarian cancers from healthy controls. Projection pursuit algorithm identified 20 biomarkers

for optimal discrimination, HGF , CA-125, sI-CAM , HE4, MIP-1a, Cytokeratin 19, CA 19-9, sE-Selectin, IP-10, LH, ng/ml, sFasL, IL-8 , TNF-a , TSH, Kallikrein 10, IGFBP-1, tPAI 1, mesothelin, IL-6 , and SMRP (listed in order of importance). This classification resulted in 86% correctly identified samples (FIGURE 6B,E).

- 5 **Classification of ovarian cancer vs. breast cancer.** To evaluate ovarian cancer-specificity of 22-marker assay that was used for discrimination between ovarian cancer and healthy controls, this panel was used to screen a blinded set consisting of sera from 120 patients with breast cancer and 86 patients with ovarian cancer. This classification resulted in 90% correctly identified breast cancers
10 at 98% correctly identified ovarian cancers (FIGURE 6C,F).

8.3 DISCUSSION

- Using the ADEPT algorithm, we have identified a combination of 22 serum proteins that offered high sensitivity of 96% in the test set and 90% in the
15 independent blinded validation set at 98% specificity. The difference in the results between the test and independent validation sets is likely due to different representation of ovarian cancer histotypes in the two subpopulations.. Independent validation set has higher representation of adenocarcinoma NOS histology than training/test sets, and lower representation of endometrioid, clear cell, serous, and
20 mucinous histotypes. The fact that the multimarker assay offers 90% sensitivity at 98% specificity in this set indicates general utility of the assay for most histotypes of ovarian cancer. Furthermore, even at 90%, the 22-marker panel offered substantially better sensitivity than that reported so far (70-72%) using limited number of markers
in comparable studies (17-18).

- 25 The most powerful biomarker panel consisted of proteins representing different functional groups, cancer antigens (CA 125, CEA, and CA 19-9), hormones (prolactin, FSH, TSH, and ACTH), metastasis-related proteins (sVCAM-I, sICAM-I, sE-Selectin, and MMP-2), cytokines (Eotaxin, MIP-1b, MCP-1, IL-8, MIF, IP-10, and MPO), growth factor-related proteins (IGFBPI and EGFR), apoptotic cleavage
30 product (Cyfra 21-1) and HE4. The functional diversity of this multimarker panel may contribute to its power in discrimination of ovarian cancer.

TABLE VII
CHARACTERISTICS OF PATIENT POPULATION

Patient Group	Age	Histology
Healthy N=154	Range 45-84 Median 57.7 Average 60	
Ovarian Cancer N=136	Range 47-87 Median 59 Average 61.6	Adenocarcinoma, endometrioid (34/6)* Adenocarcinoma, mucinous (n=33/5) Adenocarcinoma, serous (n=31/4) Carcinoma, clear cell (n=23/8) Carcinoma, in adenofibroma (n=4/0) Adenocarcinoma NOS (not otherwise specified) (0/16) Stromal tumor (0/5) Granulosa tumor (0/6)
Benign N=79	Range 43-87 Median 63 Average 63.8	Adenofibroma (n=10) Adenoma, serous (n=1) Brenner tumor (n=2) Cyst, serous (n=1) Cyst, benign (n=1) Cystadenofibroma (n=19) Cystadenoma (n=39) Endometriosis (n=1) Fibroma (n=2) Ovary benign (n=4)

*first number relates to the training/test set (86 cancers/104 healthy), second to the independent validation set (50 cancers/50 healthy)

TABLE VIII

Expression of biomarkers in three clinical groups: healthy control patients (Control; C), ovarian cancer patients (Ovarian cancer; OC) and patients with benign tumors (Benign tumor; BT). SN – sensitivity, SP – specificity.. Zaika, Add MPO, tPAI, other markers that show differences between cases and controls. Sequence of biomarkers – as in abstract.

Biomarkers	Biomarker concentrations			Units	p-value		SN at 98% SP	Correctly identified
	Control	Ovarian cancer	Benign		OC vs C	OC vs B		
IL-8	5.7±0.40	18.7±9.79	9.2±1.49	pg/ml	0.006	ns	3	58
IL-6	10.7±1.51	36.2±5.76	39.1±7.54	pg/ml	<0.001	ns	4	68
Fas	1.4±0.09	1.3±0.13	1.1±0.05	ng/ml	0.009	ns	1.5	51
Eotaxin	124.0±5.93	84.6±7.07	85.7±6.12	pg/ml	<0.001	ns	8	68
MCP-1	322.1±12.86	300.5±35.40	295.5±18.38	pg/ml	0.003	ns	2	56
MIP-1b	88.3±28.46	347.5±197.49	153.5±37.89	pg/ml	0.007	ns	3	60
IL-2R	97.3±10.74	206.0±39.77	143.8±22.34	pg/ml	0.001	ns	1	60
TNFR-1	1.7±0.14	1.7±0.23	1.6±0.11	ng/ml	0.014	ns	7	51
CA 19-9	0.1±0.01	11.8±8.77	19.1±13.25	U/ml	<0.001	<0.001	16	70
ErbB2	9.8±0.26	7.9±0.44	8.0±0.33	ng/ml	<0.001	ns	15	70
EGFR	4.8±0.08	3.7±0.11	4.0±0.10	ng/ml	<0.001	ns	20	80
CA 125	0.7±0.07	8.2±1.59	3.8±1.32	U/ml	<0.001	<0.001	55	81
Cyfra 21-1	0.5±0.53	4.2±0.80	1.4±0.48	ng/ml	<0.001	<0.001	7	77
IGFBPI	3.2±0.47	12.5±1.69	8.8±1.17	ng/ml	<0.001	ns	10	72
Mesothelin	7.6±0.65	4.7±0.35	6.1±0.56	ng/ml	<0.001	ns	3	60
SMR	58.2±4.51	231.7±116.33	78.4±4.92	ng/ml	<0.001	ns	8	59
CA 72-4	0.00±0.00	10.0±6.53	5.1±4.52	U/ml	<0.001	0.008	30	56

TABLE VIII (continued)

MMP-2	159.7±2.73	125.6±3.82	140.9±4.57	ng/ml	<0.001	ns	22	73
MMP-3	9.8±0.68	6.6±0.43	7.7±0.75	ng/ml	<0.001	ns	7	64
E-Selectin	25.2±1.66	18.8±1.03	21.4±1.66	pg/ml	0.002	ns	2	58
sV-CAM	746.8±19.51	479.5±17.26	536.9±21.06	pg/ml	<0.001	ns	20	80
sI-CAM	140.2±6.10	100.2±4.50	124.2±7.75	pg/ml	<0.001	0.005	35	75
MIF	121.7±15.52	452.1±61.64	447.0±53.94	pg/ml	<0.001	ns	12	65
HE4	332.3±147.88	1856.6±401.25	550.0±154.97	pg/ml	<0.001	<0.001	15	80
IP-10	16.7±1.81	13.2±1.58	18.0±2.37	pg/ml	0.012	0.016	3.5	55
AFP	2.4±0.41	1.7±0.20	1.5±0.19	pg/ml	<0.001	ns	3	60
Prolactin	32.8±10.86	350.5±67.44	253.8±27.28	ng/ml	<0.001	ns	17	85
FSH	42.2±2.45	25.3±2.18	26.2±1.93	ng/ml	<0.001	ns	12	69
LH	24.9±1.19	18.1±1.55	19.4±1.68	ng/ml	<0.001	ns	10	65
TSH	1.8±0.23	3.8±0.40	3.3±0.34	ng/ml	<0.001	ns	10	70
GH	862.4±473.32	1578.3±316.88	1809.6±384.41	pg/ml	<0.001	ns	7	75
ACTH	11.4±1.79	42.4±10.15	28.1±5.69	pg/ml	0.003	ns	12	58

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Various references are cited herein, the contents of which are hereby incorporated by reference in their entireties.

WE CLAIM:

1. A method of diagnosing ovarian cancer in a human subject, comprising (i) obtaining a serum sample from a subject being evaluated for possible ovarian cancer, and (ii) identifying, in the serum sample, a change in the serum levels of at least two of the following molecules relative to a healthy control subject:
 - PRL (prolactin);
 - IGFBP1 (insulin-like growth factor binding protein 1);
 - GH (growth hormone);
 - HE4 (human epididymis protein 4);
 - MIF (macrophage migration inhibitory factor);
 - IL-6 (interleukin-6);
 - IL-8 (interleukin-8);
 - IL-2R (interleukin-2 receptor)
 - SMRP (soluble mesothelin-related peptides);
 - MCP-1 (monocyte chemoattractant protein-1);
 - MIP-1 α (macrophage inflammatory protein-1 α);
 - MIP-1 β (macrophage inflammatory protein-1 β);
 - CA-19-9 (carbohydrate antigen 19-9);
 - CA-125 (cancer antigen 125);
 - Cyfra 21-1 (soluble fragment of cytokeratin-19);
 - CA 72-4 (cancer antigen 72-4);
 - leptin;
 - tPAI-1 (tissue plasminogen activator inhibitor-1);
 - TSH (thyroid stimulating hormone);
 - ACTH (adrenocorticotrophic hormone);
 - CEA (carcinoembryonic antigen);
 - eotaxin;
 - MMP-2 (matrix metalloproteinase-2);
 - MMP-3 (matrix metalloproteinase-3);
 - sV-CAM (vascular cell adhesion molecule);
 - FSH (follicle stimulating hormone);
 - EGFR (epidermal growth factor receptor);
 - ErbB2 (erythroblastic leukemia viral oncogene homolog 2);
 - adiponectin;

- sI-CAM (soluble intercellular adhesion molecule);
- sE-selectin;
- MPO (myeloperoxidase);
- IP-10 (interferon gamma induced protein 10);
- 5 LH (leutinizing hormone); and/or
- full length mesothelin;

wherein at least one of said changes are changes in levels of molecules selected from the group consisting of TSH, GH, ACTH, eotaxin, MMP-2, MMP-3, ErbB2, adiponectin, sI-CAM, sV-CAM, sE-selectin, and full-length mesothelin;

- 10 wherein the presence of such changes indicates the presence of ovarian cancer in the subject.

- 2. A method of diagnosing ovarian cancer in a human subject, comprising (i) obtaining a serum sample from a subject being evaluated for possible ovarian cancer, and (ii) identifying, in the serum sample, at least two of the following
- 15 changes in serum levels relative to a healthy control subject:

- an increase in PRL (prolactin);
- an increase in IGFBP1 (insulin-like growth factor binding protein 1);
- an increase in GH (growth hormone);
- an increase in HE4 (human epididymis protein 4);
- 20 an increase in MIF (macrophage migration inhibitory factor);
- an increase in IL-6 (interleukin-6);
- an increase in IL-8 (interleukin-8);
- an increase in IL-2R (interleukin-2 receptor)
- an increase in SMRP (soluble mesothelin-related peptides);
- 25 an increase in MCP-1 (monocyte chemoattractant protein-1);
- an increase in MIP-1 α (macrophage inflammatory protein-1 α);
- an increase in MIP-1 β (macrophage inflammatory protein-1 β);
- an increase in CA-19-9 (carbohydrate antigen 19-9);
- an increase in CA-125 (cancer antigen 125);
- 30 an increase in Cyfra 21-1 (soluble fragment of cytokeratin-19);
- an increase in CA 72-4 (cancer antigen 72-4);
- an increase in leptin;
- an increase in tPAI-1 (tissue plasminogen activator inhibitor-1);
- an increase in TSH (thyroid stimulating hormone);

- an increase in ACTH (adrenocorticotrophic hormone);
- an increase in CEA (carcinoembryonic antigen);
- a decrease in eotaxin;
- a decrease in MMP-2 (matrix metalloproteinase-2);
- 5 a decrease in MMP-3 (matrix metalloproteinase-3);
- a decrease in sV-CAM (vascular cell adhesion molecule);
- a decrease in FSH (follicle stimulating hormone);
- a decrease in EGFR (epidermal growth factor receptor);
- a decrease in ErbB2 (erythroblastic leukemia viral oncogene homolog 2);
- 10 a decrease in adiponectin;
- a decrease in sI-CAM (soluble intercellular adhesion molecule);
- a decrease in sE-selectin;
- a decrease in MPO (myeloperoxidase);
- a decrease in IP-10 (interferon gamma induced protein 10);
- 15 a decrease in LH (leutinizing hormone); and/or
- a decrease in full length mesothelin;

wherein at least one of said changes are changes in levels of molecules selected from the group consisting of TSH, GH, ACTH, eotaxin, MMP-2, MMP-3, ErbB2, adiponectin, sI-CAM, sV-CAM, sE-selectin, and full-length mesothelin;

- 20 wherein the presence of such changes indicates the presence of ovarian cancer in the subject.

3. The method of claim 1 which identifies at least a change in the serum concentration, relative to a healthy subject, of PRL, CEA, HE4, and CA 125 wherein the presence of such changes indicates the presence of ovarian cancer in the

25 subject.

4. The method of claim 1 which identifies at least an increase in the serum concentration, relative to a healthy subject, of PRL, CEA, HE4, and CA 125 wherein the presence of such increases indicates the presence of ovarian cancer in the subject.

30 5. The method of claim 1 which identifies at least a change in the serum concentrations, relative to a healthy subject, of CA 125; HE4; CA72-4; FSH; eotaxin; IP-10, wherein the presence of such changes indicates the presence of ovarian cancer in the subject.

6. The method of claim 2 which identifies at least an increase in the serum concentrations, relative to a healthy subject, of CA 125, HE4, and CA72-4, and a decrease in the serum concentrations, relative to a healthy subject, of FSH; eotaxin; and IP-10, wherein the presence of such increases and decreases indicates the presence of ovarian cancer in the subject.

7. The method of claim 1 which identifies at least a change in the serum concentrations, relative to a healthy subject, of CA 125, EGFR, HE4, sVCAM, wherein the presence of such changes indicates the presence of ovarian cancer in the subject.

8. The method of claim 2 which identifies at least an increase in the serum concentrations, relative to a healthy subject, of CA 125 and HE4, and a decrease in the serum concentrations, relative to a healthy subject, of EGFR and sVCAM, wherein the presence of such increases and decreases indicates the presence of ovarian cancer in the subject.

9. The method of claim 1 which identifies at least a change in the serum concentrations, relative to a healthy subject, of CA125, prolactin, sV-CAM1, HE4, MMP-2, TSH, eotaxin, IGFBPI, sI-CAM-1, FSH, Cyfra-21-1, MIP-1 β , CEA, MCP-1, IL-8, MPO, CA 19-9, E-selectin, IP-IO, EGFR, ACTH, and MIF, wherein the presence of such changes indicates the presence of ovarian cancer in the subject.

10. A method of diagnosing ovarian cancer in a human subject, comprising (i) obtaining a serum sample from a subject being evaluated for possible ovarian cancer, and (ii) identifying, in the serum sample, a change in the serum levels, relative to a healthy subject, of a panel of markers comprising of PRL, CEA, HE4, and CA 125, wherein the presence of such changes indicates the presence of ovarian cancer in the subject.

11. A method of diagnosing ovarian cancer in a human subject, comprising (i) obtaining a serum sample from a subject being evaluated for possible ovarian cancer, and (ii) identifying, in the serum sample, a change in the serum levels, relative to a healthy subject, of a panel of markers comprising of CA 125; HE4; CA72-4; FSH; eotaxin; IP-10, wherein the presence of such changes indicates the presence of ovarian cancer in the subject.

12. A method of diagnosing ovarian cancer in a human subject, comprising (i) obtaining a serum sample from a subject being evaluated for possible ovarian cancer, and (ii) identifying, in the serum sample, a change in the serum levels,

relative to a healthy subject, of a panel of markers comprising CA 125, EGFR, HE4, sVCAM,, wherein the presence of such changes indicates the presence of ovarian cancer in the subject.

13. A method of diagnosing ovarian cancer in a human subject,
 5 comprising (i) obtaining a serum sample from a subject being evaluated for possible ovarian cancer, and (ii) identifying, in the serum sample, a change in the serum levels, relative to a healthy subject, of a panel of markers comprising CA125, prolactin, sV-CAM1, HE4, MMP-2, TSH, eotaxin, IGFBPI, sI-CAM-1, FSH, Cyfra-21-1, MIP-1 β , CEA, MCP-1, IL-8, MPO, CA 19-9, E-selectin, IP-IO, EGFR, ACTH, and MIF,
 10 wherein the presence of such changes indicates the presence of ovarian cancer in the subject.

14. The method of any of claims 1 -13, wherein the subject has exhibited one or more symptom consistent with ovarian cancer selected from the group consisting of abdominal bloating, pelvic pain, abdominal pain, loss of appetite,
 15 early satiety after eating, urgent need to urinate, and frequent urges to urinate.

15 The method of any of claims 1-14, further comprising recommending that the subject undergo a further test selected from the group consisting of a laparoscopy, a laparotomy, a pelvic ultrasound, a tissue biopsy, and a combination thereof.

20 16. The method of any of claims 1-15, further comprising performing a further test selected from the group consisting of a laparotomy, a laparoscopy, a pelvic ultrasound, a tissue biopsy, and a combination thereof.

17. The method of any one of claims 1-16, wherein the changes in serum levels identified in step (ii) are among a plurality of serum levels of molecules
 25 being tested in the sample, where at least 50 percent of the molecules being tested are selected from the following list of ovarian cancer -associated molecules: PRL (prolactin); IGFBBP1 (insulin-like growth factor binding protein 1); GH (growth hormone); HE4 (human epididymis protein 4); MIF (macrophage migration inhibitory factor); IL-6 (interleukin-6); IL-8 (interleukin-8); IL-2R (interleukin-2 receptor);
 30 SMRP (soluble mesothelin-related peptides); MCP-1 (monocyte chemoattractant protein-1); MIP-1 α (macrophage inflammaotry protein-1 α); MIP-1 β (macrophage inflammaotry protein-1 β); CA-19-9 (carbohydrate antigen 19-9); CA-125 (cancer antigen 125); Cyfra 21-1 (soluble fragment of cytokeratin-19); CA 72-4 (cancer antigen 72-4); leptin; tPAI-1 (tissue plasminogen activator inhibitor-1); TSH (thyroid

stimulating hormone); ACTH (adrenocorticotrophic hormone); CEA (carcinoembryonic antigen); eotaxin; MMP-2 (matrix metalloproteinase-2); MMP-3 (matrix metalloproteinase-3); sV-CAM (vascular cell adhesion molecule); FSH (follicle stimulating hormone); EGFR (epidermal growth factor receptor); ErbB2 (erythroblastic leukemia viral oncogene homolog 2); adiponectin; sI-CAM (soluble intercellular adhesion molecule); sE-selectin; MPO (myeloperoxidase); IP-10 (interferon gamma induced protein 10); LH (leutinizing hormone); and full length mesothelin.

18. A kit for diagnosing ovarian cancer, said kit comprising a means for determining the serum level of a plurality of molecules, where at least 50 percent of the molecules being tested are selected from the following list of ovarian cancer -associated molecules: PRL (prolactin); IGFBP1 (insulin-like growth factor binding protein 1); GH (growth hormone); HE4 (human epididymis protein 4); MIF (macrophage migration inhibitory factor); IL-6 (interleukin-6); IL-8 (interleukin-8); IL-2R (interleukin-2 receptor); SMRP (soluble mesothelin-related peptides); MCP-1 (monocyte chemoattractant protein-1); MIP-1 α (macrophage inflammatory protein-1 α); MIP-1 β (macrophage inflammatory protein-1 β); CA-19-9 (carbohydrate antigen 19-9); CA-125 (cancer antigen 125); Cyfra 21-1 (soluble fragment of cytokeratin-19); CA 72-4 (cancer antigen 72-4); leptin; tPAI-1 (tissue plasminogen activator inhibitor-1); TSH (thyroid stimulating hormone); ACTH (adrenocorticotrophic hormone); CEA (carcinoembryonic antigen); eotaxin; MMP-2 (matrix metalloproteinase-2); MMP-3 (matrix metalloproteinase-3); sV-CAM (vascular cell adhesion molecule); FSH (follicle stimulating hormone); EGFR (epidermal growth factor receptor); ErbB2 (erythroblastic leukemia viral oncogene homolog 2); adiponectin; sI-CAM (soluble intercellular adhesion molecule); sE-selectin; MPO (myeloperoxidase); IP-10 (interferon gamma induced protein 10); LH (leutinizing hormone); and full length mesothelin.

19. The kit of claim 18, wherein said means comprises, for each molecule to be tested, a capture agent selected from the group consisting of an antibody, a portion of an antibody, a single chain antibody, a non-immunoglobulin receptor for the molecule, a peptide ligand for the molecule, and an oligonucleotide ligand for the molecule.

20. The kit of claim 19, wherein said capture agent is bound to a solid support.

21. The method of any of claims 1-17, wherein identification of changes in the following markers indicates an increased likelihood of the diagnosis of stage III or stage IV ovarian cancer:

- CA 125, HE4, EGFR and PRL;
- 5 CA125, HE4, PRL and GH;
- CA 125, HE4, MMP-3 and TSH; or
- CA 125, HE4, eotaxin, and PRL.

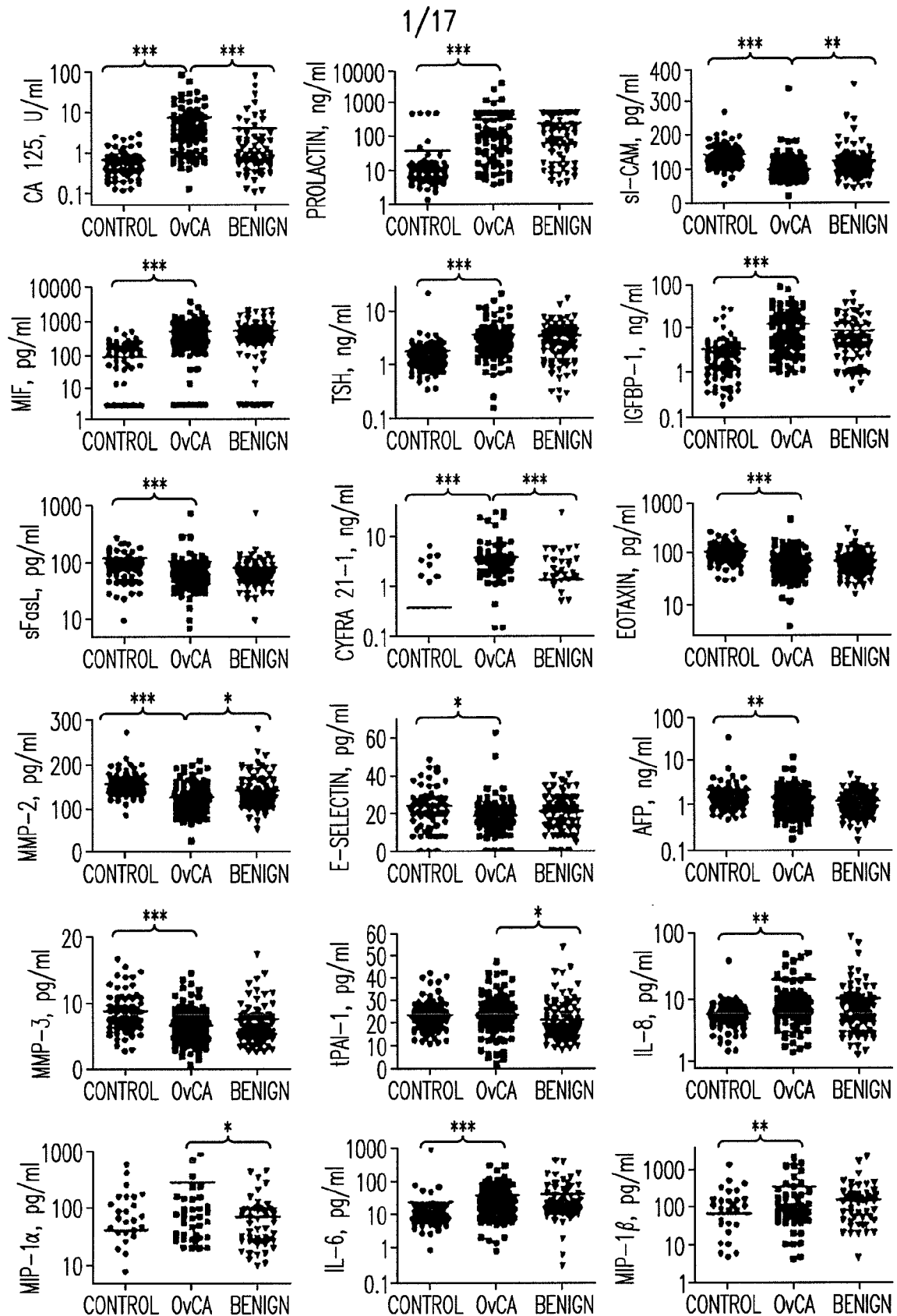


FIG.1A

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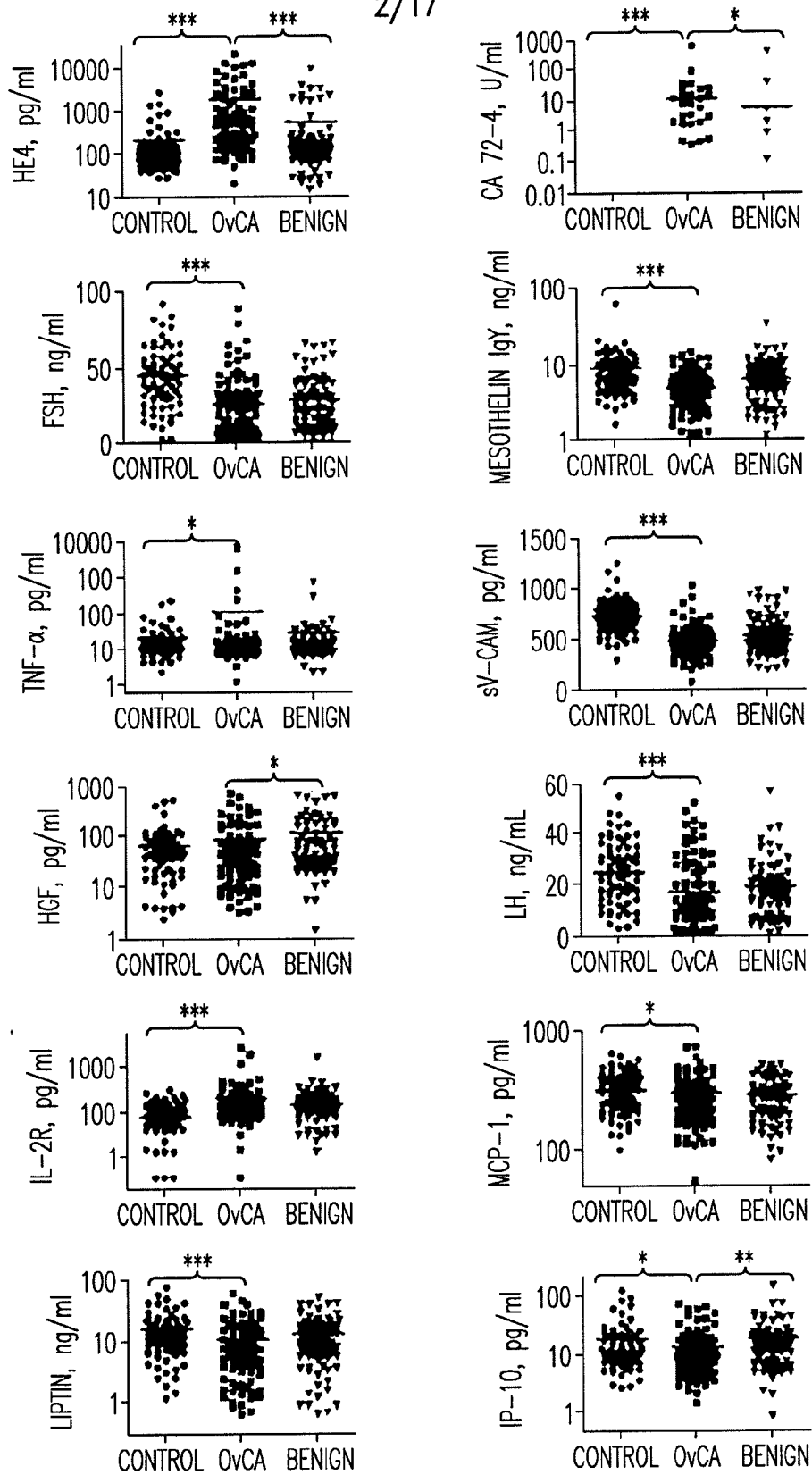


FIG.1A-1

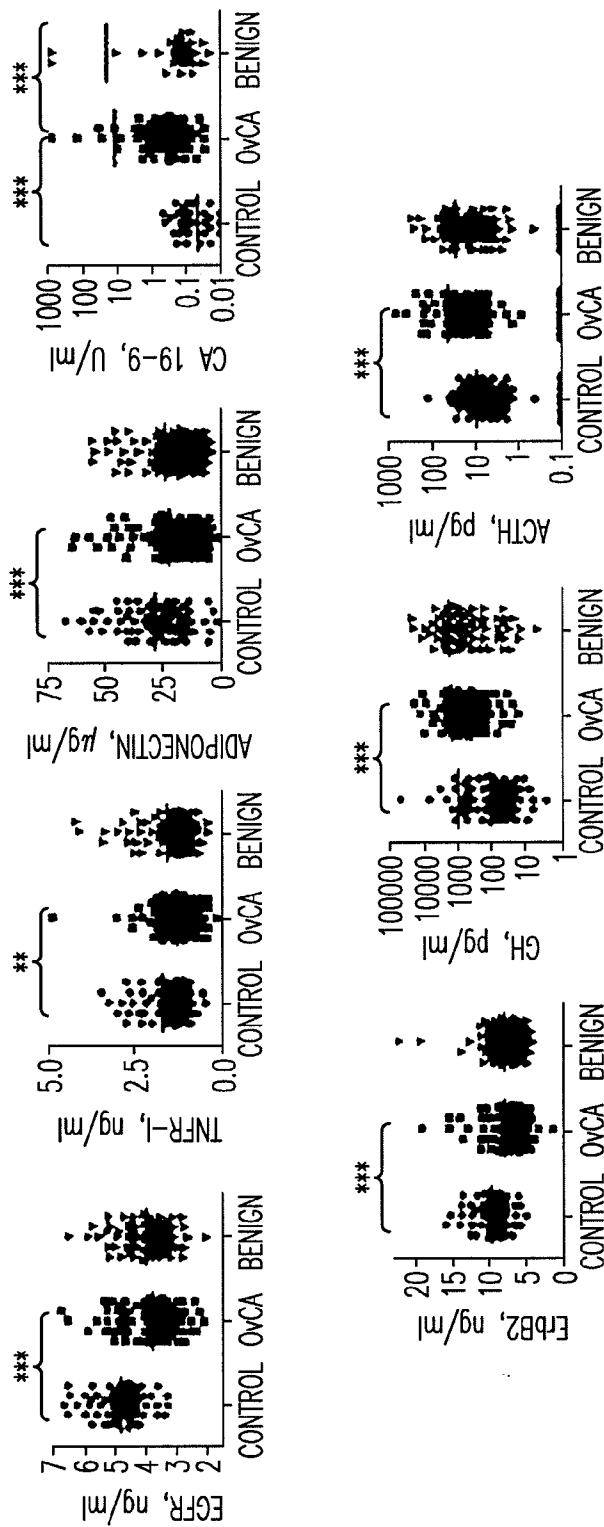


FIG. 1A-2

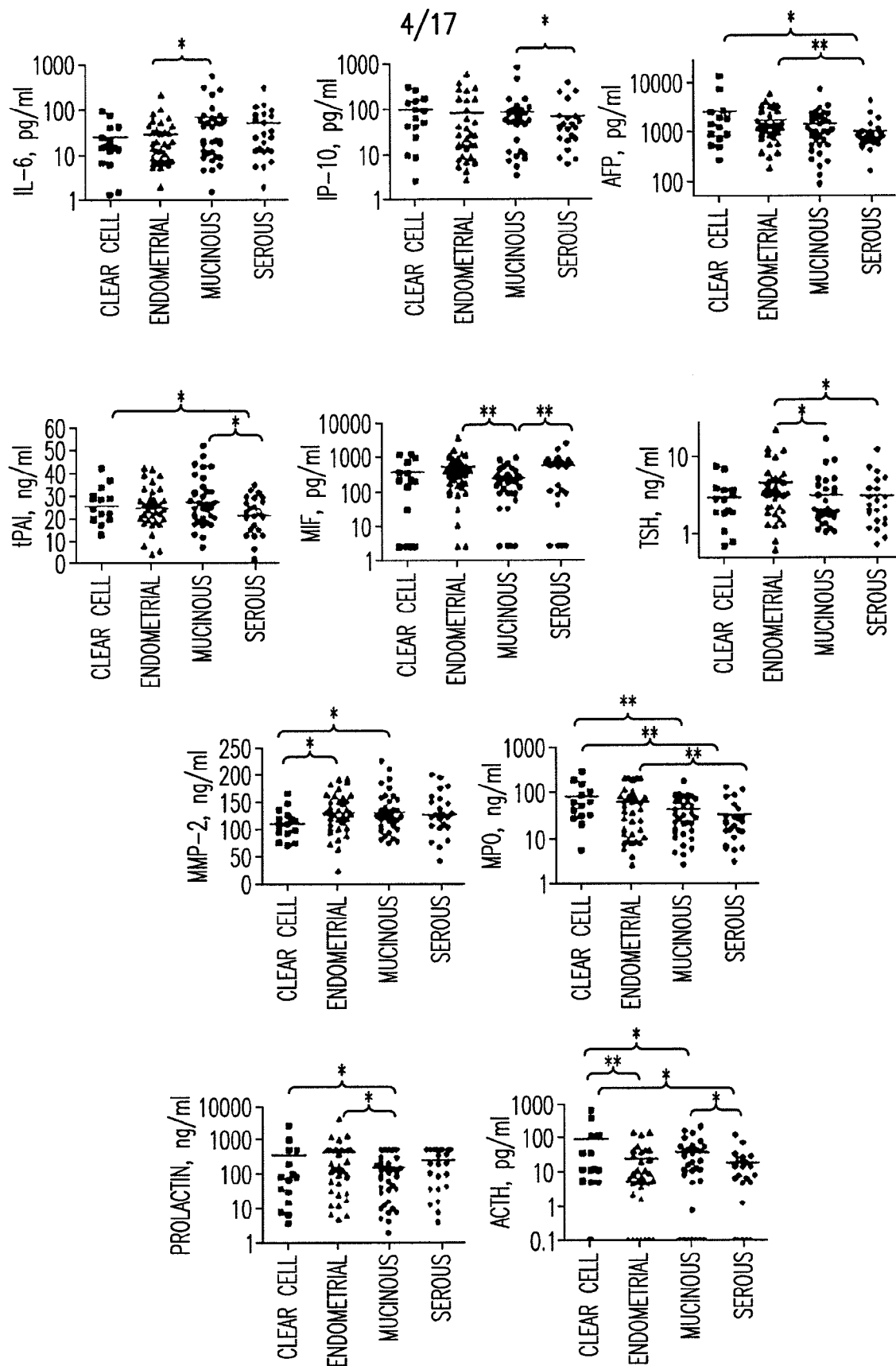


FIG. 1B

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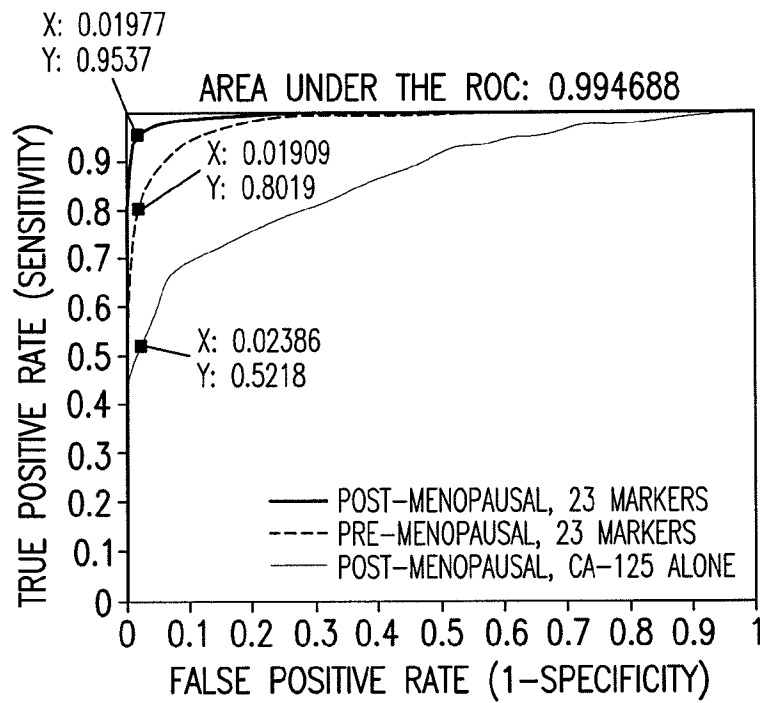


FIG. 2A

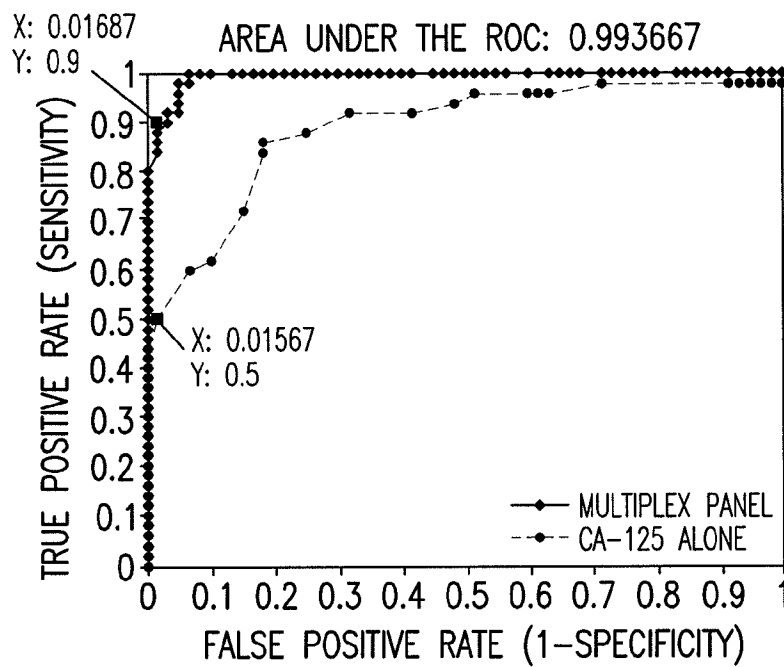


FIG. 2B

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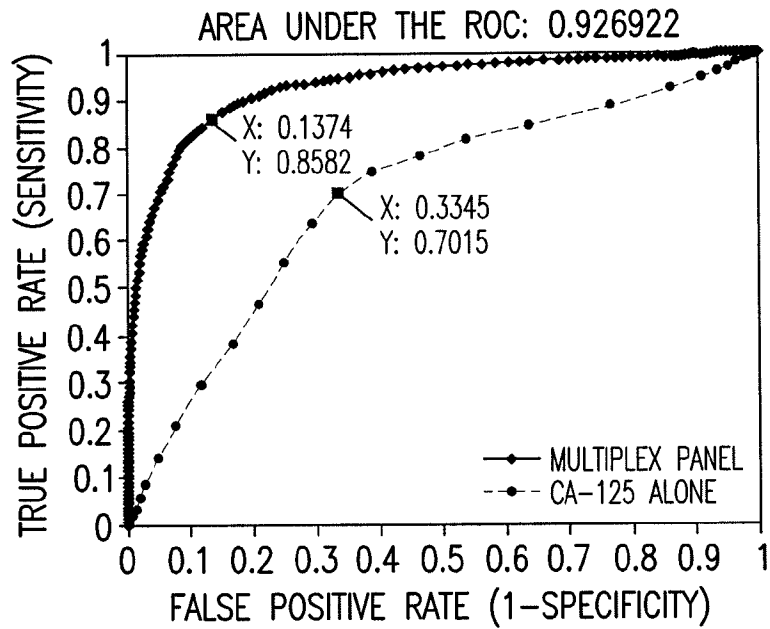


FIG. 2C

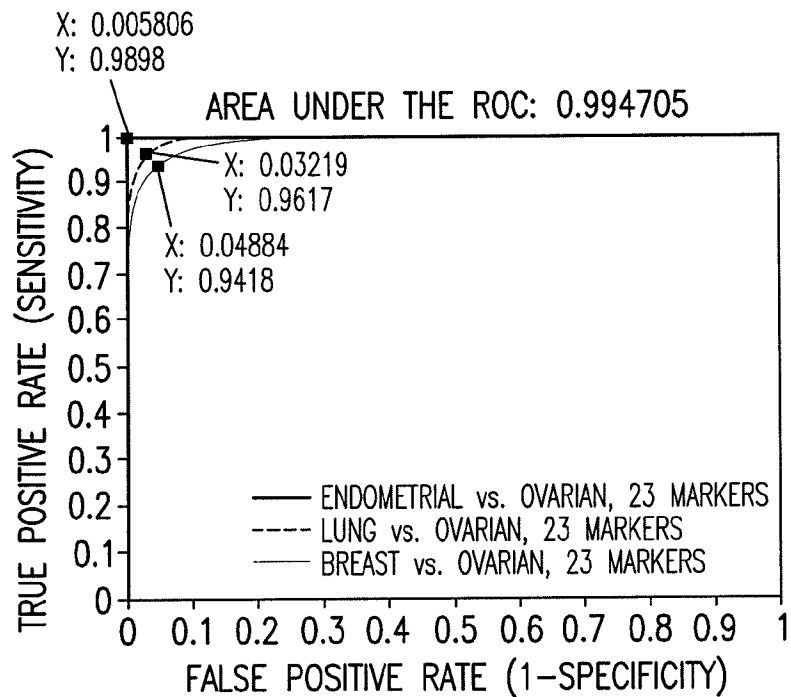


FIG. 2D

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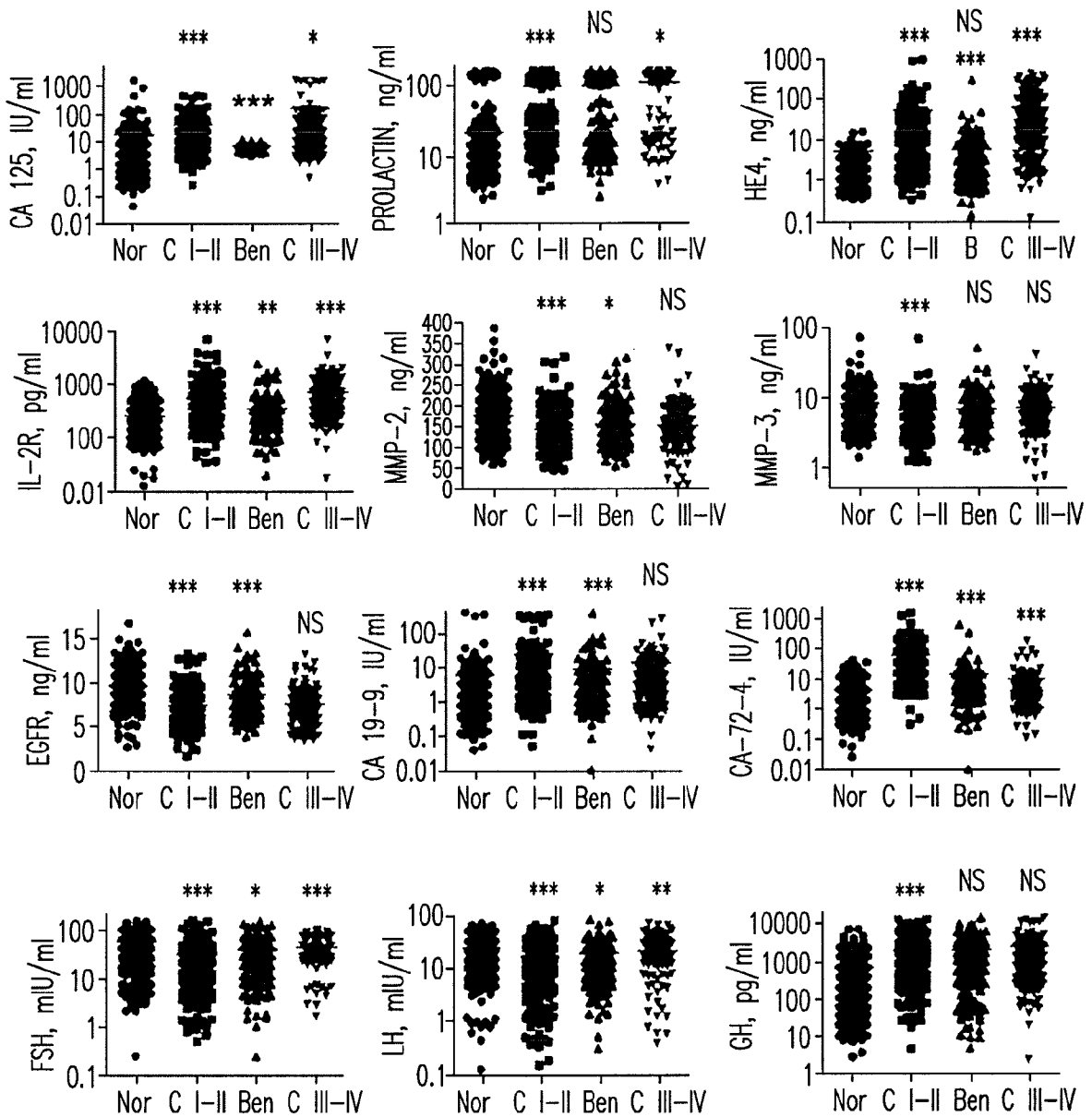


FIG.3A

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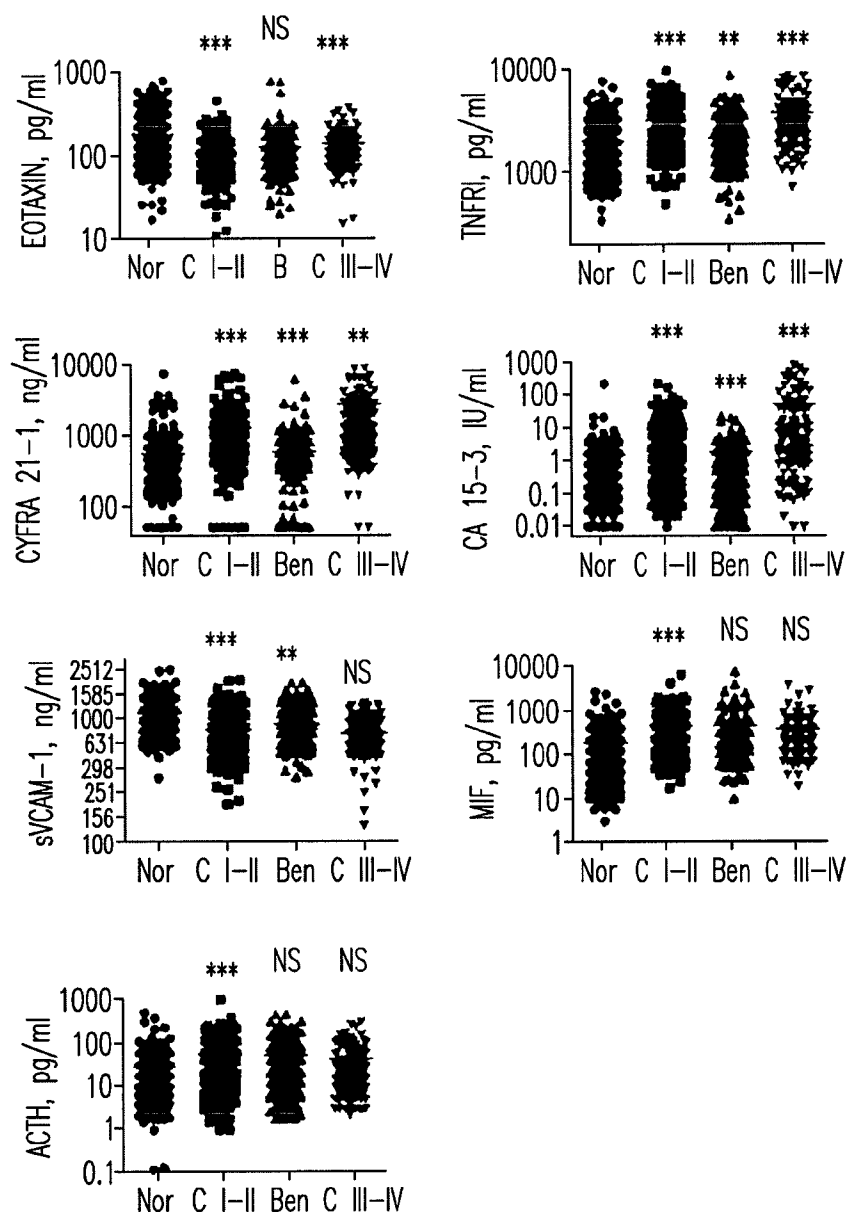


FIG.3A-1

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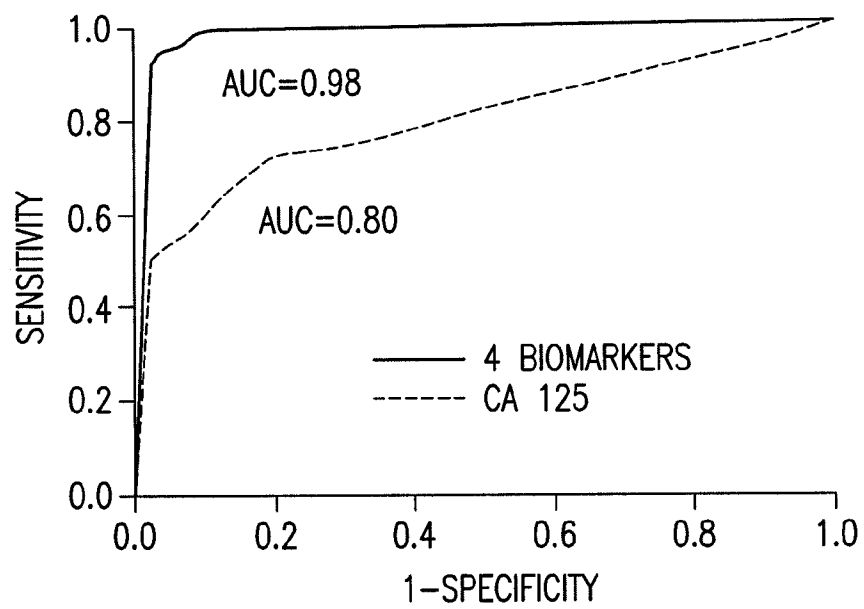


FIG.3B

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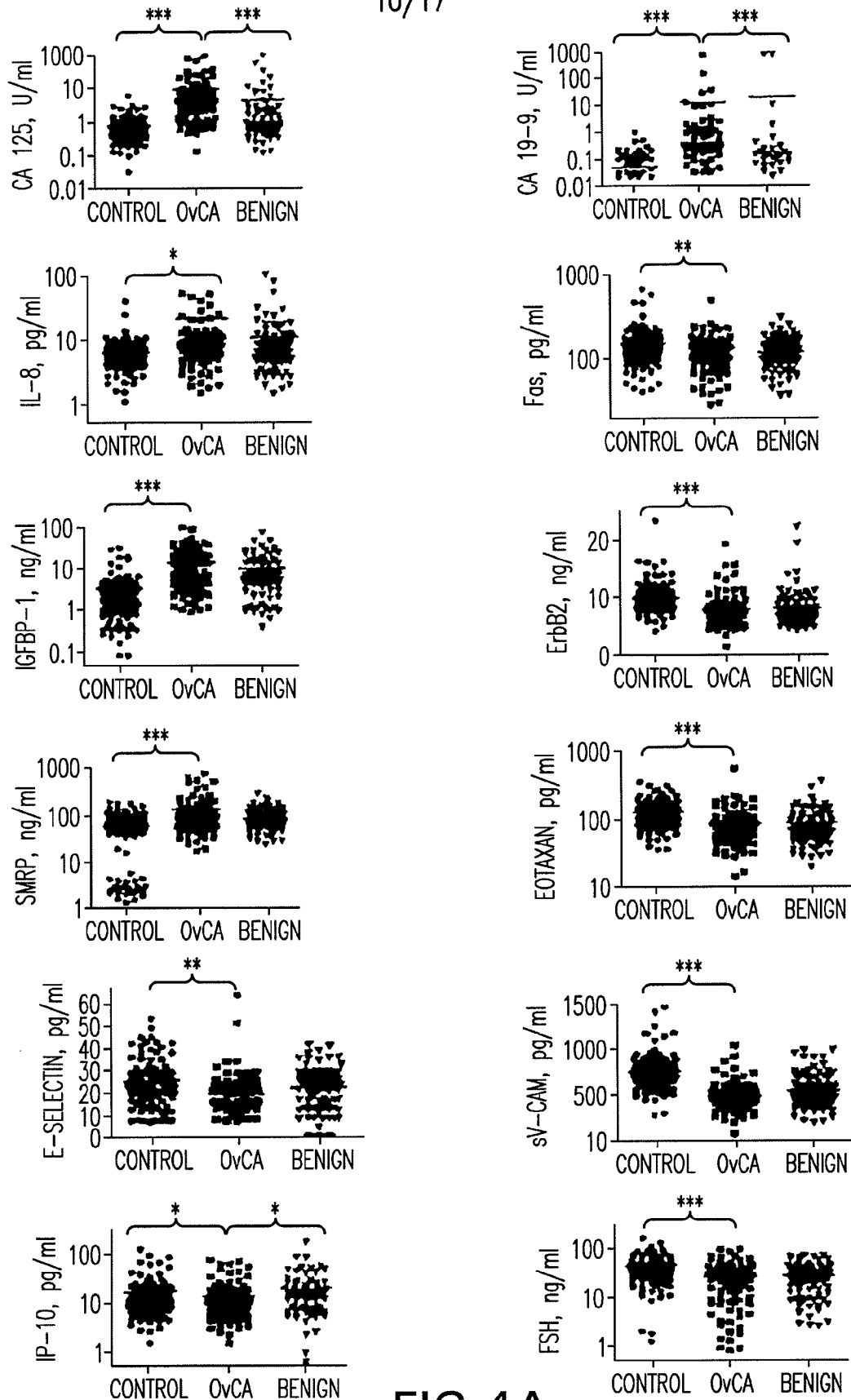


FIG.4A

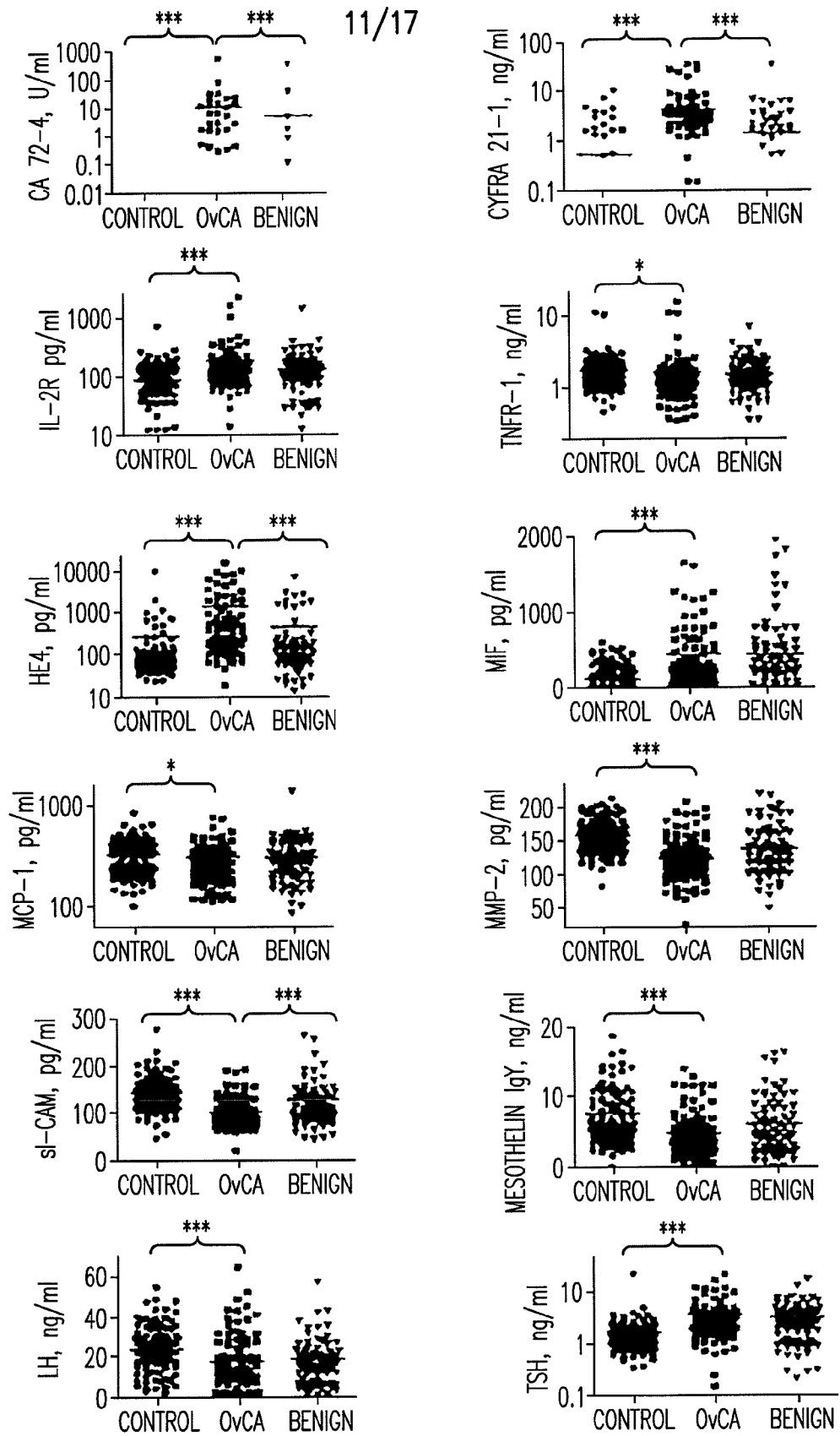


FIG.4A-1

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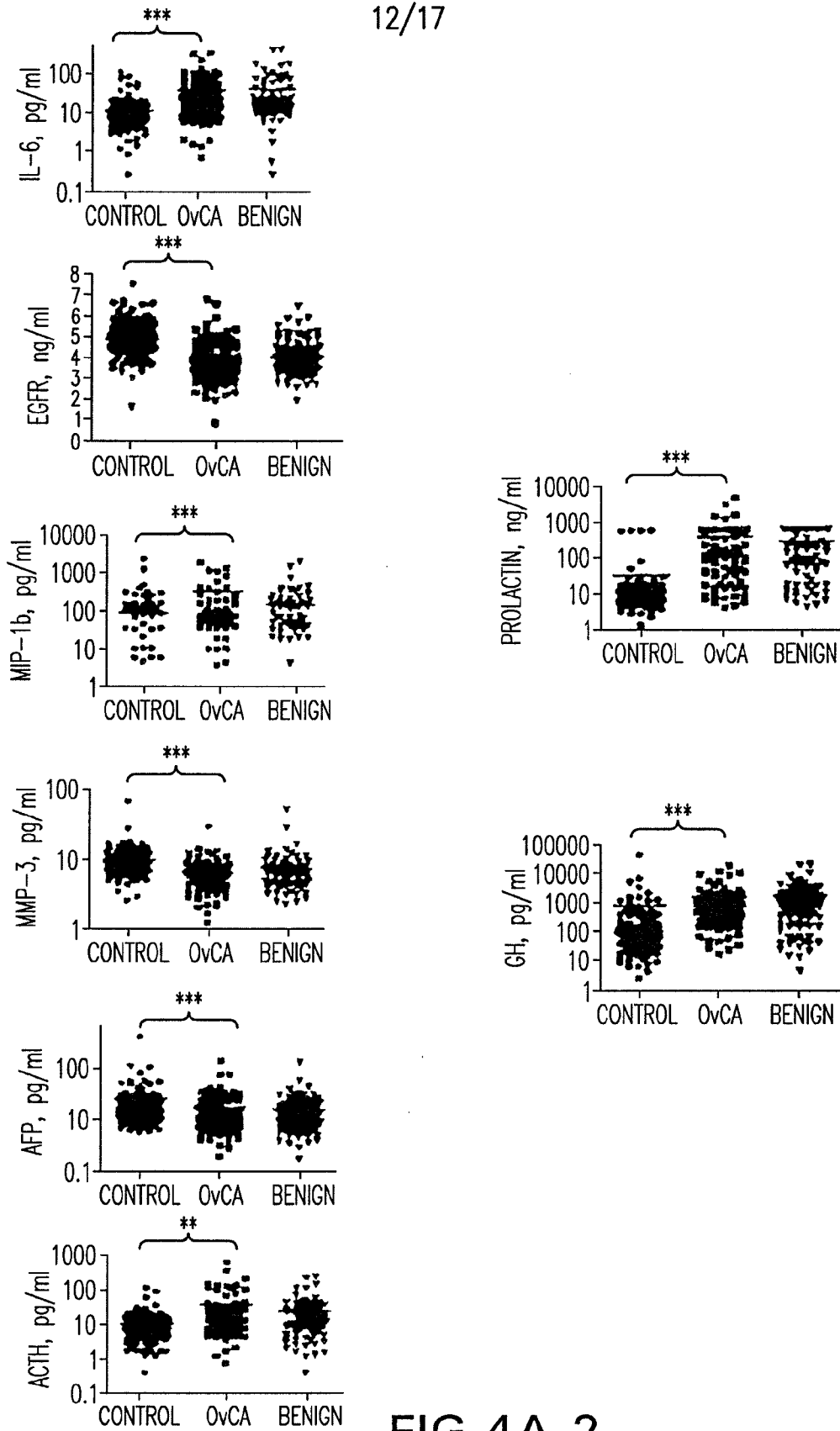


FIG.4A-2

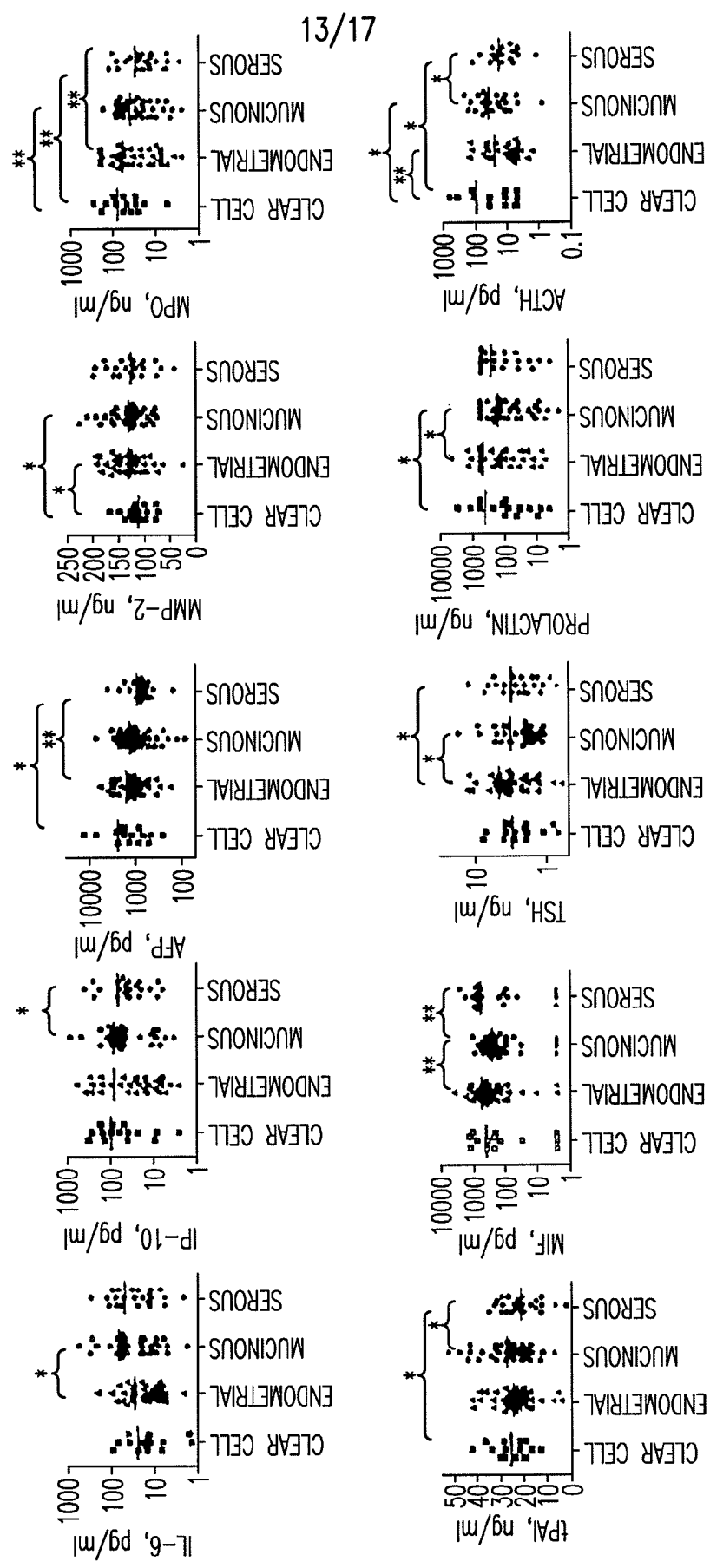
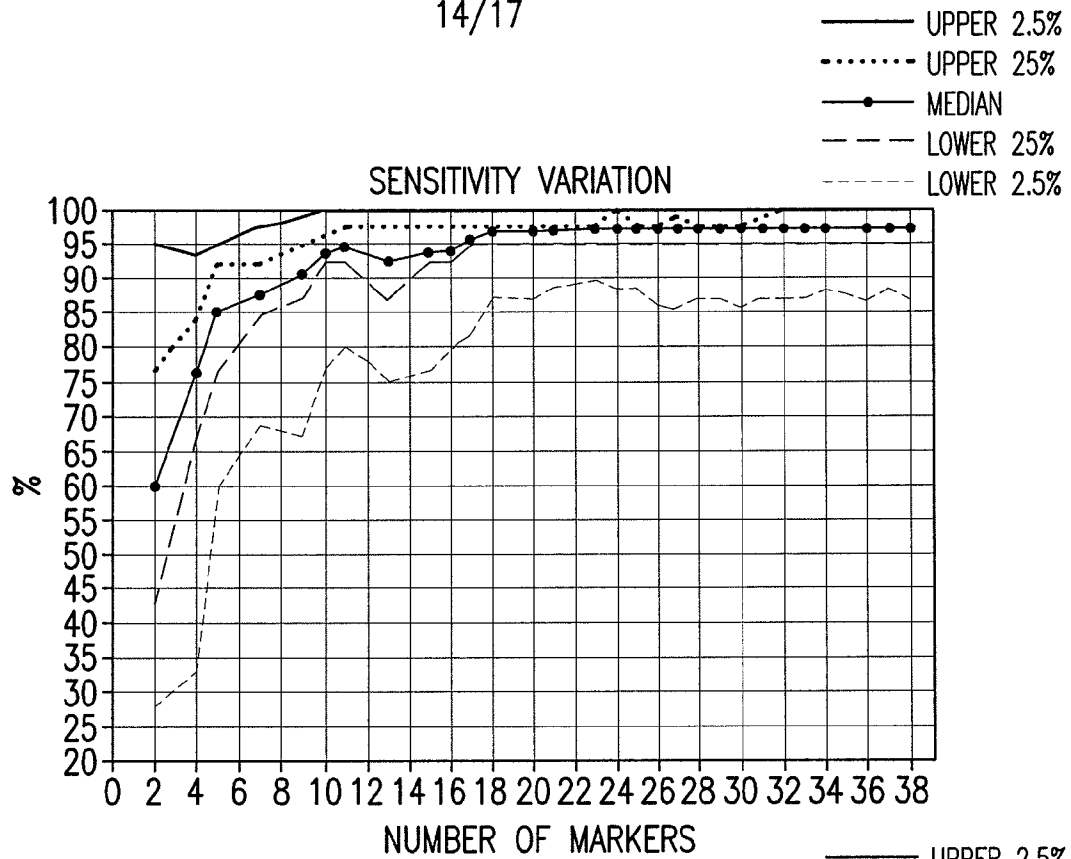
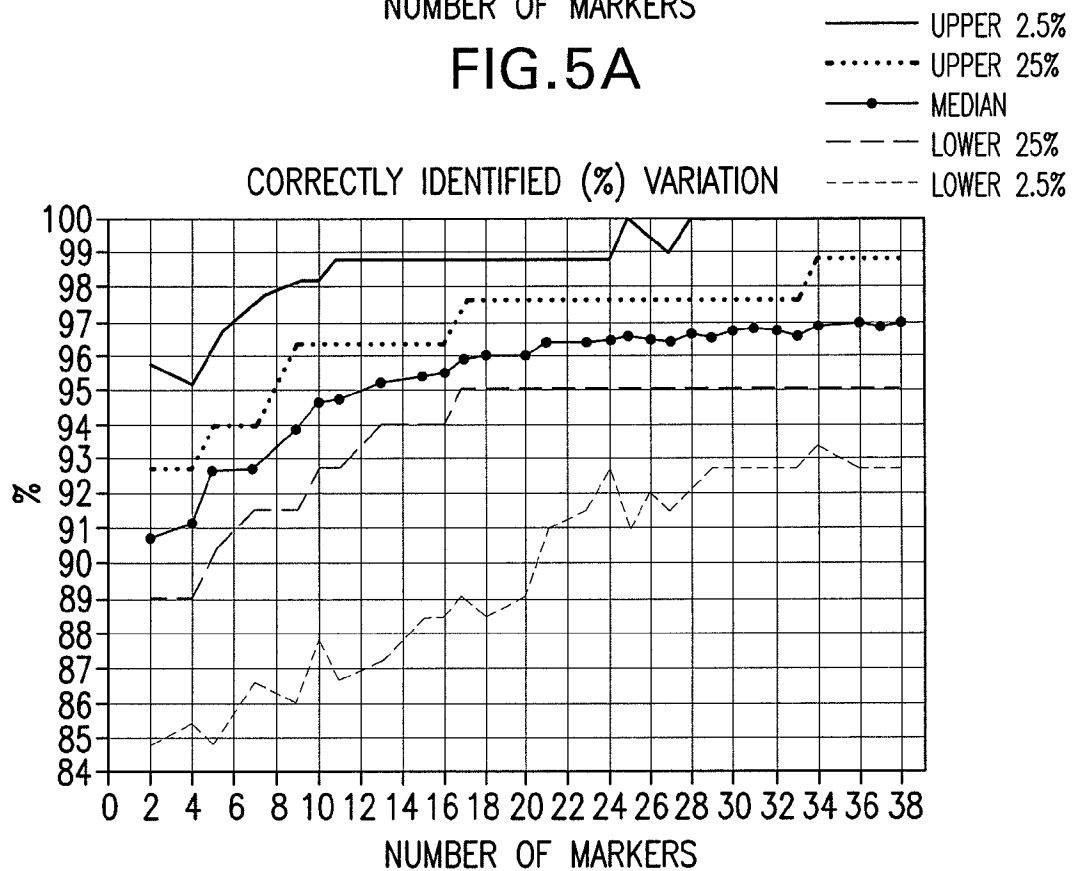


FIG. 4B

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**FIG. 5A****FIG. 5B**

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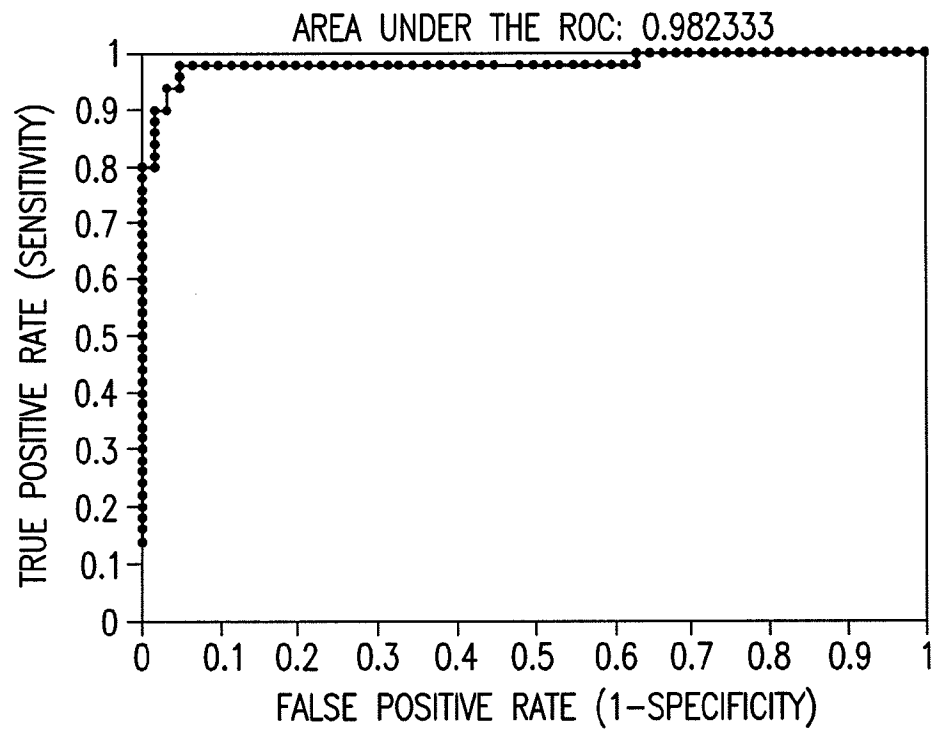


FIG. 6A

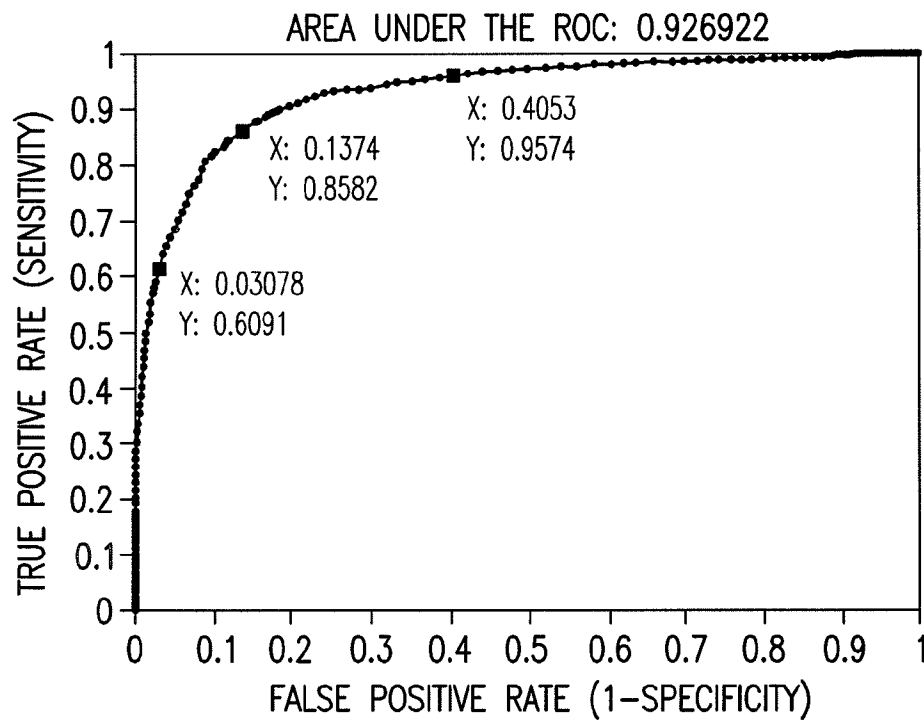


FIG. 6B

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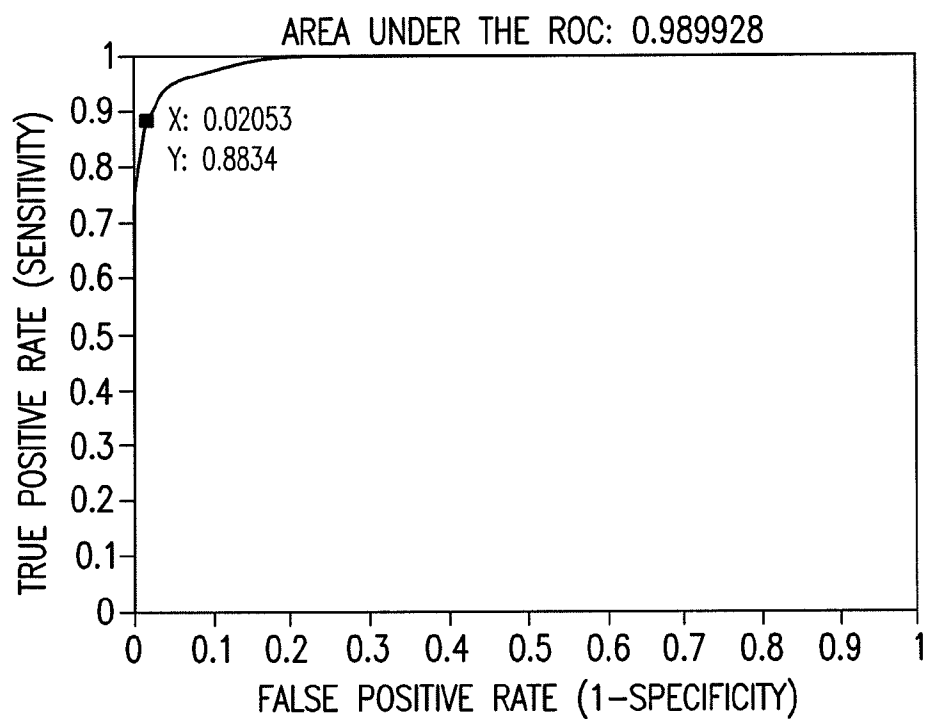


FIG.6C

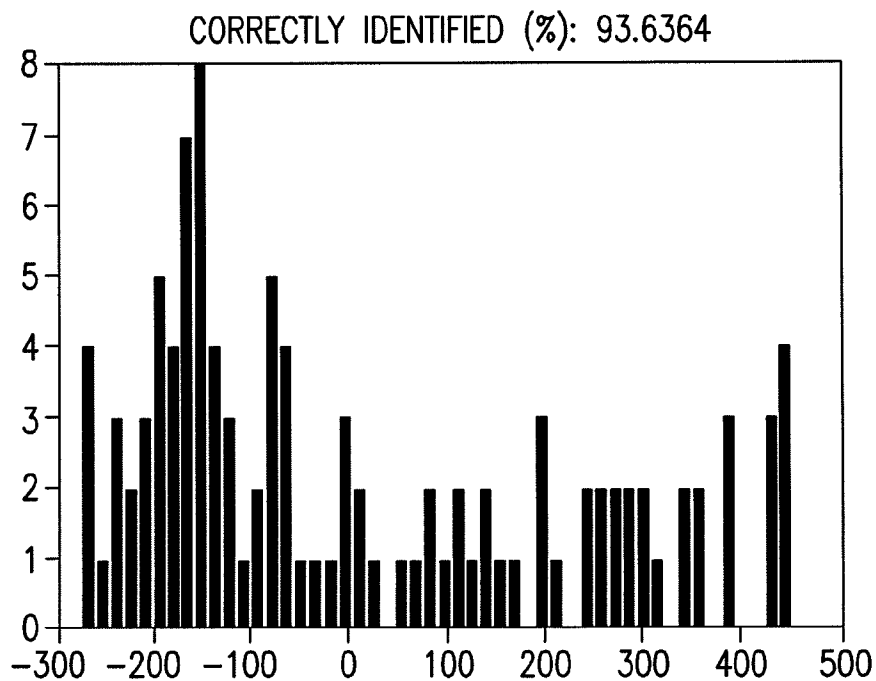


FIG.6D

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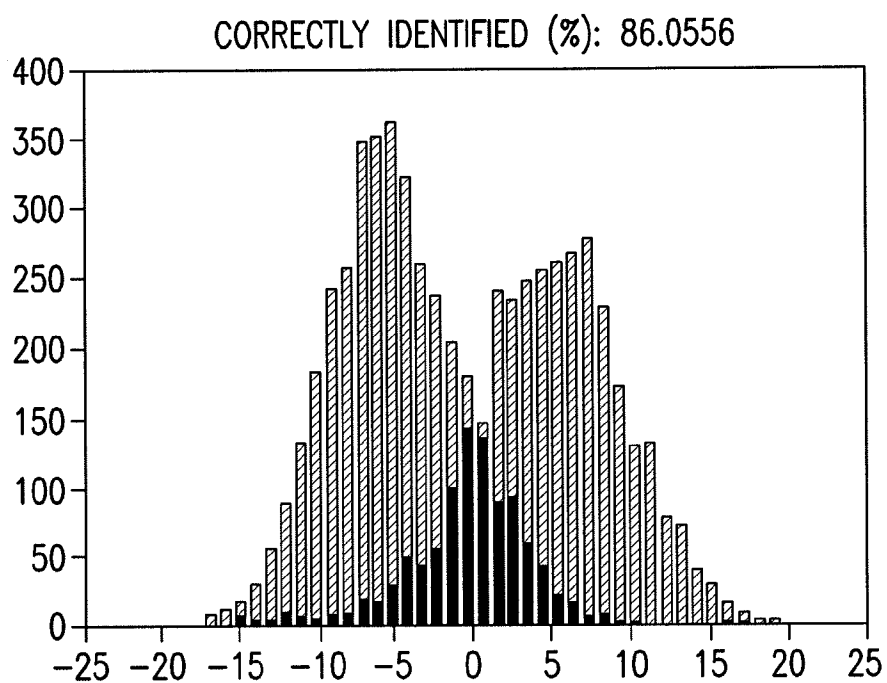


FIG. 6E

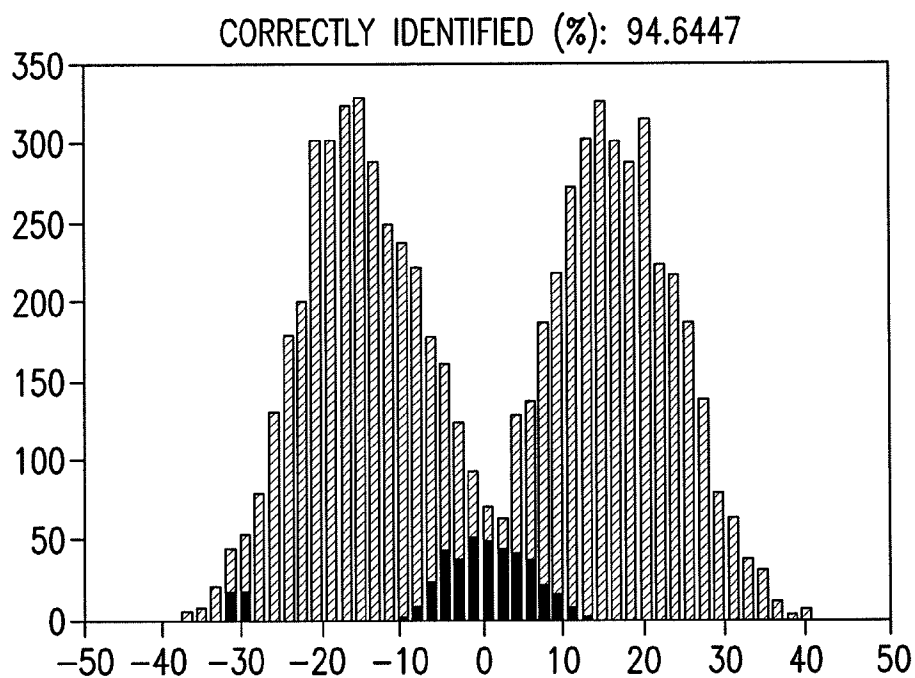


FIG. 6F

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US08/57882

A. CLASSIFICATION OF SUBJECT MATTER

IPC: C12Q 1/00(2006.01)

USPC: 435/4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2007/0042405 A1 (LOKSHIN) 22 February 2007 (22.02.2007), entire document.	1-13, 18-20
X	US 2005/0214826 A1 (MOR et al) 29 September 2005 (29.09.2005), entire document.	1-13, 18-20

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

04 July 2008 (04.07.2008)

Date of mailing of the international search report

21 AUG 2008

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

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Authorized officer

Thaïan N. Yon

Telephone No. 571-272-0500

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US08/57882

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 14-17 and 21
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US08/57882

Continuation of B. FIELDS SEARCHED Item 3:

CAPLUS, MEDLINE, EMBASE, BIOSIS, LIFESCI, WEST search terms: ovarian cancer, markers, prolactin, growth hormone, tsh, acth, eotaxin, MMP-2, MMP-3, sV-CAM, ErbB2, adiponectin