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(54) Titre : PROCEDE DE DETECTION OU DE SURVEILLANCE DU CANCER DE LA PROSTATE
(54) Title: METHOD FOR DETECTING OR MONITORING PROSTATE CANCER

(57) Abrégé/Abstract:

The present invention provides methods identifying subjects having prostate cancer (PCa) by detecting in microparticles a pair of biomarkers. The methods disclosed can be used to distinguish subjects having PCa from those having non-malignant prostate pathologies, including benign prostatic hyperplasia. Methods for monitoring prostate cancer and assessing efficacy of prostate cancer therapies are also disclosed. Kits for detecting prostate cancer using the methods disclosed are also provided.

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(54) Title: METHOD FOR DETECTING OR MONITORING PROSTATE CANCER

(57) Abstract: The present invention provides methods identifying subjects having prostate cancer (PCa) by detecting in micro-particles a pair of biomarkers. The methods disclosed can be used to distinguish subjects having PCa from those having non-malignant prostate pathologies, including benign prostatic hyperplasia. Methods for monitoring prostate cancer and assessing efficacy of prostate cancer therapies are also disclosed. Kits for detecting prostate cancer using the methods disclosed are also provided.



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METHOD FOR DETECTING OR MONITORING PROSTATE CANCER

CROSS REFERENCE TO PRIOR APPLICATIONS

[0001] This application claims priority under the Paris Convention from US Application Number 61/635,692, filed on April 19, 2012 and US Application Number 61/791,035, filed on March 15, 2013.

FIELD OF THE INVENTION

[0002] This invention relates generally to biochemical assays in the field of medicine. In particular, this invention is directed to methods and related materials for detecting and monitoring the progression of cancer, in particular prostate cancer, in human subjects.

BACKGROUND OF THE INVENTION

[0003] Prostate cancer (PCa) is a global health concern. It accounted for 10% of all cancer-related deaths in North America in 2010 (Jemal et al., Cancer Statistics 60:277-300, 2010). The number of men afflicted with PCa is increasing rapidly as the population of males over the age of 50 grows. Thus, strategies for detecting PCa in its early stages are urgently needed.

[0004] Conventional PCa screening involves assessing familial history of the disease and screening methods including digital rectal examination (DRE), transrectal ultrasound, and prostate specific antigen (PSA) testing. A subject's physician then uses assessment and screening data to determine whether a prostate biopsy is recommended. Unfortunately, current PCa screening methods result in a high rate of false positives. Large multicenter clinical trials using strict biopsy criteria (i.e., abnormal DRE results and/or a PSA > 4 ng/ml) have found a negative biopsy rate of approximately 70% (Thompson et al., New Eng. J. Med. 349:215-224, 2003 and Andriole et al., New Eng. J. Med. 362:1192-1202, 2010).

[0005] Biopsies are costly procedures that cause patients pain and anxiety and present a risk to patient health. For example, transrectal guided biopsies cause side effects ranging from temporary erectile dysfunction and blood in the urine, stool and ejaculate, to life-threatening sepsis in a minority of patients (Challacombe et al., BJU Intl. 108:1233-1234, 2011 and Zaytoun et al., Urology 77:910-914, 2011). Means to avoid unnecessary biopsies would benefit patient health and reduce health care costs.

1 [0006] Blood-based tests are advantageous for several reasons, including low invasive
2 sample collection (standard blood draw), low cost and amenability to high throughput
3 analyses. However, the standard blood test for PCa, namely PSA measurement, has a high
4 false positive rate and low specificity. Although PSA is a prostate-specific marker, it is not a
5 PCa-specific marker. Non-malignant conditions, particularly benign prostatic hyperplasia
6 (BPH), can elevate PSA levels in patient serum. BPH is an enlargement of the prostate which
7 can interfere with the normal flow of urine. PSA levels can be elevated in BPH patients due
8 to increased organ volume and inflammation due to associated urinary tract infections.
9 However, BPH is not known to increase a subject's risk of cancer. Because PSA screening
10 does not differentiate between PCa and BPH, even well-controlled studies cite an AUC of
11 approximately 0.6 for detection of PCa based on PSA testing (Aubin et al., J. Urology
12 184:1947-1952, 2010).

13 [0007] Prostate cancer antigen 3 (PCA3, also referred to as DD3) is specific to human
14 prostate tissue and is overexpressed in prostate cancer (Bussemakers et al., Cancer Res.
15 59:5975-5979, 1999). Urine tests for PCA3 have lower sensitivity but higher specificity
16 relative to serum PSA tests and a better positive and negative predictive value than PSA
17 (Vlaeminck-Guillem et al., Prog. Urol. 18:259-265, 2008). However, gathering the required
18 urine sample for a PCA3 test is invasive relative to a blood draw. A PCA3 test requires
19 collection of the first portion of urine produced following prostate massage with DRE.

20 [0008] C35 is a protein encoded by C17orf37, which is up-regulated in prostate, breast,
21 ovarian, liver and hepatocellular cancers and colorectal metastases. Advantageously, C35
22 exhibits a relative lack of expression in healthy tissues. However, currently there are no
23 prostate cancer screening tests that target C35 (Evans et al., Mol. Cancer Ther. 5:291902930,
24 2006; Dasgupta et al., Oncogene 13:2860-2872, 2009; Wong et al., AACR 101st Annual
25 Meeting 2010; Kilari et al., J. Clin. Oncol. 31:suppl 6; abstract 212, 2013).

26 [0009] A marker that distinguishes between PCa and BPH would be advantageous for
27 PCa screening methods. Such markers have been identified. For example, Ghrelin is a 28
28 amino acid peptide that is a natural growth hormone secretagogue (GHS)
29 (GS(octanoyl)FLSPEHRQVQQRKESK (SEQ ID NO:1). Ghrelin is known to be co-
30 expressed with its receptor GHSR in human PCa cells. An imaging probe, fluorescein-
31 ghrelin(1-18), that targets receptors for ghrelin can delineate PCa cells from prostate cells
32 having benign disease, including BPH (Lu et al., Prostate 72:825-833, 2012).

1 [0010] Unfortunately, using currently known methodologies, screening for Ghrelin or
2 C35 positive prostate cells would require a biopsy sample from prostate tissue.

3 [0011] Another method that has been used for identifying markers in serum is to test for
4 circulating microvesicles derived from tumor cells. Microvesicles are a type of microparticle
5 (MP), 100 nm–1 μ m in diameter, which directly bud from the plasma membrane (Morel et
6 al., Curr. Opin. Hematol. 11:156–164, 2004; Cocucci et al., Trends Cell Biol. 19:43–51,
7 2009). Microparticles are released by different cells, including tumour cells (Théry et al., Nat.
8 Rev. Immunol. 9:581–593, 2009). The emission of microvesicles, such as exosomes and
9 MPs, is suggested to be involved with tumor progression and metastasis (Schorey, J. Cell.
10 Sci, 123:1603-1611, 2010).

11 [0012] A bead-based method of detecting prostate cancer microvesicles is provided by
12 Caris® Life Sciences, wherein fluorescent beads bound to antibodies to PSMA, PSCA and
13 B7-H3 are used to capture PCa microvesicles. (Kiebel et al., poster, American Urological
14 Association, 2011). However, the bead-based assay does not enumerate PCa microparticles,
15 but rather provides a measurement of fluorescent intensity of the entire sample analyzed.
16 Enumeration of PCa microparticles is desirable, at least because it would provide an indicator
17 of tumor load by relying on the actual number of antigen-positive MPs rather than relative
18 fluorescence of the sample, which may include the binding of soluble protein complexes
19 specific for the antibodies used in the assay.

20 [0013] Use of MPs to detect disease in a subject is further complicated by the fact that the
21 presence of an antigen on a MP does not necessarily classify the cell of origin of the MP in
22 question. For example, in blood, soluble antigens derived from one cell type may adhere to
23 MPs derived from another cell type. Moreover, MPs derived from one cell type may fuse
24 with the membrane of different cell types, which subsequently release MPs (Simak and
25 Gelderman, Transfusion Med. Rev. 20:1-26, 2006). Thus, definitively identifying the origin
26 of circulating MPs has proven challenging.

27 [0014] There is a need in the art to develop a method of detecting PCa that has high
28 specificity and sensitivity. There is also a need for PCa detection method having the capacity
29 to distinguish between PCa and BPH. PCa detection methods having the capacity for
30 enumerative analysis are also desirable.

1 SUMMARY OF THE INVENTION

2 [0015] The present invention is broadly summarized as relating to biomarkers suitable for
3 identifying subjects having prostate cancer (PCa). In particular, the biomarkers are present on
4 the surface of prostate cancer microparticles. In one aspect, the invention provides a method
5 for distinguishing patients having PCa from those having non-malignant prostate pathologies,
6 including benign prostatic hyperplasia (BPH), wherein the method comprises identifying the
7 above-mentioned biomarkers.

8 [0016] In a first aspect, the present invention provides a method for detecting prostate
9 cancer in a sample obtained from a subject.

10 [0017] In some embodiments of the first aspect, the method comprises analyzing a bodily
11 fluid sample to detect microparticles having at least first and second biomarkers on their
12 surface in the bodily fluid sample. In some embodiments, the first biomarker is expressed by
13 prostate epithelial cells and the second biomarker is expressed by prostate cancer cells but not
14 by benign prostatic hyperplasia or other non-malignant prostate cells.

15 [0018] In some embodiments of the first aspect, the method comprises comparing the
16 amount of microparticles positive for both the first and second biomarkers with a reference
17 value. In some embodiments, the reference value is derived from a non-malignant prostatic
18 sample. In such embodiments, a detected value above the reference value is indicative of
19 prostate cancer and a detected value equal to or below the reference value is indicative of a
20 non-malignant disease state.

21 [0019] In some embodiments, the reference value is derived from a malignant prostatic
22 sample. In such embodiments, a detected value equal to or above the reference value is
23 indicative of prostate cancer and a detected value below the reference value is indicative of a
24 non-malignant disease state.

25 [0020] In some embodiments of the first aspect, the method comprises diagnosing the
26 subject on the basis of the results obtained in the comparing step.

27 [0021] In some embodiments of the first aspect, the bodily fluid is blood.

28 [0022] In some embodiments of the first aspect, at least two biomarkers are used. In one
29 embodiment, a first biomarker is prostate specific membrane antigen (PSMA), prostate stem

1 cell antigen (PSCA), STEAP1 or STEAP2. In preferred embodiments, the first biomarker is
2 PSMA.

3 [0023] In some embodiments of the first aspect, a second biomarker is Ghrelin or C35.
4 In preferred embodiments, the second biomarker is Ghrelin.

5 [0024] In some embodiments of the first aspect, the method further comprises
6 effectuating a treatment based on the diagnosis determined.

7 [0025] In some embodiments of the first aspect, the analysis of bodily fluid is conducted
8 using a flow cytometry assay. In some embodiments, the flow cytometry assay is
9 fluorescence activated cell sorting (FACS). In some embodiments, the flow cytometry assay
10 is carried out using a nanoscale flow cytometer. In some embodiments, the flow cytometry
11 assay comprises exposing the bodily fluid sample to a composition. The composition in the
12 assay comprising a first labeled binding probe that is specific to the first biomarker and a
13 second labeled binding probe that is specific to the second biomarker. In some embodiments,
14 the labels of the first and second probes are distinguishable. In preferred embodiments, the
15 first labeled binding probe is anti-PSMA-RPE IgG and the second labeled probe is Ghrelin-
16 Cy5 or Ghrelin-FITC.

17 [0026] In some embodiments of the first aspect, analysis is carried out with reference to
18 negative controls of the first and second binding probes using first and second negative
19 control binding probes. In preferred embodiments, the first negative control binding probe is
20 mouse IgG-RPE and the second negative control binding probe is des-acyl Ghrelin-Cy5 or
21 des-acyl Ghrelin-FITC.

22 [0027] In some embodiments of the first aspect, the reference value represents the
23 amount of microparticles positive for the first and second biomarkers in a sample obtained
24 from a subject having a non-malignant prostate or benign prostatic hyperplasia (BPH) and the
25 difference is an increase. In preferred embodiments, the reference value is in a range of
26 14,000 to 22,000 PCMP counts/ μ L.

27 [0028] In a second aspect, the present invention provides a diagnostic assay for prostate
28 cancer.

29 [0029] In some embodiments of the second aspect, the diagnostic assay comprises
30 analyzing a bodily fluid sample to detect microparticles having first and second biomarkers

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1 on their surface in the bodily fluid sample, wherein the first biomarker is expressed by
2 prostate epithelial cells and the second biomarker is expressed by prostate cancer (PCa) cells
3 but not by benign prostatic hyperplasia or other non-malignant prostate cells; comparing the
4 amount of microparticles positive for both the first and second biomarkers with a reference
5 value, wherein if the reference value is derived from a non-malignant prostatic sample then a
6 detected value above the reference value is indicative of prostate cancer and a detected value
7 equal to or below the reference value is indicative of a non-malignant disease state and,
8 wherein if the reference value is derived from a malignant prostatic sample then a detected
9 value equal to or above the reference value is indicative of prostate cancer and a detected
10 value below the reference value is indicative of a non-malignant disease state; and diagnosing
11 the subject on the basis of the results obtained the comparison step.

12 **[0030]** In a third aspect, the present invention provides a method for monitoring prostate
13 cancer in a subject.

14 **[0031]** In some embodiments of the third aspect, the method for monitoring prostate
15 cancer comprises analyzing a first bodily fluid sample, wherein the first sample was obtained
16 from the subject at a first time point, to detect microparticles having at least first and second
17 biomarkers on their surface in the bodily fluid sample. In some embodiments, the first
18 biomarker is expressed by prostate epithelial cells and the second biomarker is expressed by
19 prostate cancer (PCa) cells but not by benign prostatic hyperplasia or other non-malignant
20 prostate cells.

21 **[0032]** In some embodiments of the third aspect, the method for monitoring prostate
22 cancer comprises comparing the amount of microparticles positive for both the first and
23 second biomarkers with a reference value, wherein if the reference value is derived from a
24 non-malignant prostatic sample then a detected value above the reference value is indicative
25 of prostate cancer and a detected value equal to or below the reference value is indicative of a
26 non-malignant disease state and, wherein if the reference value is derived from a malignant
27 prostatic sample then a detected value equal to or above the reference value is indicative of
28 prostate cancer and a detected value below the reference value is indicative of a non-
29 malignant disease state.

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1 [0033] In some embodiments of the third aspect, the method for monitoring prostate
2 cancer comprises diagnosing the subject on the basis of the results obtained in the comparing
3 step.

4 [0034] In some embodiments of the third aspect, the method for monitoring prostate
5 cancer comprises effectuating a treatment regimen based diagnosis obtained in the diagnosing
6 step.

7 [0035] In some embodiments of the third aspect, the method for monitoring prostate
8 cancer comprises analyzing a second bodily fluid sample, wherein the second sample was
9 obtained from the subject at a second time point, to detect microparticles having at least first
10 and second biomarkers on their surface in the bodily fluid sample, wherein the first
11 biomarker is expressed by prostate epithelial cells and the second biomarker is expressed by
12 prostate cancer (PCa) cells but not by benign prostatic hyperplasia or other non-malignant
13 prostate cells.

14 [0036] In some embodiments of the third aspect, the method for monitoring prostate
15 cancer comprises comparing the amount of microparticles positive for both the first and
16 second biomarkers with a reference value, wherein if the reference value is derived from a
17 non-malignant prostatic sample then a detected value above the reference value is indicative
18 of prostate cancer and a detected value equal to or below the reference value is indicative of a
19 non-malignant disease state and, wherein if the reference value is derived from a malignant
20 prostatic sample then a detected value equal to or above the reference value is indicative of
21 prostate cancer and a detected value below the reference value is indicative of a non-
22 malignant disease state.

23 [0037] In some embodiments of the third aspect, the method for monitoring prostate
24 cancer comprises comparing the amount of microparticles positive for the first and second
25 biomarker in the second bodily fluid sample with a the value obtained in first comparing step
26 wherein an increase in the amount of microparticles positive for the first and second
27 biomarkers in the second sample relative to the value obtained in the first comparing step is
28 indicative of a worsened disease state and a decrease in the amount of microparticles positive
29 for the first and second biomarkers in the second sample relative to the value obtained in the
30 first comparing step is indicative of an improved disease state.

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1 [0038] In some embodiments of the third aspect, the method for monitoring prostate
2 cancer comprises diagnosing any change in the subject's disease state on the basis of the
3 results obtained by comparing the amount of dual positive microparticles in the first and
4 second samples.

5 [0039] In a fourth aspect, the present invention provides a method for assessing efficacy
6 of a therapy on a subject having prostate cancer.

7 [0040] In some embodiments of the fourth aspect, the method for assessing efficacy of a
8 therapy on a subject having prostate cancer comprises: analyzing a bodily fluid sample from a
9 subject, wherein the subject has been subjected to a prostate cancer therapy, to detect
10 microparticles having at least first and second biomarkers on their surface in the bodily fluid
11 sample, wherein the first biomarker is expressed by prostate epithelial cells and the second
12 biomarker is expressed by prostate cancer (PCa) cells but not by benign prostatic hyperplasia
13 or other non-malignant prostate cells.

14 [0041] In some embodiments of the fourth aspect, the method for assessing efficacy of a
15 therapy on a subject having prostate cancer comprises comparing the amount of
16 microparticles positive for both the first and second biomarkers with a reference value,
17 wherein if the reference value is derived from a non-malignant prostatic sample then a
18 detected value above the reference value is indicative of prostate cancer and a detected value
19 equal to or below the reference value is indicative of a non-malignant disease state and,
20 wherein if the reference value is derived from a malignant prostatic sample then a detected
21 value equal to or above the reference value is indicative of prostate cancer and a detected
22 value below the reference value is indicative of a non-malignant disease state; and

23 [0042] In some embodiments of the fourth aspect, the method for assessing efficacy of a
24 therapy on a subject having prostate cancer comprises diagnosing the efficacy of the therapy
25 as good if the value obtained in the comparing step indicates a non-malignant disease state or
26 poor if the value obtained in the comparing step indicates prostate cancer.

27 [0043] In a fifth aspect, the present invention provides a kit for detecting prostate cancer
28 in a bodily fluid sample.

29 [0044] In some embodiments of the fifth aspect, the kit comprises a first binding
30 probe specific to a biomarker that is expressed by prostate epithelial cells and a second

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1 binding probe specific to a biomarker that is expressed by prostate cancer (PCa) cells but not
2 by benign prostatic hyperplasia or other non-malignant prostate cells.

3 [0045] In some embodiments of the fifth aspect, the first binding probe anti-PSMA-RPE
4 IgG. In some embodiments, the second binding probe is Ghrelin-Cy5 or Ghrelin-FITC.

5 [0046] In some embodiments of the fifth aspect, the kit comprises a first negative control
6 binding probe specific to mouse IgG. In some embodiments, the kit comprises a second
7 negative control binding probe specific to des-acyl Ghrelin.

8 [0047] In some embodiments of the fifth aspect, the first negative control binding probe
9 is the monoclonal antibody mouse IgG-RPE. In some embodiments, the second negative
10 control binding probe is des-acyl Ghrelin-Cy5 or des-acyl Ghrelin-FITC. In preferred
11 embodiments of the fifth aspect, the kit comprises a first and second sealed container,
12 wherein the first sealed container comprises anti-PSMA-RPE IgG and Ghrelin-Cy5 or
13 Ghrelin-FITC and the second sealed container comprises mouse IgG-RPE and des-acyl
14 Ghrelin-Cy5 or des-acyl Ghrelin-FITC.

15 [0048] In some embodiments of the fifth aspect, the kit comprises a carrier, wherein a
16 carrier is a box, carton, or tube. In some embodiments, the carrier comprises one or more
17 sealed containers, wherein the one or more sealed container is a vial, tube, ampoule, bottle,
18 pouch or envelope.

19 [0049] In some embodiments of the fifth aspect, the kit comprises one or more media,
20 media ingredients or reagents for measurement of at least one of the first and second
21 biomarkers. In some embodiments, the one or more reagents are buffers or probes.

22 [0050] In some embodiments of the fifth aspect, the kit comprises one or more
23 instructions or protocols for carrying out the methods of the present invention.

24 BRIEF DESCRIPTION OF THE DRAWINGS

25 [0051] Features of the invention will become more apparent in the following detailed
26 description in which reference is made to the appended drawings wherein:

27 [0052] FIG 1. depicts nanoscale flow cytometry of prostate cancer microparticles in
28 plasma from healthy volunteers. The top panel reveals the size distribution of dual-positive
29 microparticles (events that bind anti-PSMA-RPE IgG and Ghrelin-Cy5 peptide) present in the

1 red gate of the bottom histoplot in this representative patient plasma sample. Events in the red
2 gate represent dual-positive events that are not present in the isotype stained control of the
3 same but separately stained plasma sample.

4 **[0053]** FIG 2. depicts nanoscale flow cytometry of prostate cancer microparticles in
5 plasma from patients with benign prostatic hyperplasia. The top panel reveals the size
6 distribution of dual-positive microparticles (events that bind anti- PSMA-RPE IgG and
7 Ghrelin-Cy5 peptide) present in the red gate of the bottom histoplot in this representative
8 patient plasma sample. Events in the red gate represent dual-positive events that are not
9 present in the isotype stained control of the same but separately stained plasma sample.

10 **[0054]** FIG 3. depicts nanoscale flow cytometry of prostate cancer microparticles in
11 plasma from patients with localized prostate cancer. The top panel reveals the size
12 distribution of dual-positive microparticles (events that bind anti-PSMA- RPE IgG and
13 Ghrelin-Cy5 peptide) present in the red gate of the bottom histoplot in this representative
14 patient plasma sample. Events in the red gate represent dual-positive events that are not
15 present in the isotype stained control of the same but separately stained plasma sample.

16 **[0055]** FIG 4. depicts nanoscale flow cytometry of prostate cancer microparticles in
17 plasma from patients with metastatic prostate cancer. The top panel reveals the size
18 distribution of dual-positive microparticles (events that bind anti-PSMA- RPE IgG and
19 Ghrelin-Cy5 peptide) present in the red gate of the bottom histoplot in this representative
20 patient plasma sample. Events in the red gate represent dual-positive events that are not
21 present in the isotype stained control of the same but separately stained plasma sample.

22 **[0056]** FIG 5. is a graphic representation showing counts of prostate cancer
23 microparticles (PCMPs) in patients with BPH and patients with PCa. PCMPs are defined as
24 dual-positive for anti-PSMA-RPE IgG and Ghrelin-Cy5 peptide. All patients had
25 PSA>4ng/mL.

26 **[0057]** FIG 6. is a graphic representation showing counts of prostate microparticles
27 (PSMA+ve only) in patients with BPH and patients with PCa. Prostate microparticles are
28 defined as sub-micron events that bind only the anti-PSMA-RPE IgG. PSA>4 ng/mL for all
29 patient plasmas and N>20 each group.

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1 [0058] FIG 7. depicts monitoring of changes in patient PCMP levels before and after
2 prostatectomy. For each sample ID (HL XXX), fold difference is recited in the right column.
3 Values in red (marked by an up arrow) indicate an increase in PCMP concentration after
4 prostatectomy. Values in black (without arrows) indicate a decrease in PCMP concentration
5 after prostatectomy.

6 DETAILED DESCRIPTION OF THE INVENTION

7 [0059] The definitions of certain terms as used in this specification are provided below.
8 Unless defined otherwise, all technical and scientific terms used herein generally have the
9 same meaning as commonly understood by one of ordinary skill in the art to which this
10 invention belongs.

11 [0060] As used herein, the term "about" will be understood by persons of ordinary skill in
12 the art and will vary to some extent depending upon the context in which it is used. If there
13 are uses of the term which are not clear to persons of ordinary skill in the art, given the
14 context in which it is used, "about" will mean up to plus or minus 10% of the enumerated
15 value.

16 [0061] As used herein, the terms "diagnose", "diagnosing" and "diagnostic" refer to the
17 process of determining a disease state or disorder in a subject. In determining disease state a
18 diagnostician might classify one or more characteristics of a subject, such as, for example,
19 symptoms and/or biomarkers. A "diagnostic assay" is referred to herein as a tool that a
20 diagnostician might use to narrow the diagnostic possibilities.

21 [0062] As used herein, the term "subject" refers to a mammal, such as, for example, a
22 human, non-human primate, mouse, rat, dog, cat, horse, or cow. In some embodiments, a
23 subject is human and might be referred to as a patient. A subject can be one who has been
24 previously diagnosed or identified as having a disease, and optionally one who has already
25 undergone, or is undergoing, a therapeutic intervention for a disease. Alternatively, a subject
26 can also be one who has not been previously diagnosed as having a disease.

27 [0063] As used herein, the terms "prostate cancer" and "prostate malignancy" refers to a
28 prostate containing tumor-forming prostate epithelial cells. Conversely, a "non-malignant
29 prostate", as used herein, refers to a prostate that does not contain tumor-forming prostate
30 epithelial cells.

1 [0064] As used herein, the term benign prostatic hyperplasia or "BPH" refers to an
2 increase in size of a prostate due to an increase in the number of prostate cells. BPH is not
3 known to cause cancer, including prostate cancer, or to increase the risk of cancer, including
4 prostate cancer.

5 [0065] As used herein, the terms "bodily fluid sample" and "fluid sample" refer to a
6 specimen obtained from a subject. In some embodiments, the sample comprises blood, a
7 fraction of blood or urine.

8 [0066] As used herein, the terms "detect", "detection" and "detecting" refer to a
9 quantitative or qualitative determination of a property of an entity, for example, quantifying
10 the amount or concentration of a molecule or the activity level of a molecule. The term
11 "concentration" or "level" can refer to an absolute or relative quantity. Measuring a molecule
12 may also include determining the absence or presence of the molecule. Various methods of
13 detection are known in the art, for example fluorescence analysis. In this regard, biomarkers
14 can be measured using fluorescence detection methods or other methods known to the skilled
15 artisan.

16 [0067] As used herein, the terms "microparticle" or "MP" refer to small membrane bound
17 vesicles (i.e., generally 100 nm to 1 μ m in diameter) that directly bud from the plasma
18 membrane of various cells, including tumor cells, or are storage vesicles released by prostate
19 cells or prostate cancer cells by exocytosis. Microparticles circulate in blood that is derived
20 from cells in contact with the bloodstream, such as, for example, endothelial cells.
21 Microparticles are useful in various embodiments of the present invention, at least because
22 they retain at least some of the membrane protein characteristics of their parent cells.

23 [0068] As used herein, the term "biomarker" refers to a molecule whose measurement
24 provides information regarding the state of a subject, or a feature of a subject, such as, for
25 example, an organ, tissue, system or cell. For example, the disease state of a subject can be
26 assessed using a biomarker. Measurements of a biomarker may be used alone or combined
27 with other data obtained regarding a subject, or feature thereof, in order to determine the state
28 of the subject, or feature thereof. In one embodiment, the biomarker is "differentially present"
29 in a sample taken from a subject of one disease state (e.g., having a disease) as compared
30 with another disease state (e.g., not having the disease). In one embodiment, the biomarker is
31 "differentially present" in a sample taken from a subject undergoing no therapy or one type of

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1 therapy as compared with another type of therapy. Alternatively, the biomarker may be
2 "differentially present" even if there is no known difference in disease state, e.g., the
3 biomarkers may allow the detection of asymptomatic risk.

4 **[0069]** As used herein, the terms "specific" and "specificity" refer to the nature of the
5 binding of a biomarker with its binding probe. "Specific binding" or "selective binding"
6 refers to a probe that binds a biomarker with a specificity sufficient to differentiate between
7 the biomarker and other components or contaminants of a test sample.

8 **[0070]** As used herein, the term "reference value" refers to a baseline value. In some
9 embodiments, a baseline value can represent the amount of MPs in a composite sample from
10 an effective number of subjects who do not have the disease of interest but are positive for
11 both of the biomarkers of interest. In some embodiments, a reference value can also comprise
12 the amount of MPs in a composite sample from an effective number of subjects who have the
13 disease of interest, as confirmed by an invasive or non-invasive technique.

14 **[0071]** As used herein, the terms "indicative of", "associated with" and "correlated to"
15 refer to the determination of a relationship between one type of data with another or with a
16 state. In some embodiments, correlating the measurement with disease comprises comparing
17 the amount of MPs positive for a pair of biomarkers with a reference value. In some
18 embodiments, correlating the measurement with disease comprises determining the subject's
19 disease state.

20 **[0072]** As used herein, the terms "treatment", "treatment regimen", "therapy" and
21 "therapeutic treatment" refer to an attempted remediation of a health problem. In some
22 embodiments, treatment can be selected from, administering a disease-modulating drug to a
23 subject, administering disease-modulating radiation to a subject, surgery or scheduling a
24 further appointment with a medical practitioner. Treatment refers to one or more of initiating
25 therapy, continuing therapy, modifying therapy or ending therapy.

26 **[0073]** As used herein, the terms "prophylaxis" and "prophylactic" refer to measures taken
27 to prevent disease. Prophylactic treatment includes, for example, measures to reverse,
28 prevent or slow physiological features that are precursors to disease.

29 **[0074]** As used herein, the terms "binding probe" or "binding ligand" refer to compounds
30 that are used to detect the presence of, or to quantify, relatively or absolutely, a target

1 molecule or target sequence and that will bind to the target molecule or sequence, either
2 directly or indirectly. Generally, a binding probe allows attachment of a target molecule or
3 sequence to the probe for the purpose of detection. In some embodiments, the target molecule
4 or sequence is a biomarker. It follows that the composition of the binding probe will depend
5 on the composition of the biomarker. Binding probes for a variety of biomarkers are known
6 or can be generated using known techniques. For example, when the biomarker is a protein,
7 the binding probes include proteins, such as, for example, antibodies or fragments thereof or
8 small molecules.

9 **[0075]** As used herein, the terms “label” and “labeled” refer to a composition detectable
10 by spectroscopic, photochemical, biochemical, immunochemical, chemical, or other physical
11 means. A compound that is labeled has at least one molecule, element, isotope or chemical
12 compound attached to it to enable the detection of the compound. For example, useful labels
13 include fluorescent dyes, which might also be referred to as fluorophores.

14 **[0076]** As used herein, the term “fluorophore” refers to a molecule or part of a molecule
15 that absorbs energy at one wavelength and re-emits energy at another wavelength. Detectable
16 properties of fluorophores include fluorescence intensity, fluorescence lifetime, emission
17 spectrum characteristics, energy transfer, and the like. Fluorophores are of use in the present
18 invention, at least due to their strong signals, which provide a signal-to-noise ratio sufficient
19 to allow interpretation of the signals. Suitable fluorophore for use in the present invention
20 include, but are not limited to, fluorescent lanthanide complexes, including those of
21 Europium and Terbium, fluorescein, rhodamine, tetramethylrhodamine, eosin, erythrosin,
22 coumarin, methyl-coumarins, pyrene, Malacite green, stilbene, Lucifer Yellow, Cascade
23 Blue, Texas Red, Alexa dyes and others described in the 6th Edition of the Molecular Probes
24 Handbook by Richard P. Haugland.

25 **[0077]** As used herein, the terms “nanoscale flow cytometer” or “nanoscale flow
26 cytometry” refer to a flow cytometry device, or a process of using said flow cytometry
27 device, that can analyze events that are 1000 nm-100nm in diameter.

28 **[0078]** As used herein, the term “antibody” refers to a protein comprising one or more
29 polypeptides substantially encoded by all or part of immunoglobulin genes known to the
30 skilled artisan. The immunoglobulin genes recognized by a skilled artisan include, for
31 example in humans, the kappa, lambda and heavy chain genetic loci, which together

1 compose myriad variable region genes, and constant region genes mu, delta, gamma, epsilon
2 and alpha, which encode IgM, IgD, IgG, IgE, and IgA isotypes respectively. Antibody herein
3 is meant to include full length antibodies and antibody fragments, and may refer to a natural
4 antibody from any organism, an engineered antibody or an antibody generated recombinantly
5 for experimental, therapeutic or other purposes as further defined below. The term "antibody"
6 refers to both monoclonal and polyclonal antibodies. Antibodies can be antagonists, agonists,
7 neutralizing, inhibitory or stimulatory.

8 [0079] As used herein the term "negative control" refers to an element or group used in
9 an experiment to ensure that a negative result is produced when a negative result is expected.
10 For example, a negative control binding probe, as referred to herein, is a probe that should
11 not bind to the MP being examined, because the probe's target is not present in the sample
12 being examined. Thus, when assayed, if a negative control binding probe successfully binds
13 to a MP, then then it can be inferred that a confounding variable acted on the experiment,
14 suggesting that the positive results are likely not due the intended specific binding.

15 [0080] As used herein, the term "monitoring" refers to observation of a disease over time.
16 Monitoring of a subject's disease state can be performed by continuously measuring certain
17 parameters and/or performing a medical test repeatedly. In some embodiments of the present
18 invention, a subject's disease state is monitored by obtaining bodily fluid samples repeatedly,
19 assaying the samples using the method disclosed herein and comparing assay results with one
20 another and with a reference value to identify any change in the subject's disease state.

21 [0081] As used herein, the term "disease state" refers to any distinguishable manifestation
22 of a particular disease, including non-disease. For example, disease state includes, without
23 limitation, the presence or absence of a disease, the risk of developing a disease, the stage of
24 a disease, the progression or remission of a disease over time and the severity of disease. The
25 term "worsened disease state" refers to the progression of a disease over time. The term
26 "improved disease state" refers to remission of disease over time.

27 [0082] As used herein, the term "efficacy" refers to the capacity of an intervention to
28 produce a therapeutic effect. For example, a PCa treatment having good efficacy might
29 significantly reduce or eliminate from a subject detectable tumor-forming prostate epithelial
30 cells. In contrast, a PCa treatment having a poor efficacy might not reduce in a subject the
31 level of detectable tumor-forming prostate epithelial cells.

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1 [0083] As used herein, the term “kit” refers to a collection of elements that together are
2 suitable for a defined use.

3 [0084] As used herein, the term “invasive” refers to a medical procedure in which a part
4 of the body is entered. In some embodiments, entry into the body might cause a subject to
5 feel pain during or following the procedure. For example, surgical procedures involving
6 incisions are invasive. Herein, a standard blood draw is not considered to be invasive.

7 [0085] The present invention generally relates to a non-invasive means of screening a
8 subject for PCa. The invention is based on the inventors’ observations that i) MPs found in
9 mammalian plasma can be identified and enumerated using, for example, flow cytometry, ii)
10 prostate cells or prostate cancer cells undergo extravasation, apoptosis or necrosis, releasing
11 prostate MPs into the circulatory system; and iii) prostate MPs can be distinguished as
12 cancerous or non-cancerous by quantifying the MPs positive for a pair of surface biomarkers
13 using flow cytometry. The pair of biomarkers includes a biomarker specific to prostate cells
14 that are not typically found in healthy individuals, such as, for example, prostate-specific
15 membrane antigen (PSMA) and a biomarker that is specific to PCa cells. In some aspects of
16 the invention, the PCa-specific biomarker is not significantly expressed in non-malignant
17 prostate cells, including BPH cells, and is not present on the surface of non-malignant
18 prostate MPs, including BPH MPs. In some aspects of the invention, the PCa-specific
19 biomarker is present at a level below a reference value in prostate and BPH MPs.

20 [0086] Some embodiments of the present invention involve a method for diagnosing PCa
21 in a subject. In some embodiments, the method comprises obtaining a bodily fluid sample
22 from the subject, preferably a blood sample. In some embodiments of the method, the blood
23 sample can be fractionated to obtain platelet poor plasma. The sample is then analyzed by, for
24 example, a flow cytometry assay that specifically detects MPs positive for first and second
25 biomarkers in the sample. The first biomarker is expressed in prostate epithelial cells.

26 [0087] The first biomarker is preferably PSMA, which is known to be specifically
27 expressed on the surface of prostate cells and some prostate cancer cells. It is contemplated
28 that PSCA, STEAP1 or STEAP2 could also be used as the first biomarker, at least because
29 PSCA, STEAP1 and STEP2 are known to be expressed in prostate epithelial cells and
30 therefore predicted to be present in prostate microparticles. It follows that the presence of
31 PSMA, PSCA, STEAP1 or STEAP2 would be sufficient to identify MPs of prostatic origin,

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1 as exemplified by prostate MPs positive for PSMA. However, the presence of PSMA on the
2 surface of a MP alone cannot identify the MP as being a PCa MP, at least because BPH cells
3 and MPs have a detectable level of PSMA on their surfaces. Further, the inventors are
4 unaware of any evidence to suggest that PSCA, STEAP1 or STEAP2 would be useful for
5 distinguishing PCa cells from non-malignant prostate cells.

6 [0088] The second biomarker is expressed by PCa cells but is not significantly expressed
7 by BPH or other non-malignant prostate cells. As indicated above, PSMA is not sufficient to
8 distinguish between subjects having PCa and BPH. A subpopulation of subjects having BPH
9 has MPs that are PSMA positive. Another sub-population of subjects having BPH has a low
10 amount of MPs that are PSMA positive. Such low levels of PSMA are below the reference
11 value disclosed herein.

12 [0089] In some embodiments of the present invention, the second biomarker is Ghrelin.
13 Ghrelin is a hunger-stimulating peptide and hormone that has a G protein-coupled receptor
14 called the growth hormone secretagogue receptor. Ghrelin is expressed on the surface of a
15 variety of cells. However, Ghrelin is not significantly expressed in non-malignant prostate
16 cells, including BPH cells, nor is it present on the surface of non-malignant prostate MPs,
17 including BPH MPs.

18 [0090] Detection of MPs positive for both PSMA and Ghrelin allows for specific
19 identification of samples originating from subjects having PCa.

20 [0091] It is contemplated herein that the second biomarker could be C35. C35 is specific
21 to cancer cells, including prostate cancer cells and not expressed in corresponding healthy
22 cells. Thus, detection of MPs positive for C35 would be indicative of cancer. It follows that
23 detection of MPs positive for Ghrelin or C35 and at least one of PSMA, PSCA, STEAP1 or
24 STEAP2 would allow for specific identification of samples originating from subjects having
25 PCa.

26 [0092] In some embodiments of the present invention, the amount of MPs having both
27 the first and second biomarkers on their surface is compared with a reference value.

28 [0093] The reference value can be a baseline amount that represents the amount of
29 microparticles having both the first and second biomarkers on their surface that are found in a
30 given volume of sample from a subject who do not have the disease of interest. Where a

1 reference value is indicative of a subject having a non-malignant prostate, a value greater
2 than said reference value would be indicative of prostate cancer. It is also contemplated
3 herein that a reference value could, in contrast, represent the amount of microparticles
4 positive for both first and second biomarkers that are found in a given volume of sample from
5 a subject having the disease of interest. Where a reference value is indicative of a subject
6 having prostate cancer, a value less than said reference value would be indicative of a non-
7 malignant prostate. In some embodiments of the present invention, the reference value is in a
8 range of 14,000 to 22,000 PCMP counts/ μ L and a value above 14,000 to 22,000 PCMP
9 counts/ μ L is indicative of prostate cancer.

10 [0094] In some embodiments of the present invention, the method can yield a result
11 indicative of prostate cancer. Treatments for prostate cancer are known in the art. A
12 treatment for prostate cancer can be selected from, for example, administering a
13 chemotherapeutic agent to a subject, administering disease-modulating radiation to a subject,
14 surgery or scheduling a further appointment with a medical practitioner.

15 [0095] In some embodiments of the present invention, the method can yield a result
16 indicating that prostate cancer is not present in the patient sample. In such instance, further
17 monitoring of the patient may be recommended by way of further tests or visits to a medical
18 practitioner over time.

19 [0096] In some embodiments of the present invention, the preferred flow cytometry assay
20 comprises exposing the sample to a composition, the composition comprising a first labeled
21 binding probe that is specific to the first biomarker and a second labeled binding probe that is
22 specific to the second biomarker. It is contemplated that flow cytometry instruments known
23 to the skilled artisan are suitable for use with the present invention, at least for example,
24 instruments suitable for standard flow cytometry, nanoscale flow cytometry or FACS. In
25 some embodiments, the first and second binding probes are labeled with fluorophores. When
26 selecting suitable fluorophores it is important that the excitation wavelength of the
27 fluorophore conjugated to the first binding probe is distinct from the excitation wavelength of
28 the fluorophore conjugated to the second binding probe.

29 [0097] Suitable fluorophores for use in the present invention include, but are not limited
30 to, fluorescent lanthanide complexes, including those of Europium and Terbium, fluorescein,
31 rhodamine, tetramethylrhodamine, eosin, erythrosin, coumarin, methyl-coumarins, pyrene,

1 Malacite green, stilbene, Lucifer Yellow, Cascade Blue, Texas Red, Alexa dyes and others
2 described in the 6th Edition of the Molecular Probes Handbook by Richard P. Haugland. In
3 some embodiments of the present invention R-phycoerythrin (RPE) is conjugated to the first
4 binding probe and fluorescein isothiocyanate (FITC) is conjugated to the second binding
5 probe.

6 [0098] In some embodiments of the present invention, negative controls are used in the
7 method of detecting prostate cancer to allow for enumeration of microparticles that are
8 positive for the first and/or second biomarkers.

9 [0099] In some embodiments, the first negative control is mouse IgG-RPE and the second
10 negative control is and des-acyl Ghrelin-Cy5 or des-acyl Ghrelin-FITC.

11 [00100] In some embodiments of the methods of the present invention, a portion of the
12 sample of the bodily fluid is removed from the sample and exposed to a composition
13 comprising binding probes specific to the first and second negative controls. The exposed
14 sample is then analyzed by a flow cytometry assay that specifically detects microparticles
15 having both the first and second biomarkers on their surface in the bodily fluid sample. If
16 any microparticles are found to bind to one or more of the negative control probes, then a
17 confounding variable might be responsible for any fluorescent microparticles that are
18 identified in the disclosed assay for detecting microparticles having both first and second
19 biomarkers on their surface. If the fluorescence of the negative control probes is not
20 observed, then confounding variables can be eliminated as possible cause for positive results
21 that are found in the disclosed assay for detecting microparticles having both first and second
22 biomarkers on their surface.

23 [00101] In some embodiments of the present invention, a diagnostic assay for prostate
24 cancer is provided, wherein the assay comprises the method set forth above, and disclosed
25 further in the examples herein.

26 [00102] In some embodiments of the present invention, the method provides a less-
27 invasive method for detecting prostate cancer in a subject, relative to biopsy methods and
28 PCA3 assays known in the art. Advantageously, some embodiments of the present invention
29 provide a method for detecting prostate cancer that results in fewer false positive than current
30 blood-based PSA tests. Further, in addition to being amenable to high throughput, methods
31 of the present invention involve identifying dual-positive microparticles (e.g., Ghrelin+ and

1 PSMA+). In contrast, many existing technologies such as ELISA and western
2 immunoblotting, cannot address two parameters simultaneously. In some embodiments, the
3 methods of the present invention also provide enumeration of single or dual positive
4 microparticles when negative controls are also analyzed. In some embodiments, enumeration
5 allows a diagnostician to assess the impact of a therapeutic intervention by enumerating the
6 total change in an amount of prostate cancer microparticles before and therapy.

7 **[00103]** In some embodiments of the present invention, a method for monitoring prostate
8 cancer in a subject is provided. In some monitoring methods of the present invention a first
9 fluid sample is obtained from the subject at a first time point. The first sample is then
10 subjected to analysis and comparison to a reference value, as set forth above and described
11 further in the examples below. A treatment regimen can then be effectuated based on the
12 value obtained from the first sample. The treatment might involve, for example, drug,
13 radiation or surgical intervention or it might involve further monitoring as discussed below.

14 **[00104]** In some embodiments, the monitoring method of the present invention further
15 comprises, for example, obtaining a second bodily fluid sample from the subject at a second
16 time point. The second sample is then subjected to analysis and comparison to a reference
17 value, as set forth above and described further in the examples below. The reference value
18 obtained in the second sample is then compared to the reference value, to determine if
19 prostate cancer is present, and the value obtained from the first sample to determine if the
20 subject's disease state has improved, worsened or remained constant since the first time
21 point.

22 **[00105]** In some embodiments, monitoring using the method of the present invention can
23 involve the collecting, analyzing and comparing the analytical results from a series of
24 samples taken from the patient over a series of time periods.

25 **[00106]** In some embodiments, the monitoring method of the present invention also
26 provides an opportunity to assess the efficacy of one or more treatments that were provided to
27 the subject during the time between samples obtained from the subject. A subsequent
28 reference value indicating improved disease state would be indicative of a treatment having
29 good efficacy. A subsequent reference value indicating worsened disease state would be
30 indicative of a treatment having poor efficacy.

1 [00107] In some embodiments of the present invention, a method is provided for assessing
2 efficacy of a therapy on a subject having prostate cancer, wherein repeated sampling of a
3 patient is not required. In such methods, a bodily fluid sample is obtained from a subject that
4 has been treated with a prostate cancer therapy. The sample is then analyzed and compared
5 to a reference sample as set forth above and described further in the examples below. The
6 reference value obtained in from the sample is then compared to the reference value to
7 determine if prostate cancer is present. Such a method might be advantageous for
8 determining whether surgery, such as, for example, radical prostatectomy, has successfully
9 removed all PCa tissue.

10 [00108] In some embodiments of the present invention, a kit is provided for detecting
11 prostate cancer in a bodily fluid sample. In some embodiments, the kit comprises a first
12 binding probe that is expressed by prostate epithelial cells, such as, for example, PSMA,
13 PSCA, STEAP1 or STEAP2, and a second binding probe specific to a biomarker that is
14 expressed by prostate cancer (PCa) cells but not by BPH or other non-malignant prostate
15 cells. In some embodiments, the biomarker that is expressed by PCa cells but not by BPH or
16 other non-malignant prostate cells is Ghrelin or C35. The first biomarker must also be
17 present on the surface of microparticles derived from parent PCa cells. The second biomarker
18 must also be present on the surface of MPs derived from parent PCa cells and must not be
19 present on the surface of MPs derived from parent BPH or non-malignant prostate cells.

20 [00109] First and second binding probes might be commercially available or they might be
21 prepared by a skilled artisan, at least because the sequence and structure of PSMA, PSCA,
22 STEAP1, STEAP2, Ghrelin and C35 are known in the art.

23 [00110] In some embodiments, the kit comprises anti-PSMA-RPE IgG and Ghrelin-Cy5 or
24 Ghrelin-FITC.

25 [00111] In some embodiments, the kit of the present invention also comprises first and
26 second negative control binding probes. In some embodiments the negative control binding
27 probes are mouse IgG-RPE and des-acyl Ghrelin-Cy5 or des-acyl Ghrelin-FITC.

28 [00112] In some embodiments, the kit of the present invention provides the first and
29 second binding probes in a first sealed container. In some embodiments, the negative
30 controls are provided in a second sealed container.

1 [00113] In some embodiments, the kits of the present invention might comprise a carrier,
2 such as a box, carton, tube or the like, having disposed therein one or more sealed containers,
3 such as vials, tubes, ampoules, bottles, pouches, envelopes and the like. In some
4 embodiments, the kit might comprise one or more media or media ingredients or reagents for
5 measurement of the various biomarkers disclosed herein. For example, kits of the invention
6 may also comprise, in the same or different containers, one or more suitable buffers or
7 probes. The kits of the present invention may also comprise one or more instructions or
8 protocols for carrying out the methods of the present invention.

9 [00114] The invention will be more fully understood upon consideration of the following
10 non-limiting Examples.

11 [00115] EXAMPLES

12 [00116] The present invention is further illustrated by the following examples, which
13 should not be construed as limiting in any way.

14 [00117] Example 1: Materials and Methods

15 [00118] *Subjects:* Patients were recruited under three REB approved ethics applications,
16 REB103156, REB100960, and REB 18632E.

17 [00119] The patient group made up of patients with BPH included males who were 50+
18 years old, exhibited serum PSA levels greater than 4 ng/mL and whose prostate biopsy
19 yielded no prostate cancer based on pathology reports (N>20).

20 [00120] The patient group made up of patients with localized prostate cancer included
21 males who were 50+ years old, exhibited serum PSA levels greater than 4 ng/mL and whose
22 prostate biopsy yielded evidence of prostate cancer, with a Gleason Score of 6 or above. All
23 patients in this group were candidates for, or had been subjected to, radiation therapy or
24 prostatectomy at the time of blood collection (N>25).

25 [00121] The patient group made up of patients with metastatic prostate cancer included
26 males who were 50+ years old, had received some form of treatment (e.g., radiation therapy
27 or prostatectomy) and who had a relapse of prostate cancer years later, as evidenced by rising
28 levels of PSA, determined as PSA>2 ng/mL. A subpopulation of these patients had positive
29 radiographic bone scans indicating the presence of metastatic PCa bone lesions (N>20).

1 [00122] Patients that were monitored for changes in PCa microparticles after
 2 prostatectomy included males who were 50+ years old who had prostate cancer with a
 3 Gleason score of >6, had a pre-surgery serum PSA value of 4>ng/mL and had a tumor
 4 volume of at least 20mL (N>20).

5 [00123] *Plasma Preparation:* 7ml blood was collected from each subject into Sodium-
 6 Heparin BD Vacutainers (BD Biosciences; Cat# 3678800). To separate plasma from
 7 erythrocytes, blood was spun down at 1500 gs for 10 minutes at 24°C in an Eppendorf
 8 Centrifuge 5810 R. Plasma was removed from the vacutainer in 1mL quantities and
 9 transferred into 1.7mL microtubes tubes (Frogga Bio; Cat#1260-00). To remove residual
 10 platelets or erythrocytes microtubes were spun down at 7000rpm for 5 minutes at room
 11 temperature in Eppendorf Centrifuge 5415 C. Plasma was transferred into 1.5mL cryovials
 12 (Sarstedt; Cat# 72.694.006) in 0.5mL aliquots and stored at -80°C.

13 [00124] *Antibody Conjugation:* Anti-PSMA antibody that binds to the extracellular
 14 domain of PSMA was conjugated to a Phycoerythrin fluorophore using the Lightning-Link
 15 R-Phycoerythrin conjugation kit (Innova Biosciences; Cat# 703-0010). Antibody was
 16 aliquoted and stored at -20°C.

17 [00125] Purified mouse IgG1, κ Isotype Ctrl (Biolegend; Cat# 401402) was conjugated to
 18 a Phycoerythrin fluorophore using the Lightning-Link R-Phycoerythrin conjugation kit
 19 (Innova Biosciences; Cat# 703-0010). Antibody was aliquoted and stored at -20°C.

20 [00126] *Sample Preparation (using Ghrelin-Cy5 Peptide):* The following procedure was
 21 performed in the dark to protect light sensitive reagents. 1 μ L of Anti-PSMA-RPE antibody
 22 (408.42ug/mL) and 1 μ L of LCE 00242-Cy5 (62.5 μ M) were added to 20 μ L of patient plasma
 23 in microtube. The samples were left to incubate in the dark at room temperature for 30
 24 minutes. After incubation, samples were diluted in 600 μ L sterile double-distilled Milli-Q
 25 water.

26 [00127] The sequence of the Ghrelin-Cy5 binding probe LCE00242 is: H-GS-
 27 Dpr(octanoyl)-FLSPEHRQVQQRKES-K(Cy5)-NH₂ (SEQ ID NO:2).

28 [00128] *Sample Preparation (isotype negative control for Ghrelin-Cy5 Peptide):* The
 29 following procedure was performed in the dark to protect light sensitive reagents. 1 μ L of
 30 Mouse IgG-RPE antibody (408.42 μ g/mL) and 1 μ L of LCE 00254-Cy5 (62.5 μ M) were added

1 to 20 μ L of patient plasma in microtube. The samples were left to incubate in the dark at room
 2 temperature for 30 minutes. After incubation, samples were diluted in 600 μ L sterile double-
 3 distilled Milli-Q water.

4 **[00129]** The sequence of the des-acyl Ghrelin-Cy5 binding probe LCE00254 is: H-
 5 GSSFLSPEHRQVQQRKES-K(Cy5)-NH₂ (SEQ ID NO:3).

6 **[00130]** Sample Preparation (Using Ghrelin-FITC Peptide): The following procedure was
 7 performed in the dark due to light sensitive reagents. 1 μ L of Anti-PSMA-PE antibody
 8 (408.42 μ g/mL) and 1 μ L of Ghrelin-FITC (0.125mM) were added to 20 μ L of patient plasma
 9 in microtube. The samples were left to incubate in the dark at room temperature for 30
 10 minutes. After incubation, samples were diluted in 600 μ L sterile double-distilled Milli-Q
 11 water.

12 **[00131]** The sequence of the Ghrelin-FITC binding probe LCE0080 is: H-GS-
 13 Dpr(octanoyl)-FLSPEHRQVQQRKES-K(FITC)-NH₂ (SEQ ID NO:4).

14 **[00132]** Sample Preparation (isotype negative control of Ghrelin-FITC Peptide):
 15 1 μ L of Mouse IgG-RPE antibody (408.42 μ g/mL) and 1 μ L of LCE00203-FITC (0.125mM)
 16 were added to 20 μ L of patient plasma in microtube. The samples were left to incubate in the
 17 dark at room temperature for 30 minutes. After incubation, samples were diluted in 600 μ L
 18 sterile double-distilled Milli-Q water.

19 **[00133]** The sequence of the des-acyl Ghrelin-FITC binding probe LCE00203: H-
 20 GSSFLSPEHRQVQQRKES-K(FITC)-NH₂ (SEQ ID NO:5).

21 **[00134]** *Sample Analysis:* Samples were analyzed using the Apogee A50 Nanoscale Flow
 22 Cytometer. Each sample was run in triplicate at a flow rate of 1.39 μ L/min for a total of 2
 23 minutes.

24 **[00135]** Example 2: Microparticles positive for both PSMA and Ghrelin are indicative of
 25 prostate cancer.

26 **[00136]** A prostate cancer microparticle (PCMP) in this assay is defined as an event that
 27 exhibits a size less than 1 μ m in diameter and exhibits significant binding of both an anti-
 28 PSMA antibody pre-conjugated to a fluorophore (in this case, RPE), and Ghrelin peptide
 29 molecules (D- or L-enantiomer versions pre-conjugated to either FITC or Cy5). Incubation

1 of patient plasma (healthy volunteer) with anti-PSMA-RPE and Ghrelin-Cy5 agents yielded a
2 low number of dual positive events (FIG 1, bottom panel, events within red gate). The red
3 gate is set *a priori* following analysis of the same plasma sample that has been
4 stained separately with the isotype negative controls, mouse IgG-RPE (as a
5 negative isotypecontrol for anti-PSMA antibody) and des-acyl Ghrelin-Cy5 (wherein removal
6 of the side chain on third amino acid prevents the peptide from binding to its receptor,
7 GHSR). When these dual-positive events were gated onto the size histogram (FIG. 1, top
8 panel), resulting events exhibited a size range between 179-304 nm in diameter. These size
9 ranges are based on the analysis of silica sizing beads that exhibit consistent size diameters
10 (110nm, 179nm, 235nm, 304nm, 585nm and 880nm).

11 **[00137]** When this assay was performed on a representative plasma sample from a patient
12 with BPH, a similar result was observed, wherein a small dual-positive subpopulation (dual-
13 positive for anti-PSMA-RPE and Ghrelin-Cy5) was detected, as shown by events in the red
14 gate (FIG. 2, bottom panel). When transposed onto the sizing histogram (FIG. 2, top panel)
15 dual-positive events had a size diameter distribution from 179nm-304nm, indicating that
16 these events were indeed microparticles not background noise or soluble proteins that exhibit
17 sizes of 0.1nm-25nm, which are much smaller than MPs that are 100nm-1000nm.

18 **[00138]** When this assay was performed on a representative plasma sample from a patient
19 with localized prostate cancer (Gleason 7, PSA>4 ng/mL, N0, M0), a more abundant dual-
20 positive subpopulation was observed in the red gate (FIG. 3, bottom panel). A much larger
21 number of dual-positive events was observed relative to healthy BPH samples and, when
22 transposed (FIG. 3, top panel) prostate cancer MP size was in a range from 179nm-304nm.

23 **[00139]** When this assay was performed on a representative plasma sample from a patient
24 with metastatic prostate cancer (evidenced by biochemical failure, PSA >2, PSA nadir
25 ≤ 0.2 ng/mL and bone scan positive for bone metastases), a dense population of dual-positive
26 events was observed in the red gate (FIG. 4, bottom panel). When transposed onto the
27 sizing histogram (FIG.5, upper panel), a size range from 110nm-585nm was observed,
28 suggesting that these dual-positive events are MPs and not soluble proteins or background
29 noise.

30 **[00140]** Plasmas representing patients with BPH, localized PCa and metastatic PCa were
31 analyzed in a blinded and randomized fashion for PCMP counts (dual-positive PSMA-RPE

and Ghrelin-Cy5 events). The difference in PCMP counts between the three cohorts, was largest and most statistically significant between the BPH group and the localized/metastatic PCa groups (FIG. 5). A significantly higher count was observed between BPH patients and Localized PCa or Metastatic PCa patients (* $P < 0.01$, $N > 20$ each group, ANOVA, bon ferroni's test). There was no statistically significant difference between the Localized PCa and Metastatic PCa groups. In this experiment, a cut-off of 17,000 PCMP counts/ μL was used to distinguish patients with BPH from patients with PCa. When counts of prostate microparticles (PSMA-RPE only) were evaluated, no major differences between the groups were observed (FIG. 6). There was no statistically significant difference between any of the groups in terms of prostate microparticle counts present in plasma. Therefore, detection of PCMPs, defined as binding with both PSMA-RPE IgG and the Ghrelin-Cy5 peptide, was the only quantifiable parameter that enabled a distinction between BPH and PCa patient samples.

[00141] To monitor post-prostatectomy patient outcome (surgical removal of prostate and the tumor), blood was collected from patients before surgery and 3-weeks after prostatectomy. Upon analysis of plasmas from these serially collected whole bloods, it was found that a subpopulation of patients exhibited a fold increase in PCMP counts whereas the majority of patients exhibited a fold decrease in PCMP counts (FIG. 7).

[00142] Although the invention has been described with reference to certain specific embodiments, various modifications thereof will be apparent to those skilled in the art without departing from the purpose and scope of the invention as outlined in the claims appended hereto.

[00143] Any examples provided herein are included solely for the purpose of illustrating the invention and are not intended to limit the invention in any way. Any drawings provided herein are solely for the purpose of illustrating various aspects of the invention and are not intended to be drawn to scale or to limit the invention in any way.

WE CLAIM:

1. A method for diagnosing prostate cancer in a subject, the method comprising:
 - a) analyzing a bodily fluid sample to detect in the bodily fluid microparticles having at least first and second biomarkers on their surface, wherein the first biomarker is expressed by prostate epithelial cells and the second biomarker is expressed by prostate cancer cells but not by benign prostatic hyperplastic cells or other non-malignant prostate cells;
 - b) comparing the amount of microparticles positive for both the first and second biomarkers with a reference value,
wherein if the reference value is derived from a non-malignant prostatic sample then a detected value above the reference value is indicative of prostate cancer and a detected value equal to or below the reference value is indicative of a non-malignant disease state and,
wherein if the reference value is derived from a malignant prostatic sample then a detected value equal to or above the reference value is indicative of prostate cancer and a detected value below the reference value is indicative of a non-malignant disease state; and
 - c) diagnosing the subject on the basis of the results obtained in step (b).
2. The method of claim 1, wherein the bodily fluid is blood.
3. The method of claim 1 or 2, wherein the first biomarker is prostate-specific membrane antigen (PSMA), prostate stem cell antigen (PSCA), STEAP1 or STEAP2 and wherein the second biomarker is Ghrelin or C35.
4. The method of claim 3, wherein the first biomarker is PSMA.
5. The method of any one of claims 1 to 4, wherein the second biomarker is Ghrelin.
6. The method of any one of claims 1 to 5, wherein the analysis of bodily fluid is conducted using a flow cytometry assay.
7. The method of claim 6, wherein the flow cytometry assay is carried out using a nanoscale flow cytometer.

8. The method of claim 6 or 7, and wherein said flow cytometry assay comprises exposing the bodily fluid sample to a composition, the composition comprising a first labeled binding probe that is specific to the first biomarker and a second labeled binding probe that is specific to the second biomarker, wherein the labels of the first and second probes are distinguishable.
9. The method of claim 8, wherein the first labeled binding probe is anti-PSMA-RPE IgG.
10. The method of claim 8 or 9, wherein the second labeled probe is Ghrelin-Cy5 or Ghrelin-FITC.
11. The method of any one of claims 1 to 10, wherein the analysis is carried out with reference to at least one negative control of the first and second binding probes, wherein the at least one negative control comprises first and second negative control binding probes.
12. The method of claim 11, wherein the first negative control binding probe is mouse IgG-RPE.
13. The method of claim 11 or 12, wherein the second negative control binding probe is des-acyl Ghrelin-Cy5 or des-acyl Ghrelin-FITC.
14. The method of any one of claims 1 to 13, wherein the reference value represents the amount of microparticles positive for the first and second biomarkers in a sample obtained from a subject having a non-malignant prostate or benign prostatic hyperplasia (BPH) and wherein the detected value is an increase relative to the reference value.
15. The method of claim 14, wherein the reference value is in a range of 14,000 to 21,000 prostate cancer microparticle (PCMP) counts/ μ L.
16. The method of claim 15, wherein the reference value is about 17,000 PCMP counts/ μ L.
17. A method for monitoring prostate cancer in a subject, the method comprising:
 - a) analyzing a first bodily fluid sample, wherein the first sample was obtained from the subject at a first time point, to detect microparticles having at least first and second biomarkers

on their surface in the bodily fluid sample, wherein the first biomarker is expressed in prostate epithelial cells and the second biomarker is expressed by prostate cancer (PCa) cells but not by benign prostatic hyperplasia or other non-malignant prostate cells;

b) comparing the amount of microparticles positive for both the first and second biomarkers with a reference value,

wherein if the reference value is derived from a non-malignant prostatic sample then a detected value above the reference value is indicative of prostate cancer and a detected value equal to or below the reference value is indicative of a non-malignant disease state and,

wherein if the reference value is derived from a malignant prostatic sample then a detected value equal to or above the reference value is indicative of prostate cancer and a detected value below the reference value is indicative of a non-malignant disease state;

c) diagnosing the subject on the basis of the results obtained in step (b);

d) analyzing a second bodily fluid sample, wherein the second sample was obtained from the subject at a second time point after a treatment regimen, to detect microparticles having at least first and second biomarkers on their surface in the bodily fluid sample, wherein the first biomarker is expressed in prostate epithelial cells and the second biomarker is expressed by prostate cancer (PCa) cells but not by benign prostatic hyperplasia or other non-malignant prostate cells;

e) comparing the amount of microparticles positive for both the first and second biomarkers with a reference value,

wherein if the reference value is derived from a non-malignant prostatic sample then a detected value above the reference value is indicative of prostate cancer and a detected value equal to or below the reference value is indicative of a non-malignant disease state and,

wherein if the reference value is derived from a malignant prostatic sample then a detected value equal to or above the reference value is indicative of prostate cancer and a detected value below the reference value is indicative of a non-malignant disease state;

f) comparing the amount of microparticles positive for the first and second biomarker in the second bodily fluid sample with a the value obtained in step (b) wherein an increase in the amount of microparticles positive for the first and second biomarkers relative to the value obtained in step (b) is indicative of a worsened disease state and a decrease in the amount of microparticles positive for the first and second biomarkers relative to the value obtained in step (b) is indicative of an improved disease state; and

g) diagnosing any change in the subject's disease state on the basis of the results obtained in step (f).

18. The method of claim 17, wherein the bodily fluid is blood.

19. The method of claim 17 or 18, wherein the first biomarker is prostate-specific membrane antigen (PSMA), prostate stem cell antigen (PSCA), STEAP1 or STEAP2 and wherein the second biomarker is Ghrelin or C35.

20. The method of claim 19, wherein the first biomarker is PSMA.

21. The method of any one of claims 17 to 20, wherein the second biomarker is Ghrelin.

22. The method of any one of claims 17 to 21, wherein the analysis of bodily fluid is conducted using a flow cytometry assay.

23. The method of claim 22, wherein the flow cytometry assay is carried out using a nanoscale flow cytometer.

24. The method of claim 22 or 23, wherein said flow cytometry assay comprises exposing the bodily fluid sample to a composition, the composition comprising a first labeled binding probe that is specific to the first biomarker and a second labeled binding probe that is specific to the second biomarker, wherein the labels of the first and second probes are distinguishable.

25. The method of claim 24, wherein the first labeled binding probe is anti-PSMA-RPE IgG.

26. The method of claim 24 or 25, wherein the second labeled probe is Ghrelin-Cy5 or Ghrelin-FITC.

27. The method of any one of claims 17 to 26, wherein the analysis is carried out with reference to at least one negative control of the first and second binding probes, wherein the at least one negative control comprises first and second negative control binding probes.

28. The method of claim 27, wherein the first negative control binding probe is mouse IgG-RPE.
29. The method of claim 27 or 28, wherein the second negative control binding probe is des-acyl Ghrelin-Cy5 or des-acyl Ghrelin-FITC.
30. The method of any one of claims 17 to 29, wherein the reference value represents the amount of microparticles positive for the first and second biomarkers in a sample obtained from a subject having a non-malignant prostate or benign prostatic hyperplasia (BPH) and wherein the detected value is an increase relative to the reference value.
31. The method of claim 30, wherein the reference value is in a range of 14,000 to 21,000 prostate cancer microparticle (PCMP) counts/ μ L.
32. The method of claim 31, wherein the reference value is about 17,000 PCMP counts/ μ L.
33. A method for assessing efficacy of a therapy on a subject having prostate cancer, the method comprising:
- a) analyzing a bodily fluid sample from a subject, wherein the subject has been subjected to a prostate cancer therapy, to detect microparticles having at least first and second biomarkers on their surface in the bodily fluid sample, wherein the first biomarker is expressed in prostate cells and the second biomarker is expressed by prostate cancer (PCa) cells but not by benign prostatic hyperplasia or other non-malignant prostate cells;
 - b) comparing the amount of microparticles positive for both the first and second biomarkers with a reference value,
- wherein if the reference value is derived from a non-malignant prostatic sample then a detected value above the reference value is indicative of prostate cancer and a detected value equal to or below the reference value is indicative of a non-malignant disease state and,
- wherein if the reference value is derived from a malignant prostatic sample then a detected value equal to or above the reference value is indicative of prostate cancer and a detected value below the reference value is indicative of a non-malignant disease state; and

c) diagnosing the efficacy of the therapy as good if the value obtained in step (b) indicates a non-malignant disease state or poor if the value obtained in step (b) indicates prostate cancer.

34. The method of claim 33, wherein the bodily fluid is blood.

35. The method of claim 33 or 34, wherein the first biomarker is prostate-specific membrane antigen (PSMA), prostate stem cell antigen (PSCA), STEAP1 or STEAP2 and wherein the second biomarker is Ghrelin or C35.

36. The method of claim 35, wherein the first biomarker is PSMA.

37. The method of any one of claims 33 to 36, wherein the second biomarker is Ghrelin.

38. The method of any one of claims 33 to 37, wherein the analysis of bodily fluid is conducted using a flow cytometry assay.

39. The method of claim 38, wherein the flow cytometry assay is carried out using a nanoscale flow cytometer.

40. The method of claim 38 or 39, and wherein said flow cytometry assay comprises exposing the bodily fluid sample to a composition, the composition comprising a first labeled binding probe that is specific to the first biomarker and a second labeled binding probe that is specific to the second biomarker, wherein the labels of the first and second probes are distinguishable.

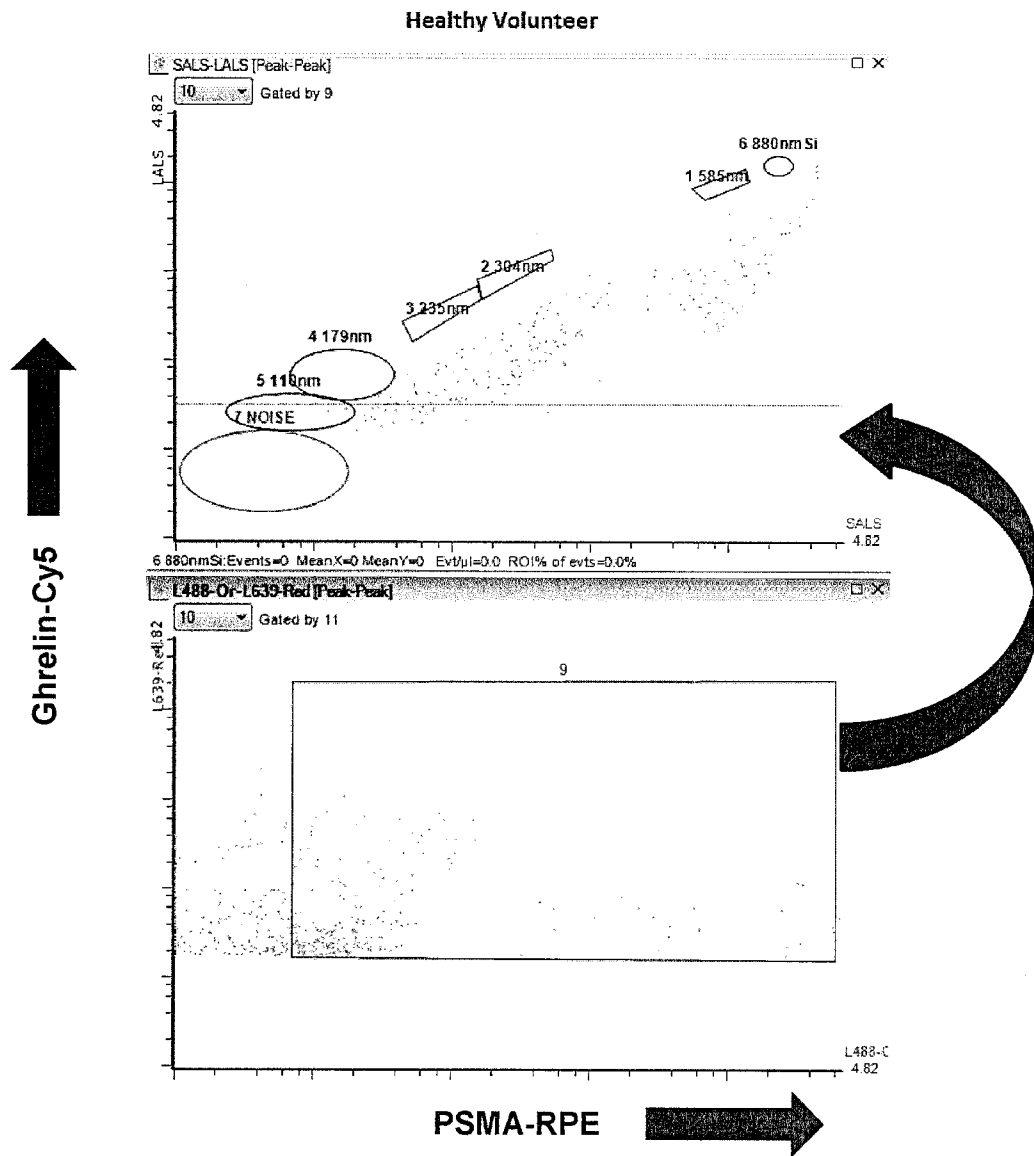
41. The method of claim 40, wherein the first labeled binding probe is anti-PSMA-RPE IgG.

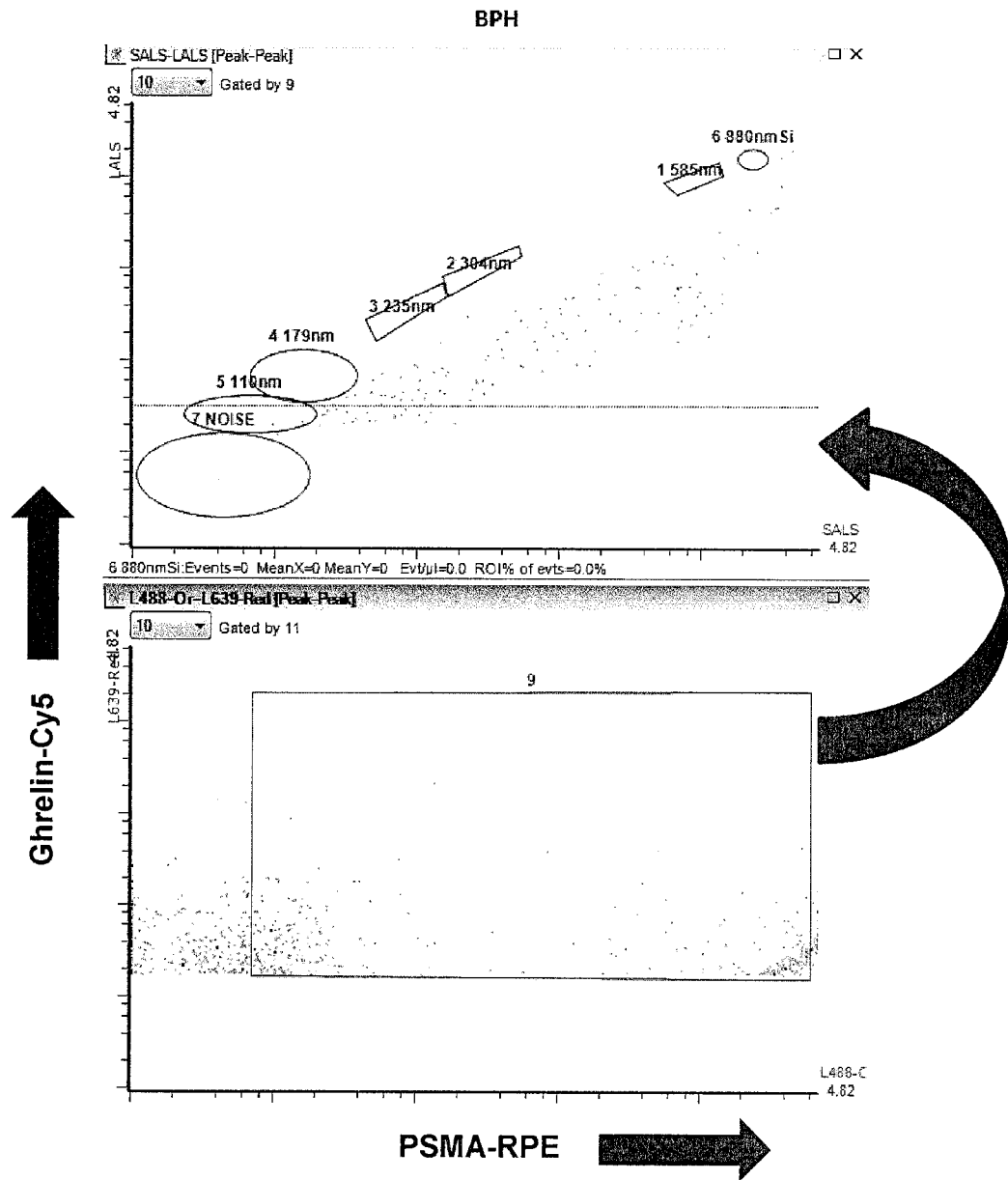
42. The method of claim 40 or 41, wherein the second labeled probe is Ghrelin-Cy5 or Ghrelin-FITC.

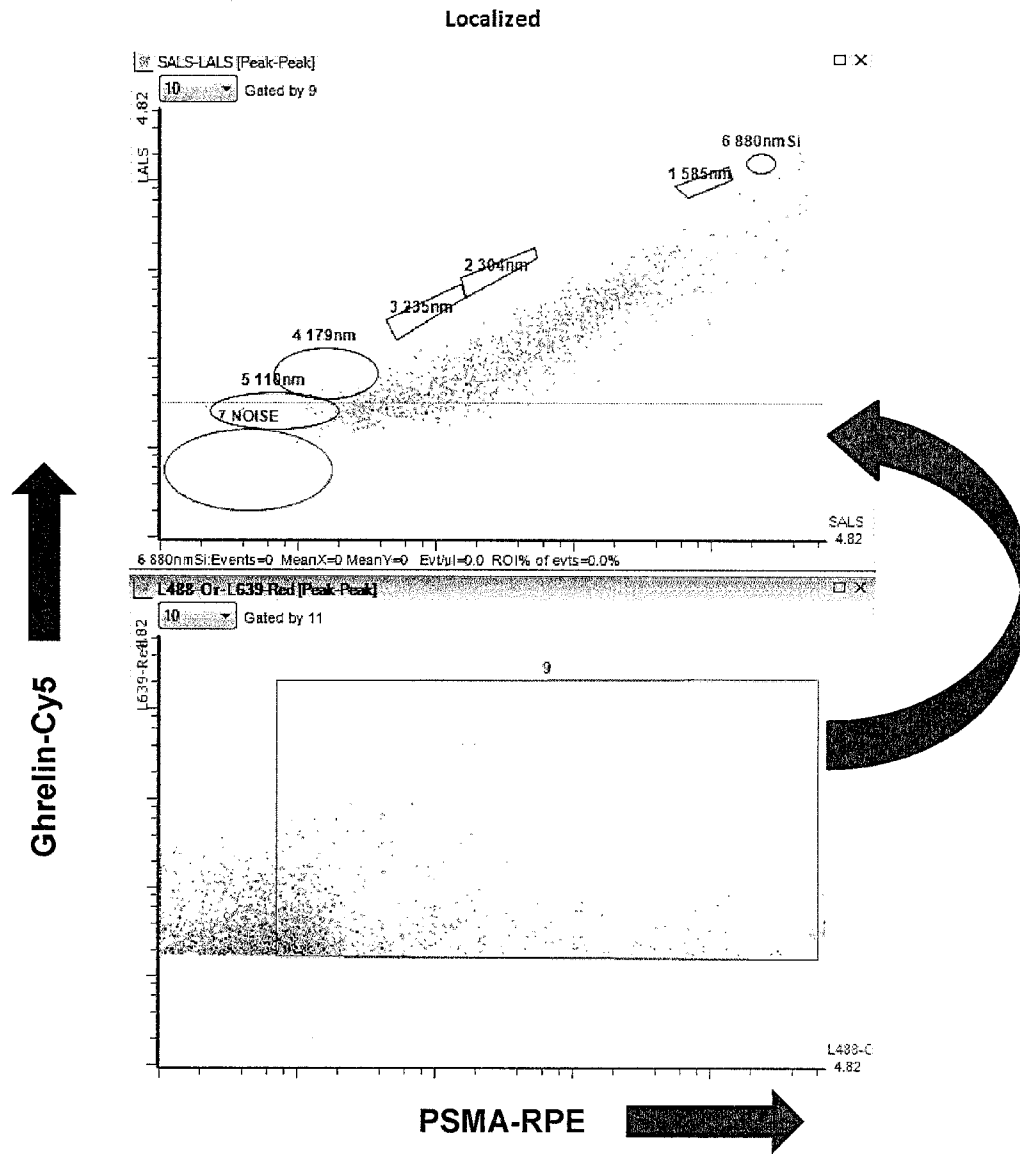
43. The method of any one of claims 33 to 42, wherein the analysis is carried out with reference to at least one negative control of the first and second binding probes, wherein the at least one negative control comprises first and second negative control binding probes.
44. The method of claim 43, wherein the first negative control binding probe is mouse IgG-RPE.
45. The method of claim 43 or 44, wherein the second negative control binding probe is des-acyl Ghrelin-Cy5 or des-acyl Ghrelin-FITC.
46. The method of any one of claims 33 to 45, wherein the reference value represents the amount of microparticles positive for the first and second biomarkers in a sample obtained from a subject having a non-malignant prostate or benign prostatic hyperplasia (BPH) and wherein the detected value is an increase relative to the reference value.
47. The method of claim 46, wherein the reference value is in a range of 14,000 to 21,000 prostate cancer microparticle (PCMP) counts/ μ L.
48. The method of claim 47, wherein the reference value is about 17,000 PCMP counts/ μ L.
49. A method of any one of claims 1 to 48, wherein the subject is a mammal.
50. The method of claim 49, wherein the mammal is a human.
51. A kit for detecting prostate cancer in a bodily fluid sample, the kit comprising
a first binding probe specific to a first biomarker that is expressed by prostate epithelial cells, and
a second binding probe specific to a second biomarker that is expressed by prostate cancer (PCa) cells but not by benign prostatic hyperplasia or other non-malignant prostate cells.
52. The kit of claim 51, wherein the first biomarker is prostate-specific membrane antigen (PSMA), prostate stem cell antigen (PSCA), STEAP1 or STEAP2 and wherein the second biomarker is Ghrelin or C35.

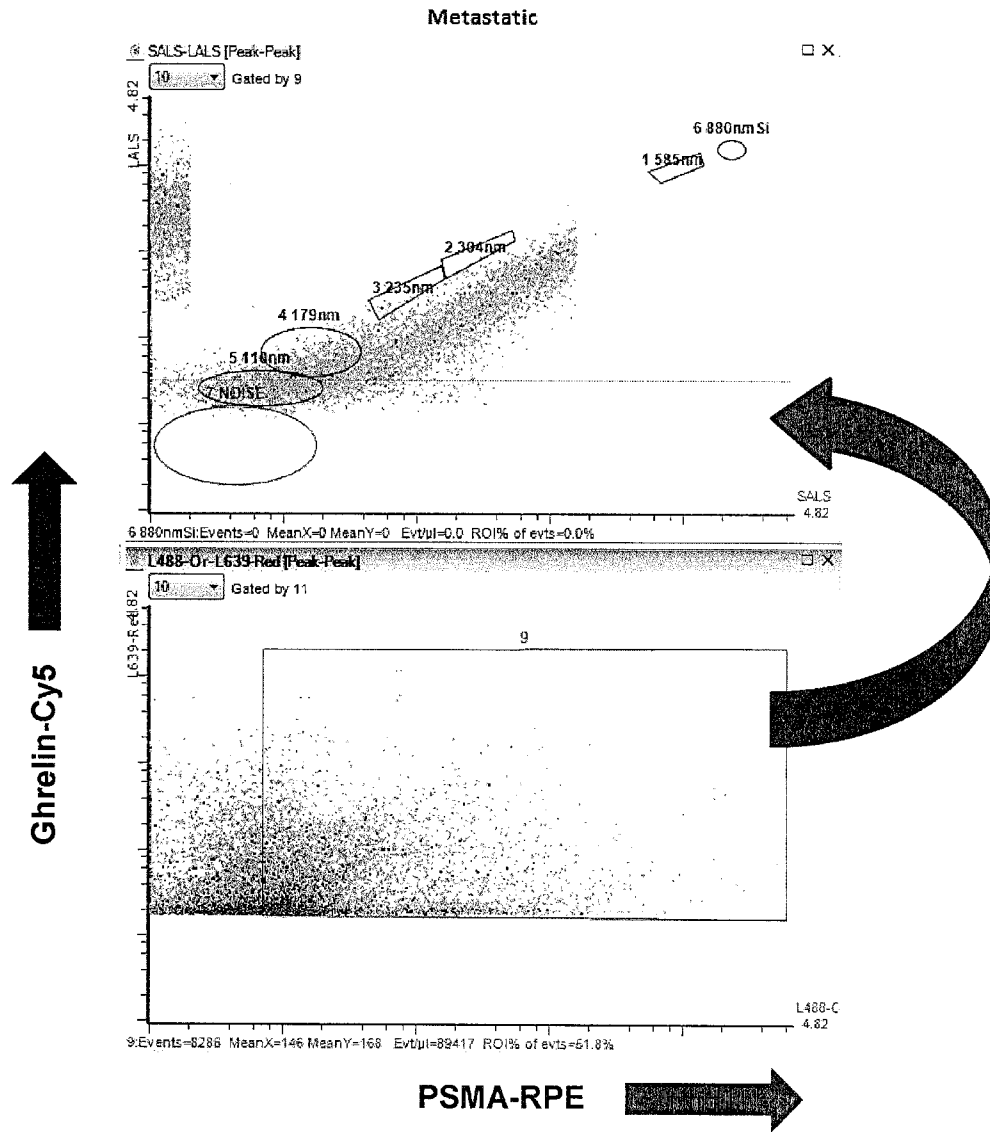
53. The kit of claim 52, wherein the first biomarker is PSMA.
54. The kit of claim 52 or 53, wherein the first binding probe is anti-PSMA-RPE IgG.
55. The kit of any one of claims 51 to 54, wherein the second biomarker is Ghrelin.
56. The kit of any one of claims 51 to 55, wherein the second binding probe is Ghrelin-Cy5 or Ghrelin-FITC.
57. The kit of any one of claims 51 to 56, further comprising a first negative control binding probe specific to mouse IgG.
58. The kit of claim 57, further comprising a second negative control binding probe specific to des-acyl Ghrelin.
59. The kit of claim 57 or 58, wherein the first negative control binding probe is monoclonal antibody mouse IgG-RPE.
60. The kit of claim 58 or 59, wherein the second negative control binding probe is des-acyl Ghrelin-Cy5 or des-acyl Ghrelin-FITC.
61. The kit of any one of claims 51 to 60, further comprising a first and second sealed container, wherein the first sealed container comprises anti-PSMA-RPE IgG and Ghrelin-Cy5 or Ghrelin-FITC and the second sealed container comprises mouse IgG-RPE and des-acyl Ghrelin-Cy5 or des-acyl Ghrelin-FITC.
62. The kit of any one of claims 51 to 61, further comprising a carrier, wherein a carrier is a box, carton, or tube.
63. The kit of claim 62, wherein the carrier comprises one or more sealed containers, wherein the one or more sealed container is a vial, tube, ampoule, bottle, pouch or envelope.

64. The kit of any one of claims 51 to 63, further comprising one or more media, media ingredients or reagents for measurement of at least one of the first and second biomarkers.
65. The kit of claim 64, wherein the one or more reagents are buffers or probes.
66. The kit of any one of claims 51 to 65, further comprising one or more instructions or protocols for carrying out the methods of any one of claims 1 to 50.









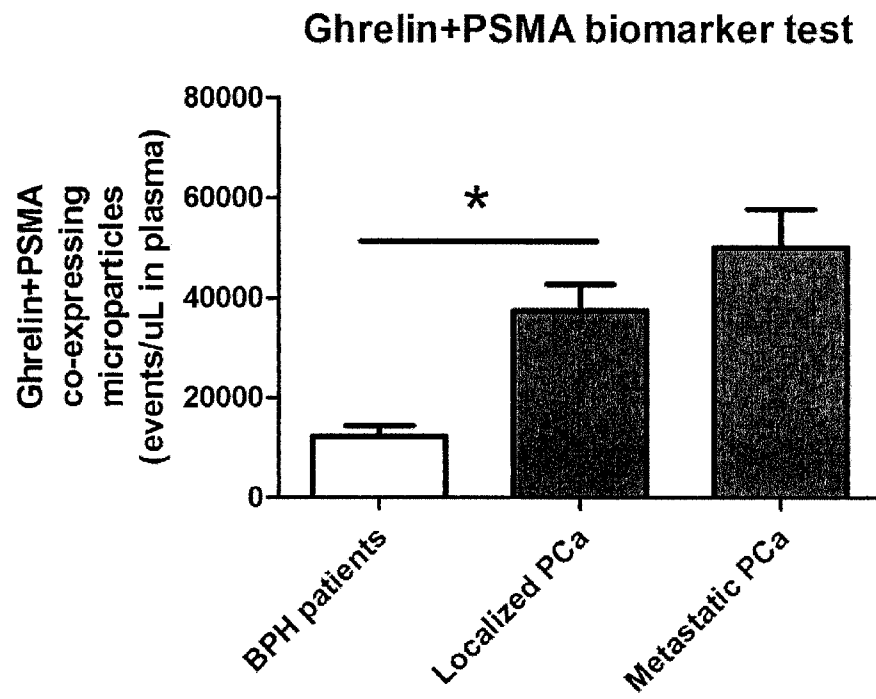


FIG. 5

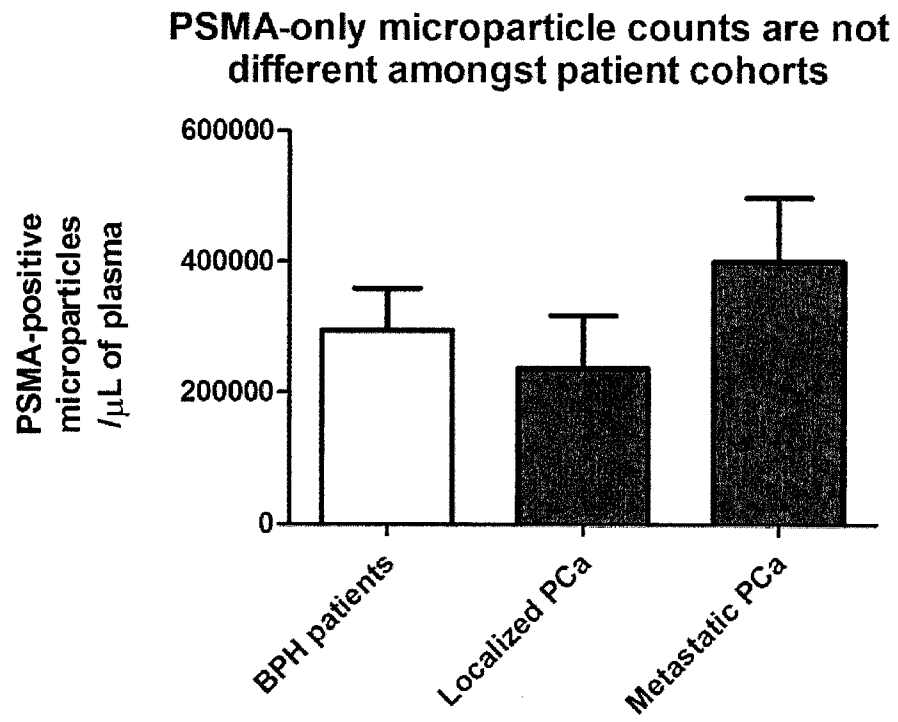


FIG. 6

Patient ID	Fold change in PCMPs after prostatectomy	
HL 101	2.5X	↑
HL 102	45X	
HL 103	2X	
HL 104	3.6X	
HL 51	2.6X	↑
HL 106	2.5X	
HL 108	0.5X	
HL 110	19.7X	
HL 111	3.7X	↑
HL 112	7.9X	
HL 109	5.9X	
HL 115	5.3X	
HL 121	9.6X	↑
HL 122	1.5X	
HL 123	5.7X	
HL 125	2.6X	
HL 126	24.6X	
HL 127	2.9X	
HL 128	2X	
HL 129	3.3X	
HL 130	1.5X	

FIG. 7