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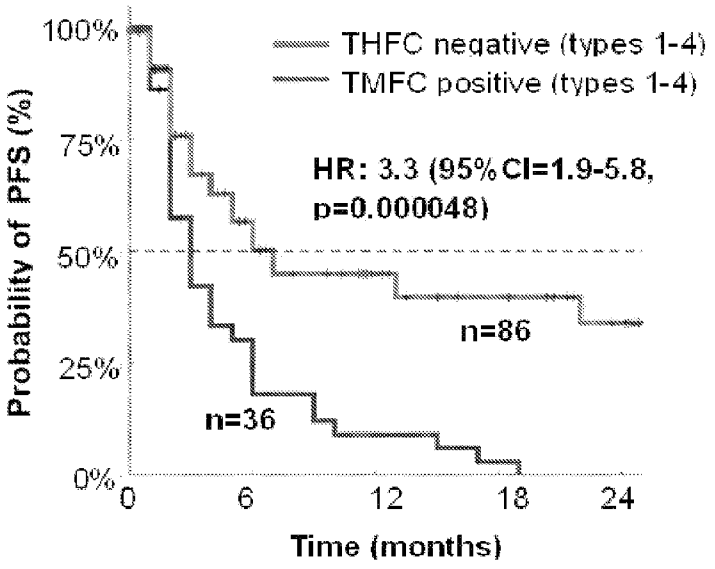
FIG. 3

A.



(57) Abstract: Methods for predicting overall survival (OS) and progression free survival (PFS) of subjects having cancer, based on the presence of Tumor Macrophage Hybrid Cells (TMHCs), a unique group of cell types that include fusions and/or clusters of different cells found in the blood of subjects having solid tumors, are provided.

WO 2024/250002 A1

[Continued on next page]

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METHODS FOR PREDICTING PROGRESSION FREE SURVIVAL AND OVERALL SURVIVAL IN SUBJECTS HAVING CANCER USING TUMOR MACROPHAGE HYBRID CELLS

BACKGROUND

Field of the Invention

[0001] The present invention generally relates to the use of biomarkers in the blood and other bodily fluids to make predictions regarding overall survival and progression free survival in subjects having cancer, such as solid tumors.

Related Art

[0002] When tumor cells break away from primary solid tumors, they penetrate into the blood or lymphatic circulation, and ultimately leave the blood stream and enter either organs or tissue to form metastasis. 90% of cancer-related deaths are caused by the metastatic process. The most common metastatic sites are the lung, liver, bone and brain. Tumor cells found in the circulation are called circulating tumor cells (CTCs). Many research publications and clinical trials show that CTCs have clinical utility in (i) providing prognostic survival and cancer recurrence information through the enumeration of CTCs in the blood stream, and (ii) providing treatment information through examination of protein expression levels, and the occurrence of gene mutations and translocations in the CTCs. However, CTCs are not consistently associated with the development and/or presence of cancer in a subject, even in stage IV cancer patients. While CTCs are found most often in stage IV of breast, prostate and colorectal cancers, they are rare in early stages of the same cancer. CTCs are also rare in other cancers.

[0003] Circulating Cancer Associated Macrophage-Like cells (CAMLs) are another cancer-related cell type that is found in the blood of subjects having cancer. CAMLs are associated with all solid tumors tested and all stages of cancer. CAMLs are polyploid and very large in size, ~20 μm to ~300 μm in size. These polyploid cells can be either CD45(-) or CD45(+) and express CD11c, CD14 and CD31, which confirms their origin as a myeloid lineage. They are often found in the process of engulfing CTCs and cell debris [14.19]. The examination of protein expression levels in CAMLs can also assist clinicians in making informed treatment decisions.

[0004] Assays associated with additional types of cancer-associated circulating cells, in blood and other body fluids, could be used to provide further important prognostic and treatment information for a subject having cancer. The present invention is directed to discovery of such cells and other important goals.

SUMMARY

[0005] The present invention is directed prognostic methods that make use of Tumor Macrophage Hybrid Cells (TMHCs), a unique group of cell types that include fusions and/or clusters of different cells that are found in the blood of subjects having solid tumors, including carcinoma, sarcoma, neuroblastoma and melanoma. These circulating cells have been shown to be associated with the presence of solid tumors in a subject having cancer. TMHCs have been found in peripheral blood of subjects having solid tumors via microfiltration using precision microfilters.

[0006] As discussed in detail below, the present inventors have discovered that the presence of TMHCs in the blood of a subject having cancer is an indicator of more aggressive disease with worse clinical outcomes. Thus, medical applications associated with TMHCs include, but are not limited to, use of the cells themselves as biomarkers to provide early detection of cancer and diagnosis of cancer, in particular, in the early detection and diagnosis of cancer relapse or recurrence, and in the determination of cancer mutation.

[0007] More specifically, and in a first embodiment, the present invention is directed to a method for predicting overall survival (OS) and/or progression free survival (PFS) of a subject having cancer. The method comprises determining the presence of TMHCs in a biological sample from a subject having cancer, wherein the presence of TMHCs predicts lower OS and/or PFS than a subject having the same cancer without the presence of TMHCs.

[0008] In a second embodiment, the invention is directed to a method for predicting presence of metastatic spread and/or metastatic progression in a subject having cancer. The method comprises determining the presence of TMHCs in a biological sample from a subject having cancer, wherein the presence of TMHCs predicts presence of metastatic spread and/or metastatic progression in the subject.

[0009] In a third embodiment, the invention is directed to a method for predicting cancer progression in a subject having cancer. The method comprises determining the presence TMHCs in a first biological sample and a second biological sample at a later date, and optional additional biological samples, obtained from a subject having cancer, wherein when TMHCs are present in the second and/or additional biological sample but not present in the first biological sample, the cancer is predicted to progress in the subject.

[0010] In a fourth embodiment, the invention is directed to a method for predicting response to treatment in a subject having cancer. The method comprises determining the presence TMHCs in a first biological sample and a second biological sample at a later date, and optional additional biological samples, obtained from a subject having cancer, wherein the first sample is obtained from the subject prior to or during cancer treatment, wherein the second sample and optional additional samples are obtained from the subject after at least one cancer treatment, wherein when TMHCs are present in the first biological sample but not present in the second and/or additional biological sample, the subject is predicted to respond to the treatment.

[0011] In each of the embodiments and aspects of the invention, the TMHCs comprise one or more of the following cell types:

(a) partial tumor macrophage fusion cells;

(b) homodimeric tumor macrophage fusion cells;

(c) cannibalistic tumor macrophage fusion cells;

(d) binucleated tumor macrophage fusion cells;

(e) heterotypic circulating tumor cell (CTC) clusters;

(f) homotypic circulating tumor cell (CTC) clusters; and

(g) epithelial-mesenchymal transition circulating tumor cell (EMT CTCs) clusters.

[0012] In aspects of the first embodiment, OS and/or PFS is over a period of at least 12 months. In other aspects of the first embodiment, OS and/or PFS is over a period of at least 24 months.

[0013] In each of the embodiments and aspects of the invention, the biological sample is one or more of blood (such as peripheral blood), lymph node, bone marrow, cerebral spinal fluid, and urine. For example, the biological sample may be antecubital-vein blood, inferior-vena-cava blood, femoral vein blood, portal vein blood, or jugular-vein blood.

[0014] In each of the embodiments and aspects of the invention, the size of the biological sample is between 5 and 15 mL.

[0015] The cancer in each of the embodiments of the invention is a Stage I cancer, Stage II cancer, Stage III cancer, Stage IV cancer, carcinoma, sarcoma, neuroblastoma, melanoma, epithelial cell cancer, lung cancer, breast cancer, prostate cancer, pancreatic cancer, bladder cancer, kidney cancer, head and neck cancer, colorectal cancer, liver cancer, ovarian cancer, osteosarcoma, esophageal, brain & ONS, larynx, bronchus, oral cavity and pharynx, stomach, testis, thyroid, uterine cervix, uterine corpus cancer or other solid tumor cancers, or a blood cancer.

[0016] The TMHCs may be isolated from the biological samples for the determining steps using one or more means selected from the group consisting of size exclusion methodology, immunocapture, red blood cell lysis, white blood cell depletion, a high-molecular weight polysaccharide such as FICOLL®, electrophoresis, dielectrophoresis, flow cytometry, magnetic levitation, and various microfluidic chips, slits, channels, hydrodynamic size-based sorting, grouping, trapping, concentrating large cells, eliminating small cells, or a combination thereof.

[0017] The TMHCs may be isolated from the biological samples using size exclusion methodology that comprises using a microfilter. The microfilter may have a pore size ranging from about 5 microns to about 20 microns. Additionally, the pores of the microfilter may have a round, race-track shape, oval, square and rectangular pore shape. The microfilter may also have precision pore geometry and uniform pore distribution.

[0018] The TMHCs may be isolated using a microfluidic chip via physical size-based sorting, hydrodynamic size-based sorting, grouping, trapping, immunocapture, concentrating large cells, or eliminating small cells based on size.

[0019] The TMHCs may be isolated from the biological samples for the determining steps using a microfiltration assay.

[0020] In relevant embodiments, the treatment is one or more of chemotherapy, single drug, combination of drugs, immunotherapy, radiation therapy, chemoradiation, radiation combined with single or multiple drugs, chemoradiation combined with single or multiple drugs, cancer vaccine, and cell therapy. As a non-limiting example, the treatment may be a cancer vaccine and the subject expresses at least one HLA allele.

[0021] In relevant embodiments, the subject may be treated with one or more of chemotherapy, single drug, combination of drugs, immunotherapy, radiation therapy, chemoradiation, radiation combined with single or multiple drugs, chemoradiation combined with single or multiple drugs, cancer vaccine, and cell therapy. As a non-limiting example, the immunotherapy may be PD-L1 immunotherapy.

[0022] TMHCs may be used independently as cancer markers, or in combination with biomarker expression by other circulating cells, such as CAMLs and other circulating tumor cells (CTCs). Suitable CTC subtypes include, but are not limited to, pathologically definable CTCs (PDCTCs), apoptotic CTCs, circulating cancer associated vascular endothelial cells (CAVEs). The presence of these additional cell types themselves and also cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), methylated DNA, proteomic, metabolomic, lipidomic and other biomarkers may provide a more complete understanding of a patient's disease.

[0023] In each aspect and embodiment of the invention, the methods further comprise administering a therapeutically effective amount of a cancer treatment to the subject. The subject may be a subject in which the OS and PFS is predicted to be lower or shorter than the OS and PFS of another subject.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] **FIG. 1** is a diagram of the different TMFC subtypes and images of TMFCs from metastatic breast cancer patient blood.

[0025] **FIG. 2** is a diagram of the different CTC cluster subtypes and images of CTC clusters from metastatic breast cancer patient blood.

[0026] **FIG. 3** shows survival times. **A.** shows progression free survival (PFS) for mBC patients with any TMFCs (types (1)-(4)) detected in blood. **B.** shows overall survival (OS) for mBC patients with any TMFCs (types (1)-(4)) detected in blood.

[0027] **FIG. 4** shows Cox Proportional Survival Analysis of PFS (**A.**) and OS (**B.**) for no CTCs (Green Line), with CTCs (Black Line) or with TMFCs (not including hyperploidy fusion cells (CAMLs)) (Red Line) at BL.

[0028] **FIG. 5** shows PFS of Homotypic vs Heterotypic CTC Clusters.

[0029] **FIG. 6** shows PFS of EMT Clusters vs ≥ 50 μm CAMLs.

DETAILED DESCRIPTION

[0030] The matters defined in the description such as a detailed construction and elements are nothing but the ones provided to assist in a comprehensive understanding of the invention. Accordingly, those of ordinary skill in the art will recognize that various changes and modifications of the embodiments described herein can be made without departing from the scope and spirit of the invention.

[0031] Cancer is one of the most feared illness in the world, affecting all populations and ethnicities in all countries. Approximately 40% of both men and women will develop cancer in their lifetime. In the United States alone, at any given time there are more than 12 million cancer patients, with 1.7 million new cancer cases and more than 0.6 million deaths estimated yearly. Cancer death worldwide is estimated to be about 8 million annually, of which 3 million occur in developed countries where patients have access to treatment.

[0032] Liquid biopsies provide real-time, sequential tracking of diagnostically-important circulating cells isolated from a subject having cancer. Cells such as circulating tumor cells (CTCs) are found in the peripheral blood of cancer patients and previous work has shown that CTC-based assays can be used as a substitute to tissue biopsies [1-4].

[0033] Recently, another circulating cell associated with cancer has been identified in the peripheral blood of cancer patients. This cancer stromal cell subtype has been termed a cancer associated macrophage-like cell or CAML. CAMLs have been identified in the blood using a non-affinity microfiltration based method which captures both CTCs and CAMLs, and allows for singular or parallel analysis of these cancer specific circulating cell subtypes [1, 6-16]. CAMLs are a recently defined circulating myeloid derived stromal cell, found in all the stages of invasive malignancy and in various solid malignancies (e.g. breast, prostate, non-small cell lung carcinoma (NSCLC), and pancreatic) [11, 13, 14, 17]. CAMLs are specialized myeloid polyploid cells in the blood in all stages of solid tumors. They are easy to identify by their large size (greater than 20 μm), polyploid nucleus and morphologies: round, rod shaped, with one tail or two tails 180 degrees apart. CAMLs typically express CD31, CD14, CD45 and cytokeratin, and can also express EpCAM, CD146, CD11c and tie2 [11, 13, 14, 17].

[0034] As defined herein, a third group of cancer-associated circulating cells has recently been characterized and comprises Tumor Macrophage Hybrid Cells (TMHCs). These cells are a unique group of cell types that include fusions and/or clusters of different cells that are found in the blood of subjects having solid tumors, including carcinoma, sarcoma, neuroblastoma and melanoma. These circulating cells have been shown to be associated with the presence of solid tumors in a subject having cancer. TMHCs have been found in peripheral blood of subjects having solid tumors via microfiltration using precision microfilters.

[0035] The presence of TMHCs in the blood of a subject having cancer is an indicator of more aggressive disease with worse clinical outcomes. Thus, medical applications associated with TMHCs include, but are not limited to, use of the cells themselves as biomarkers to provide early detection of cancer and diagnosis of cancer, in particular, in the early detection and diagnosis of cancer relapse or recurrence, and in the determination of cancer mutation. The simple presence of one or more of these circulating cell types in a blood sample obtained from a subject having cancer provides diagnostic information.

Tumor Macrophage Hybrid Cells (TMHCs).

[0036] Recently, it was described that macrophages and tumor cells can fuse to form tumor macrophage fusion cells (TMFCs), which are detectable within primary tumors and a patient's blood. Similarly, circulating tumor cell clusters (CTCCs) have been described which are aggregated groups of tumor cells that detached from primary tumors and circulate in the bloodstream. While circulating tumor cells (CTCs) are a well studied phenomenon, CTCCs remain relatively unexplored and ill-defined.

[0037] As defined herein, these circulating fusion cells and clustered cells are collectively termed "Tumor Macrophage Hybrid Cells" or TMHCs. Each reference to "circulating cells" is synonymous with TMHCs, and each reference to "TMHCs" is synonymous with circulating cells. The TMHCs of the present invention comprise one or more of the following cell types:

(a) partial tumor macrophage fusion cells;

(b) homodimeric tumor macrophage fusion cells;

(c) cannibalistic tumor macrophage fusion cells;

(d) binucleated tumor macrophage fusion cells;

(e) heterotypic circulating tumor cell (CTC) clusters;

(f) homotypic circulating tumor cell (CTC) clusters; and

(g) epithelial-mesenchymal transition circulating tumor cell (EMT CTCs) clusters.

[0038] Partial tumor macrophage fusion cells are cellular fusions between tumor cells and macrophages where there is some membrane interaction between the cells and both cells retaining their original phenotypes (**FIG. 1**).

[0039] Homodimeric tumor macrophage fusion cells are cellular fusions between tumor cells and macrophages where the two cells have a fused membrane and shared cytoplasm (**FIG. 1**).

[0040] Cannibalistic tumor macrophage fusion cells are cellular fusions between tumor cells and macrophages where the tumor cells has been taken up by the macrophage and both cells retaining their original phenotypes (**FIG. 1**).

[0041] Binucleated tumor macrophage fusion cells are cellular fusions between tumor cells and macrophages where both cells completely merge to become one cell with dual expression of phenotypes (**FIG. 1**).

[0042] Heterotypic CTC clusters are clusters of CTCs and immune/stromal white blood cells (WBCs) (**FIG. 2**).

[0043] Homotypic CTC clusters are clusters of only CTCs (**FIG. 2**).

[0044] Epithelial-mesenchymal transition circulating tumor cell (EMT CTCs) clusters are clusters of CTCs undergoing epithelial-mesenchymal transition (EMT), a process whereby tumor cells downregulate epithelial traits and upregulate mesenchymal traits (**FIG. 2**).

[0045] TMHCs can be visualized by colorimetric stains, such as H&E, or fluorescent staining of specific markers as shown in **FIG. 1** and **FIG. 2**.

Circulating Tumor Cells

[0046] As defined herein, CTCs associated with carcinomas express a number of cytokeratins (CKs). CK 8, 18, & 19 are the cytokeratins most commonly expressed and used in diagnostics, but surveying need not be limited to these markers alone. The surface of solid tumor CTCs usually express epithelial cell adhesion molecule (EpCAM). However, this expression is not uniform or consistent. CTCs do not express any CD45 because it is a white blood cell marker. In

assays to identify tumor-associated cells, such as CTCs and CAMLs, it is sufficient to use antibodies against markers associated with the solid tumor such as CK 8, 18 and 19, or antibodies against CD45 or DAPI.

[0047] Different subgroups of CTCs upregulate and/or down regulate phenotypes and marker expression in relation to tumor progression, tumor spread, and in response to tumor treatments. Therefore, assessing CTCs and the markers expressed by such cells in the peripheral blood can provide important information regarding the status of the cancer in the subject.

[0048] CTCs that can be used as cancer diagnostics, as well as means for screening and monitoring treatment and determining the susceptibility of a tumor in a particular subject to a particular treatment, can be divided into three subgroups. The first subgroup is pathologically definable CTCs (PDCTCs) [6-10]. PDCTCs can be characterized by: a "cancer-like" nucleus stained by DAPI; cytokeratin having a filamentous pattern; expression of one or more of CK 8, 18 and 19 (CTCs from epithelial cancers usually express at least CK 8, 18 and 19); lack of CD45 expression.

[0049] The second subgroup is apoptotic CTCs. When a CTC dies, the cytokeratin pattern degrades into dots. Therefore, early apoptotic CTC have some cytokeratin dots and later apoptotic CTCs have all of the cellular cytokeratin degraded into dots. Apoptotic CTCs can also be characterized as expressing CK 8, 18 and 19; a degrading nuclei; lack of CD45 expression.

[0050] In the third subgroup of CTCs, the cells are undergoing epithelial to mesenchymal transition (EMTCTCs) [2, 3, 5-9]. EMT is a gradual morphogenetic process, and EMTCTCs encompass cells in various stages of transition [6]. EMTCTCs can be generally described by the down regulation of epithelial proteins, e.g. EpCAM and CK, and the upregulation of mesenchymal stem cell proteins, e.g. vimentin and CD34 [13]. EMTCTC subtyping is typically performed using non-proteomic methods, i.e. mRNA expression or DNA analysis [13].

[0051] A further type of circulating cell associated with cancer that may serve as a diagnostic is the cancer-associated vascular endothelial cell or CAVE. CAVEs are a subtype of circulating endothelial cells. Tumors require blood supply provided by tumor endothelial cells. CAVES are tumor endothelial cells that have broken off from the tumor site into the blood stream. CAVEs are often found in clusters. CAVEs express cytokeratin and various subtypes endothelial cell markers such as CD31, CD146, CD144, CD105, but do not express CD14 or CD45 [18].

[0052] Combining staining techniques with morphology, pathologically-definable CTCs (PDCTC), apoptotic CTCs and CAMLs can be identified [6].

Predictive Methods

[0053] As suggested above, unique characteristics of TMHCs make them well-suited for use in clinical methodology including methods of screening and diagnosis diseases such as cancer, monitoring treatment, monitoring of disease progression and recurrence. It has been shown that the presence of TMHCs in the blood of a subject is an indicator of more aggressive disease with worse clinical outcomes.

[0054] As suggested in the Summary above, in a first embodiment, the present invention is directed to a method for predicting overall survival (OS) and/or progression free survival (PFS) of a subject having cancer. The method comprises determining the presence of TMHCs in a biological sample from a subject having cancer, wherein the presence of TMHCs predicts lower OS and/or PFS than a subject having the same cancer without the presence of TMHCs.

[0055] In a second embodiment, the invention is directed to a method for predicting presence of metastatic spread and/or metastatic progression in a subject having cancer. The method comprises determining the presence of TMHCs in a biological sample from a subject having cancer, wherein the presence of TMHCs predicts presence of metastatic spread and/or metastatic progression in the subject.

[0056] In a third embodiment, the invention is directed to a method for predicting cancer progression in a subject having cancer. The method comprises determining the presence TMHCs in a first biological sample and a second biological sample at a later date, and optional additional biological samples, obtained from a subject having cancer, wherein when TMHCs are present in the second and/or additional biological sample but not present in the first biological sample, the cancer is predicted to progress in the subject.

[0057] In a fourth embodiment, the invention is directed to a method for predicting response to treatment in a subject having cancer. The method comprises determining the presence TMHCs in a first biological sample and a second biological sample at a later date, and optional additional biological samples, obtained from a subject having cancer, wherein the first sample is obtained from the subject prior to or during cancer treatment, wherein the second sample and optional

additional samples are obtained from the subject after at least one cancer treatment, wherein when TMHCs are present in the first biological sample but not present in the second and/or additional biological sample, the subject is predicted to respond to the treatment.

[0058] As used herein, overall survival (OS) means the length of time survived by a subject having cancer from a selected date, such as the date of diagnosis, the date on which treatment began, and the date on which blood is drawn to assess cancer progression.

[0059] As used herein, progression free survival (PFS) means the length of time survived by a subject having cancer from a selected date, such as the date on which treatment began or the date on which blood is drawn to assess cancer progression, and where the cancer has not worsened or progressed.

[0060] In each of the methods of the invention, OS or PFS, or both, is over a period of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 months, or more. In one aspect of the invention, OS or PFS, or both, is over a period of at least about 12 months or at least about 24 months.

[0061] In each of the methods of the invention, when OS and/or PFS is predicted to be "lower", "shortened" or "worse", OS and/or PFS is shorter in duration then would be the case for a subject having cancer in which the CAML associated structures had not been found.

[0062] In each of the methods of the invention, it will be apparent that the amount of the biological sample in which the circulating cells (e.g., TMHCs) are assayed can vary. However, the biological sample should generally be at least about 2.5 mL. The amount of biological sample may also be at least about 3, 4, 5, 6, 7, 7.5, 8, 9, 10, 11, 12, 12.5, 13, 14, 15, 16, 17, 17.5, 18, 19, 20, 21, 22, 22.5, 23, 24, 25, 26, 27, 27.5, 28, 29, or 30 mL, or more. The amount of biological sample may also be between about 2.5 and 20 mL, between about 5 and 15 mL, or between about 5 and 10 mL. In one aspect of the invention, the biological sample is about 7.5 mL.

[0063] In each of the embodiments and aspects of the invention, the source of the biological sample may be, but is not limited to, one or more of blood (such as peripheral blood), lymph node, bone marrow, cerebral spinal fluid, and urine. When the biological sample is blood, the blood may be antecubital-vein blood, inferior-vena-cava blood femoral vein blood, portal vein

blood, or jugular-vein blood, for example. The sample may be a fresh sample or a cryo-preserved sample that is thawed [14].

[0064] In each of the embodiments and aspects of the invention, the cancer may be a Stage I cancer, Stage II cancer, Stage III cancer, Stage IV cancer, carcinoma, sarcoma, neuroblastoma, melanoma, epithelial cell cancer, lung cancer, breast cancer, prostate cancer, pancreatic cancer, bladder cancer, kidney cancer, head and neck cancer, colorectal cancer, liver cancer, ovarian cancer, osteosarcoma, esophageal, brain & ONS, larynx, bronchus, oral cavity and pharynx, stomach, testis, thyroid, uterine cervix, uterine corpus cancer or other solid tumor cancers. The skilled artisan will appreciate that the methods of the invention are not limited to particular forms or types of cancer and that they may be practiced in association with a wide variety of cancers

[0065] In each of the embodiments and aspects of the invention, TMHCs are isolated from the biological samples for the determining steps using one or more means selected from size exclusion methodology, immunocapture, red blood cell lysis, white blood cell depletion, a high-molecular weight polysaccharide such as FICOLL®, electrophoresis, dielectrophoresis, flow cytometry, magnetic levitation, and various microfluidic chips, slits, channels, hydrodynamic size-based sorting, grouping, trapping, concentrating large cells, eliminating small cells, or a combination thereof. In a particular aspect, the size exclusion methodology comprises use of a microfilter.

[0066] In one aspect of the invention, TMHCs are isolated from the biological samples using size exclusion methodology that comprises using a microfilter. Suitable microfilters can have a variety of pore sizes and shapes. The microfilter may have a pore size ranging from about 5 microns to about 20 microns. In certain aspects of the invention, the pore size is between about 5 and 10 microns; in other aspects, the pore size is between about 7 and 8 microns. The larger pore sizes will eliminate most of the WBC contamination on the filter. The pores of the microfilter may have any shape, with acceptable shapes including round, race-track shape, oval, slit, square, rectangular and/or other shapes. The microfilter may have precision pore geometry, uniform pore distribution, more than one pore geometry, and/or non-uniform distribution. The microfilter may be single layer, or multi-layers with different shapes on different layers.

[0067] In another aspect of the invention, TMHCs are isolated from the biological samples using a microfluidic chip via physical size-based sorting, slits, channels, hydrodynamic size-based sorting, grouping, trapping, immunocapture, concentrating large cells, or eliminating small

cells based on size. The circulating cell capture efficiency can vary depending on the collection method. The size of a circulating cell that can be captured on different platforms can also vary depending, for example, on the identity of the cell. Collection of circulating cells using CELLSIEVE™ microfilters provides 100% capture efficiency and high quality cells.

[0068] In another aspect of the invention, when the biological sample is peripheral blood or blood, the sample may be collected from a subject using a blood collection tube. CELLSAVE™ blood collection tubes (Menarini Silicon Biosystems Inc., San Diego, CA), for example, provide stable cell morphology and size.

[0069] In a further aspect of the invention, TMHCs are isolated from the biological samples using a CELLSIEVE™ microfilter low-pressure microfiltration assay.

[0070] TMHCs may be used independently as cancer markers, or in combination with biomarker expression by other circulating cells, such as circulating tumor cells (CTCs). Suitable CTC subtypes include, but are not limited to, pathologically definable CTCs (PDCTCs), and apoptotic CTCs. Other suitable circulating cell types include CAMLs and circulating cancer associated vascular endothelial cells (CAVEs). The presence of these additional cell types themselves and also cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), methylated DNA, proteomic, metabolomic, lipidomic and other biomarkers may provide a more complete understanding of a patient's disease.

Methods of Treatment

[0071] In each aspect and embodiment of the invention, the method optionally further comprises administering a therapeutically effective amount of a cancer treatment to the subject. The subject may be a subject in which the OS and PFS is predicted to be lower or shorter than the OS and PFS of another subject.

[0072] The identity of the cancer treatment will correspond to the particular type of cancer being treated. However, suitable cancer treatments include chemotherapy, single drugs, combination of drugs, immunotherapy, radiation therapy, chemoradiation, chemoradiation combined with single or multiple drug, chemoradiation combined with single or multiple drugs (such as immunotherapy drugs), cell therapy, and other therapies.

[0073] Additional cancer treatments include, but are not limited to, immunotherapeutic agents, chemotherapeutic agents, radiotherapeutic agents, existing cancer drugs, CCR5 antagonists and CXCR4 antagonists. Examples of cancer treatments include, but are not limited to, one or more of antibodies or antagonists that block the activity of CCL3, CCL5 (RANTES), CCL7 or CCL8; Leronlimab (PRO 140); T-VEC, AM-0010, CXCR4 antagonist, TGF-beta kinase inhibitor galunisertib, anti-CSF-1R monoclonal antibody, Abemaciclib, Faslodex, necitumumab, AZD9291, Cyramza (ramucirumab), TPIV 200, Galunisertib, cancer vaccines, cytokines, cell-based therapies, bi- and multi-specific antibodies, tumor-targeting mAbs, Rituximab, oncolytic viruses, reovirus, Blinatumomab, Sipuleucel-T, T-Vec, IL-2, IFN-α, Trastuzumab, Celuximab, bevacizumab, Tim-3, BTLA, anti-IL-10, GM-CSF, anti-angiogenesis treatment, VEGF blockade, HMGB1, Nrp1, TAM receptor tyrosine kinases, Axl , MerTK, ALT-803, IL-15, Immunosuppressive Ligand Phosphatidylserine (PS), bavituximab, bevacizumab (anti-VEGF), coblmetinib (MEK inhibitor), vemurafenib (BRAF inhibitor), erlotinib (EGFR), alectinib (ALK inhibitor), bevacizumab (anti-VEGF), pazopanib (tyrosine kinase inhibitor), dabrafenib (BRAF inhibitor), trametinib (MEK inhibitor), durvalumab (anti-PD-L1), sunitinib (RTK inhibitor), pazopanib (RTK inhibitor), sargramostim, VISTA, TIM-3, LAG-3, PRS-343, CD137 (4-1BB)/HER2 bispecific, USP7, anti-HER2, SEMA4D, CTLA-4, PD-1, PD-L1, and PD-L2. As a non-limiting example, the treatment may be a cancer vaccine and the subject expresses at least one HLA allele. As a non-limiting example, the immunotherapy may be PD-L1 immunotherapy.

[0074] The subjects mentioned in the methods of the present invention will be a human, a non-human primate, bird, horse, cow, goat, sheep, a companion animal, such as a dog, cat or rodent, or other mammal. The subject having cancer may be undergoing treatment for the cancer. Such treatments include, but are not limited to targeted agents, chemotherapy, and radiation therapy.

Companion Diagnostics

[0075] The information obtained using the methods of the invention can be used as a companion or complementary diagnostic. A companion or complementary diagnostic is a diagnostic test that can be used in combination with a therapeutic drug for a selected disease or condition. The companion diagnostic determines the suitability of the drug for treatment of the disease or condition in a particular patient, i.e. the companion diagnostic can help to predict

whether the subject will be a responder or non-responder to the effects of the drug on the disease or condition in the subject. Thus, the companion diagnostic provides information that is used in treatment decisions and that is essential for the safe and effective use of a corresponding drug or biological product.

Examples

A. Tumor Macrophage Fusion Cells (TMFCs)

[0076] Four types of Tumor Macrophage Fusion Cells (TMFCs) were categorized and enumerated, namely (1) Partial, (2) Homodimeric, (3) Cannibalistic, and (4) Binucleated, along with (5) Hyperploidy CAMLs and (6) Hyper-engorged CAMLs, as shown in **FIG. 1**, from a prospective pilot study using n = 122 metastatic breast cancer (mBC) patients that were starting new lines of treatment. Whole peripheral blood (7.5 mL) was procured, filtered and stained using cytokeratin CD45/CD14 to identify TMFCs. The presence of the various types of TMFCs & CTCs were compared to a patient's progression free survival (PFS) and overall survival (OS) hazard ratios (HRs), analyzed by censored univariate analysis based on RECIST v1.1 over 24 months.

[0077] CTCs were found in 39% of patients, Partial TMFCs in 25% of patients, Homodimeric TMFCs in 6% of patients, Cannibalistic TMFCs in 0% of patients, Binucleated TMFCs in 2% of patients, Hyperploidy CAMLs in 96% of patients.

[0078] Neither CTCs alone, Binucleated TMFCs, nor Hyperploidy CAMLs were prognostic for PFS or OS (**Table 1**).

[0079] TMFCs with Partial or Homodimeric fusions were prognostic for worse PFS and OS (**Table 1**).

[0080] Combining patients with any TMFCs into one group (minus Hyperploidy TMFCs) was highly significant for worse PFS and OS (**Fig. 3A** and **Fig. 3B**).

[0081] Thus, TMFCs were detected and described in the blood of mBC patients, demonstrating an association with poor clinical outcomes. These data suggest a TMFC involvement in the pathogenesis of cancer.

Table 1. Hazard ratio comparisons of CTCs, TMFCs and CAMLs.

HR (95% CI) P value	**Any CTC**	**Partial TMFCs**	**Homodimeric TMFCs**	**Cannibalistic TMFCs**	**Binucleated TMFCs**	**Any TMFC Minus Hyperploidy CAMLs**	**Hyperploidy (any CAML)**	**Hyper-engorged CAMLs (≥100 µm)**
Present vs Absent	47 vs 75	31 vs 91	7 vs 115	0 vs 122	2 vs 120	36 vs 86	117 vs 5	58 vs 64
PFS	1.7 (1.1-2.8) p=0.0506	3.0 (1.7-5.3) p=0.0005	8.0 (1.9-34.2) p=0.0156	N/A	1.9 (0.1-28.6) p=0.8375	3.3 (1.9-5.8) P<0.0001	3.3 (1.1-10.2) p=0.0735	1.5 (0.9-2.5) p=0.1395
OS	1.8 (1.0-3.2) p=0.0806	3.7 (1.8-7.4) p=0.0006	10.4 (1.7-64.4) p=0.0400	N/A	78.4 (0.8-7532.2) p=0.4787	4.3 (2.2-8.6) P<0.0001	3.0 (0.7-12.3) p=0.2381	1.6 (0.9-2.4) p=0.1939

B. Tumor Macrophage Fusion Cells (TMFCs)

[**0082**] In a prospective study, 7.5 mL blood samples were collected from n=137 metastatic breast cancer (mBC) patients (**Table 2**) enrolled in prospective trials from multiple institutions before starting new treatment lines for newly progressive mBC. If possible, an optional follow-up (FU) sample was collected (n=73) after baseline (BL) (median=4.9 weeks). TMFCs in this analysis did not include Hyperploidy Fusion cells (i.e. CAMLs). TMFCs & CTCs were isolated using a CellSieve^{TM} microfilter and differentiated by staining for CK, CD45, CD14, and DAPI. Cox proportional regression hazard ratios (HRs) with 95% Confidence Intervals (95%CI) for progression free survival (PFS) and overall survival (OS) by univariate & multivariate analyses were based on RECIST v1.1, determined by local institutional pathologist over 24 months (M).

[**0083**] TMFCs were detected in 26% of patient samples at BL and 27% of patient samples at FU. TMFCs were significantly prognostic for worse PFS and OS at both BL and FU. Any type of CTCs were detected in 39% at BL and 38% at FU. Any type of CTCs were significantly prognostic for worse PFS but not OS at BL. At FU, CTCs were significantly prognostic for worse PFS and OS. At BL, patients without CTCs or TMFCs had the best survival outcomes (**Fig. 4A** and **4B**), followed by patients with just CTC, and patients with TMFCs having the worst outcomes. TMFCs at BL and FU were the only significant independent parameters for PFS and OS (**Table 3**).

[**0084**] TMFCs appears to represent a subgroup of CTCs with additional prognostic value not previously analyzed. Presence of TMFCs correlates with significantly worse PFS and OS versus patients without CTCs, or versus patients with normal CTCs. Patients with drops in TMFC populations after induction of new treatments had better outcomes, indicating responses to specific treatment types.

Table 2. Demographic Table

Median Age (Range)		58 (25-92)
Race	Caucasian	79 (58%)
	Black	13 (9%)
	Asian	11 (8%)
	Hispanic	4 (3%)
	Not Reported	30 (22%)
Histology	IDC (IBC)	76 (56%)
	ILC	7 (5%)
	Other/unknown	2/52 (39%)
Hormone		
	Positive (ER+/PR+/HER2+)	72 (53%) (51/34/21)
	Negative (TNBC)	60 (44%)
unknown		5 (4%)
# Prior Lines Therapies	0	16 (12%)
	1	22 (16%)
	2	33 (34%)
	≥3	66 (48%)
Therapy Type	Chemo Alone	49 (36%)
	Hormone	8 (6%)
	Immunotherapy	49 (36%)
	Other Targeted	31 (23%)
Number of Metastatic Sites	1	71 (52%)
	≥2	66 (48%)
CTC at BL	0	83 (61%)
	≥1	54 (39%)
CTC at FU	0	45 (62%)
	≥1	28 (38%)
TMFCs at BL	0	101 (74%)
	≥1	36 (26%)
TMFCs at FU	0	53 (73%)
	≥1	20 (27%)

Table 3. Univariate & Multivariate Table

Variable			PFS HR (95%CI)	Uni PFS (p value)	Multi PFS (p value)	OS HR (95%CI)	Uni OS (p value)	Multi OS (p value)
Age <65 vs ≥65	106	31	1.3 (0.8-2.2)	0.349		1.5 (0.7-2.8)	0.360	
Race Cauc vs Other	85	22	1.4 (0.8-2.6)	0.333		2.1 (1.0-4.3)	0.072	
Histology (IDC v other)	84	13	1.1 (0.6-2.4)	0.861		0.6 (0.3-1.6)	0.462	
Hormone Pos v TNBC	72	60	1.5 (0.9-2.4)	0.169		1.6 (0.8-2.8)	0.212	
# Prior Ther. <2 v ≥2	38	89	2.3 (1.4-3.7)	0.002	0.886	1.5 (0.8-2.8)	0.328	

Hormone Ther. vs Other	8	129	1.5 (0.6-3.7)	0.516		1.3 (0.4-3.8)	0.850	
# Met Sites 1 vs ≥2	71	66	1.6 (0.9-2.7)	0.154		1.0 (0.5-2.1)	0.926	
CTCs+ (BL)	54	83	2.3 (1.4-3.8)	<0.001	0.092	1.7 (1.0-3.2)	0.096	0.589
CTCs+ (FU)	28	45	4.1 (1.9-8.8)	<0.001	0.344	3.2 (1.3-8.2)	0.027	0.149
TMFCs+ (BL)	36	101	3.7 (2.1-6.6)	<0.001	0.950	3.1 (1.6-6.1)	<0.001	0.014
TMFCs+ (FU)	20	53	5.7 (2.4-13.8)	<0.001	0.042	8.5 (2.9-24.6)	<0.001	0.993
Increase in CTCs	21	52	4.0 (1.7-9.5)	0.003	0.371	4.7 (1.4-12.7)	0.021	0.798
Increase in TMFCs	18	55	4.5 (1.8-11.1)	0.002	0.123	7.6 (2.6-22.4)	<0.001	0.821

C. Circulating Tumor Cell Clusters (CTCCs)

[0085] Circulating tumor cell clusters (CTCCs) are aggregated groups of tumor cells that detached from primary tumors and circulate in the bloodstream. However, while Circulating Tumor Cells (CTCs) are a well studied phenomenon, CTCCs remain relatively unexplored and ill-defined, with only initial studies evaluating their clinical utility. Adding to the CTCC complexity is that various subtypes exist (**Fig. 2**), including homotypic clusters made of only tumor cells and heterotypic CTCCs made of CTCs attached to immune/stromal white blood cells (WBCs). Furthermore, CTCs can undergo Epithelial-Mesenchymal Transition (EMT), a process where tumor cells downregulate epithelial traits and upregulate mesenchymal traits, and also form clustered EMTs (CEMTs). Further, CTCs can fuse with macrophages forming Cancer-Associated Macrophage-Like cells (CAMLs) when in circulation. Single CTCs, EMTs, and CAMLs, were enumerated, as well as homotypic CTCCs, heterotypic CTCCs and EMT CTCCs from the blood of metastatic breast cancer (mBC) patients to quantify these CTC populations and assess their clinical utility by median progression free survival (mPFS) and median overall survival (mOS) over 24 months.

[0086] The 6 populations were enumerated from a prospective pilot study of n=79 mBC patients. Whole peripheral blood (7.5 mL) was filtered and stained with cytokeratin (CK) & CD45/CD14 to identify CTCs. CTCs were defined as having an intact DAPI nucleus and strong filamentous CK. Homotypic CTCCs were defined as ≥2 CTCs attached together. Heterotypic CTCCs were defined as ≥1 CTC attached to ≥1 WBC. EMTs were defined as having DAPI nuclei and weak non-filamentous CK. EMT CTCCS were defined as ≥2 EMTs. CAMLs were

defined as having an enlarged polynucleated DAPI, and positive for CD45/CD14 or non-filamentous CK.

[0087] Single CTCs were found in 57% of patients (n=34/79), homotypic CTCCs 15% (n=12/79), heterotypic CTCCs 66% (n=27/79), EMTs 56% (n=44/79), EMT CTCCs 23% (n=18/79), any CAML in 97% (n=77/79), and Giant ≥ 50 µm CAMLs in 84% (n=66/79) (**Table 4**).

[0088] Over 24 months, patients with heterotypic CTCCs, homotypic CTCCs and Giant ≥ 50 µm CAMLs had the worst PFS, followed by any CTCs, EMTs, and EMT CTCCs (**Figs. 5 & 6**). Both CTCCs and EMT CTCCs were rare in HER2+ patients at 8.7% (n=2/23) and 17.4% (n=4/23), respectively.

[0089] Despite no established definition, CTC Clusters appear to represent an array of subtypes with different biological and clinical meanings. The CTC cluster subtypes from the blood of mBC patients were stratified and enumerated, and compared to clinical outcomes. CAML hyperploidy and CTC clustering appears to indicate poor prognosis.

Table 4. Hazard ratio comparisons of CTCs and CTC cluster types.

HR (95% CI) P value	**Any CTC**	**Homotypic cluster**	**Heterotypic cluster**	**EMTs**	**EMT CTCCs**	**CAMLs (≥50 µm)**
Present vs Absent	34 vs 45	12 vs 67	27 vs 52	35 vs 44	18 vs 61	66 vs 13
PFS	2.0 (1.2-3.4) p=0.0202	5.5 (2.0-15.8) p=0.0032	2.7 (1.5-4.9) p=0.0027	1.3 (0.7-2.2) p=0.4942	0.9 (0.5-1.7) p=0.8949	2.3 (1.2-4.3) P=0.0151
OS	2.0 (1.0-4.0) p=0.0849	2.0 (0.6-6.3) p=0.4139	3.8 (1.7-8.3) p=0.0019	1.4 (0.7-2.9) p=0.4025	2.0 (0.9-4.7) p=0.1502	3.3 (1.5-7.5) P=0.0069

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19. International Patent Application Publication No. WO 2016/33103, dated March 3, 2016.

What is claimed is:

1. A method for predicting overall survival (OS) and/or progression free survival (PFS) of a subject having cancer, comprising determining the presence of Tumor Macrophage Hybrid Cells (TMHCs) in a biological sample from a subject having cancer, wherein the presence of TMHCs predicts lower OS and/or PFS than a subject having the same cancer without the presence of TMHCs.

2. A method for predicting presence of metastatic spread and/or metastatic progression in a subject having cancer, comprising determining the presence of TMHCs in a biological sample from a subject having cancer, wherein the presence of TMHCs predicts presence of metastatic spread and/or metastatic progression in the subject.

3. A method for predicting cancer progression in a subject having cancer, comprising determining the presence TMHCs in a first biological sample and a second biological sample at a later date, and optional additional biological samples, obtained from a subject having cancer, wherein when TMHCs are present in the second and/or additional biological sample but not present in the first biological sample, the cancer is predicted to progress in the subject.

4. A method for predicting response to treatment in a subject having cancer, comprising determining the presence TMHCs in a first biological sample and a second biological sample at a later date, and optional additional biological samples, obtained from a subject having cancer, wherein the first sample is obtained from the subject prior to or during cancer treatment, wherein the second sample and optional additional samples are obtained from the subject after at least one cancer treatment, wherein when TMHCs are present in the first biological sample but not present in the second and/or additional biological sample, the subject is predicted to respond to the treatment.

5. The method of any one of claims 1-4, wherein the TMHCs comprise one or more of the following cell types:
(a) partial tumor macrophage fusion cells;
(b) homodimeric tumor macrophage fusion cells;

(c) cannibalistic tumor macrophage fusion cells;
(d) binucleated tumor macrophage fusion cells;
(e) heterotypic circulating tumor cell (CTC) clusters;
(f) homotypic circulating tumor cell (CTC) clusters; and
(g) epithelial-mesenchymal transition circulating tumor cell (EMT CTCs) clusters.

6. The method of claim 1, wherein OS and/or PFS is over a period of at least 12 months.

7. The method of claim 1, wherein OS and/or PFS is over a period of at least 24 months.

8. The method of any one of claims 1-4, wherein the biological sample is one or more of blood, peripheral blood, lymph node, bone marrow, cerebral spinal fluid, and urine. For example, the biological sample may be antecubital-vein blood, inferior-vena-cava blood, femoral vein blood, portal vein blood, or jugular-vein blood.

9. The method of any one of claims 1-4, wherein the size of the biological sample is between 5 and 15 mL.

10. The method of any one of claims 1-4, wherein the cancer is a Stage I cancer, Stage II cancer, Stage III cancer, Stage IV cancer, carcinoma, sarcoma, neuroblastoma, melanoma, epithelial cell cancer, lung cancer, breast cancer, prostate cancer, pancreatic cancer, bladder cancer, kidney cancer, head and neck cancer, colorectal cancer, liver cancer, ovarian cancer, osteosarcoma, esophageal, brain & ONS, larynx, bronchus, oral cavity and pharynx, stomach, testis, thyroid, uterine cervix, uterine corpus cancer or other solid tumor cancers, or a blood cancer.

11. The method of any one of claims 1-4, wherein TMHCs are isolated from the biological samples for the determining steps using one or more means selected from the group consisting of size exclusion methodology, immunocapture, red blood cell lysis, white blood cell depletion, a high-molecular weight polysaccharide such as FICOLL®, electrophoresis, dielectrophoresis, flow cytometry, magnetic levitation, and various microfluidic chips, slits, channels, hydrodynamic size-based sorting, grouping, trapping, concentrating large cells, eliminating small cells, or a combination thereof.

12. The method of any one of claims 1-4, wherein TMHCs are isolated from the biological samples using size exclusion methodology that comprises using a microfilter.

13. The method of any one of claims 1-4, wherein TMHCs are isolated using a microfluidic chip via physical size-based sorting, hydrodynamic size-based sorting, grouping, trapping, immunocapture, concentrating large cells, or eliminating small cells based on size.

14. The method of any one of claims 1-4, wherein TMHCs are isolated from the biological samples using a microfiltration assay.

15. The method of any one of claims 1-4, wherein the treatment is one or more of chemotherapy, single drug, combination of drugs, immunotherapy, radiation therapy, chemoradiation, radiation combined with single or multiple drugs, chemoradiation combined with single or multiple drugs, cancer vaccine, and cell therapy.

16. The method of any one of claims 1-4, wherein the subject is treated with one or more of chemotherapy, a single drug, a combination of drugs, immunotherapy, radiation therapy, chemoradiation, radiation combined with single or multiple drugs, chemoradiation combined with single or multiple drugs, cancer vaccine, and cell therapy.

17. The method of any one of claims 1-4, further comprising administering a therapeutically effective amount of a cancer treatment to the subject.

18. The method of claim 17, wherein the subject is a subject in which the OS and/or PFS is predicted to be lower or shorter than the OS and/or PFS of another subject.

FIG. 1

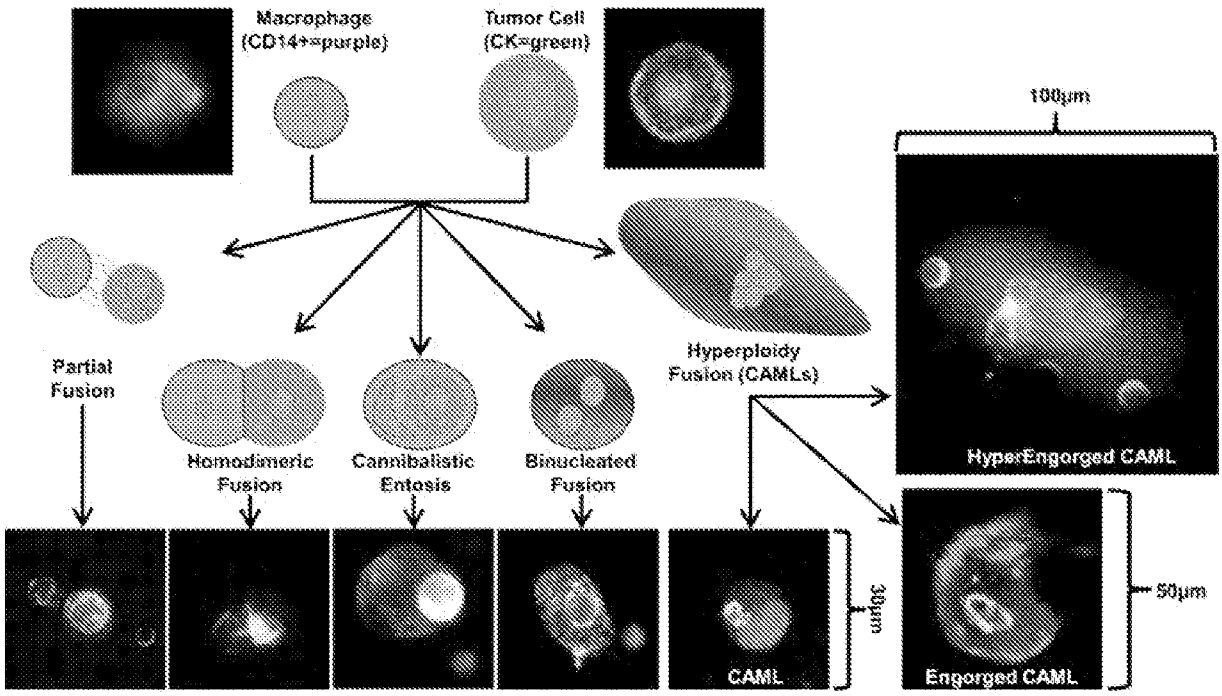

Macrophage
(CD14+=purple)
Tumor Cell
(CK=green)
100µm
Partial
Fusion
Homodimeric
Fusion
Cannibalistic
Entosis
Binucleated
Fusion
Hyperploidy
Fusion (CAMLs)
HyperEngorged CAML
CAML
30µm
Engorged CAML
50µm

FIG. 2

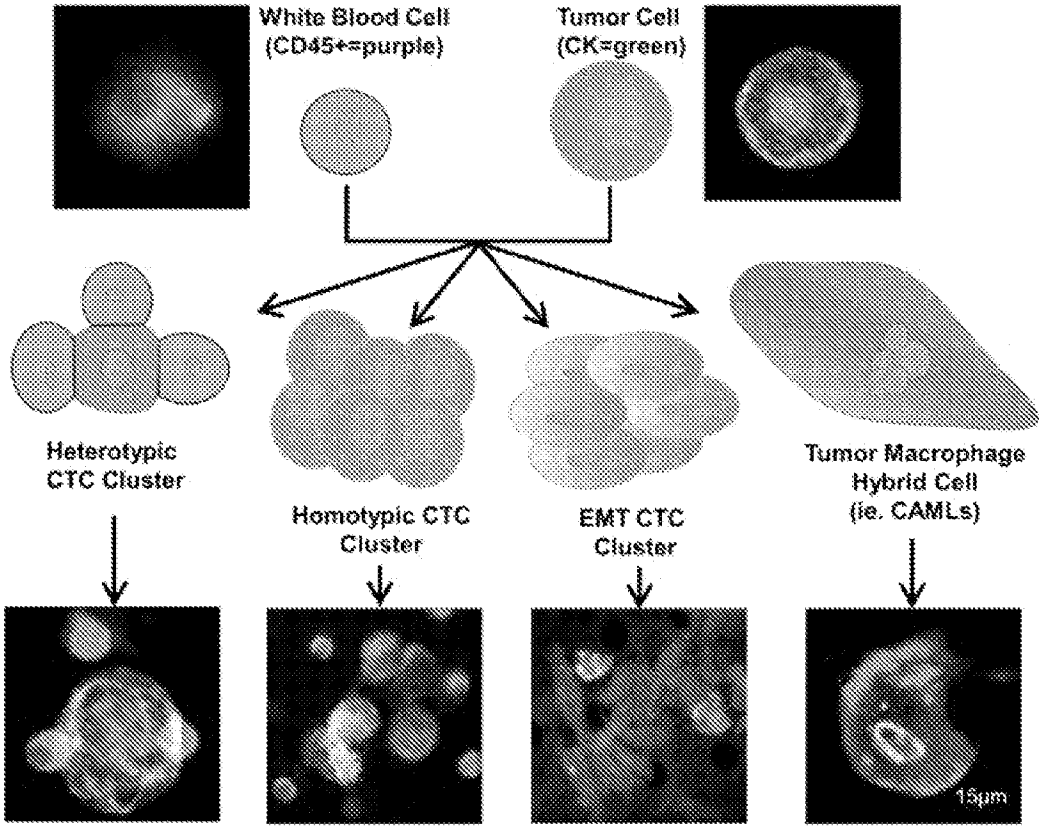
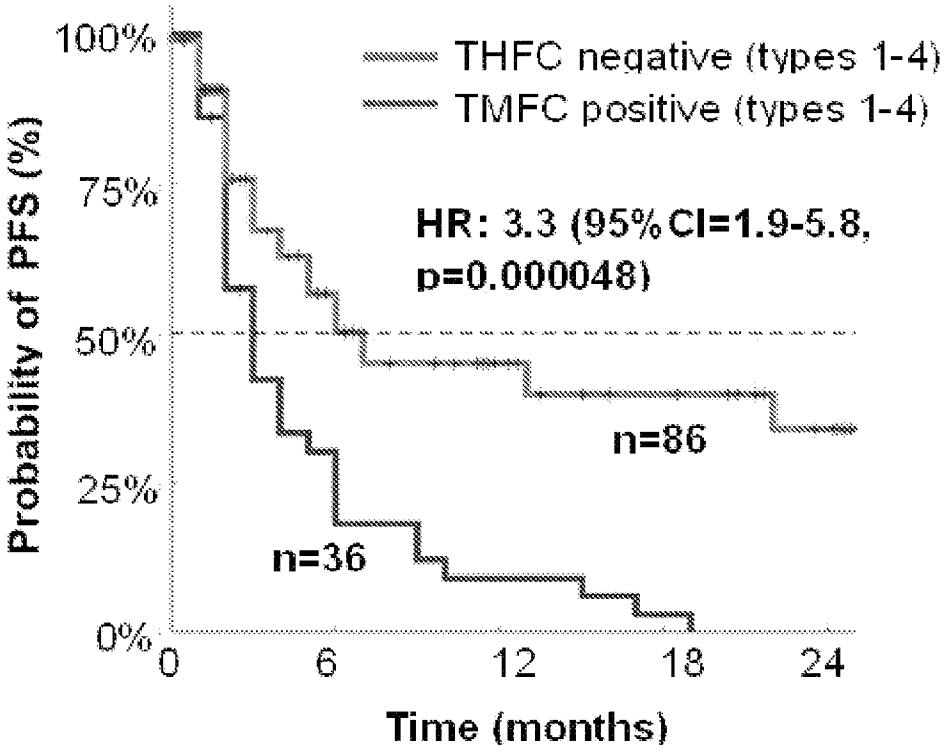

White Blood Cell
(CD45+=purple)
Tumor Cell
(CK=green)
Heterotypic
CTC Cluster
Homotypic CTC
Cluster
EMT CTC
Cluster
Tumor Macrophage
Hybrid Cell
(ie. CAMLs)
15μm

FIG. 3

A.


100%
75%
50%
25%
0%
Probability of PFS (%)
THFC negative (types 1-4)
TMFC positive (types 1-4)
HR: 3.3 (95% CI=1.9-5.8,
p=0.000048)
n=86
n=36
0
6
12
18
24
Time (months)

B.

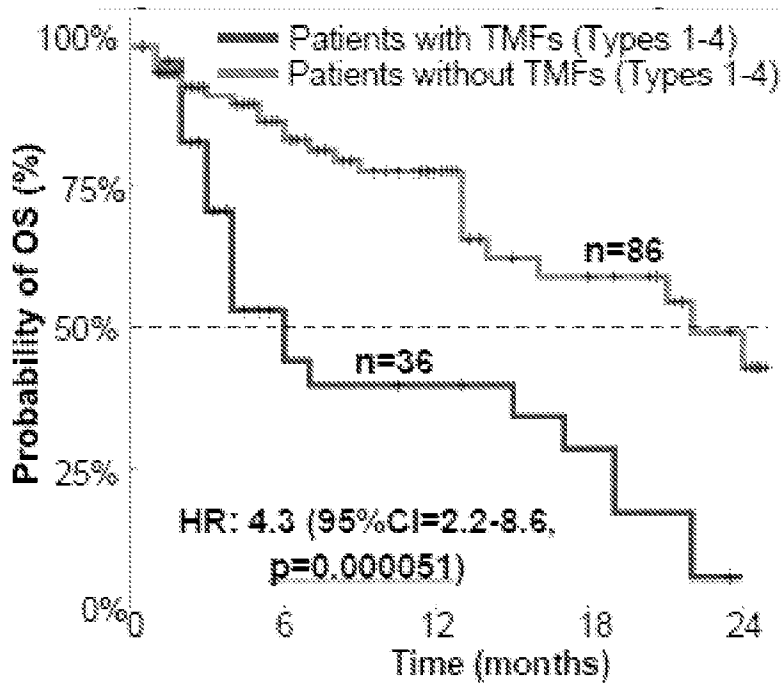
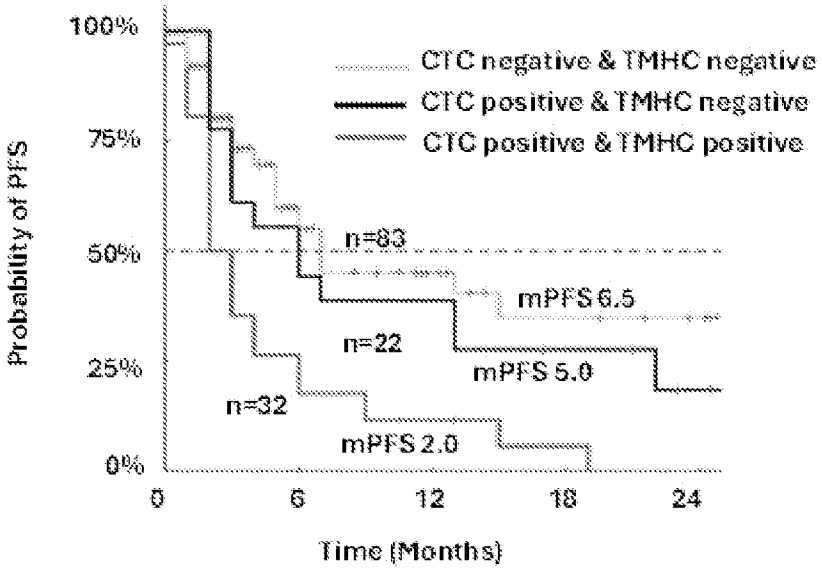

100%
75%
50%
25%
0%
Probability of OS (%)
Patients with TMFs (Types 1-4)
Patients without TMFs (Types 1-4)
n=86
n=36
HR: 4.3 (95%CI=2.2-8.6,
p=0.000051)
0
6
12
18
24
Time (months)

FIG. 4

A.


100%
75%
50%
25%
0%
Probability of PFS
CTC negative & TMHC negative
CTC positive & TMHC negative
CTC positive & TMHC positive
n=83
mPFS 6.5
n=22
mPFS 5.0
n=32
mPFS 2.0
0
6
12
18
24
Time (Months)

B.

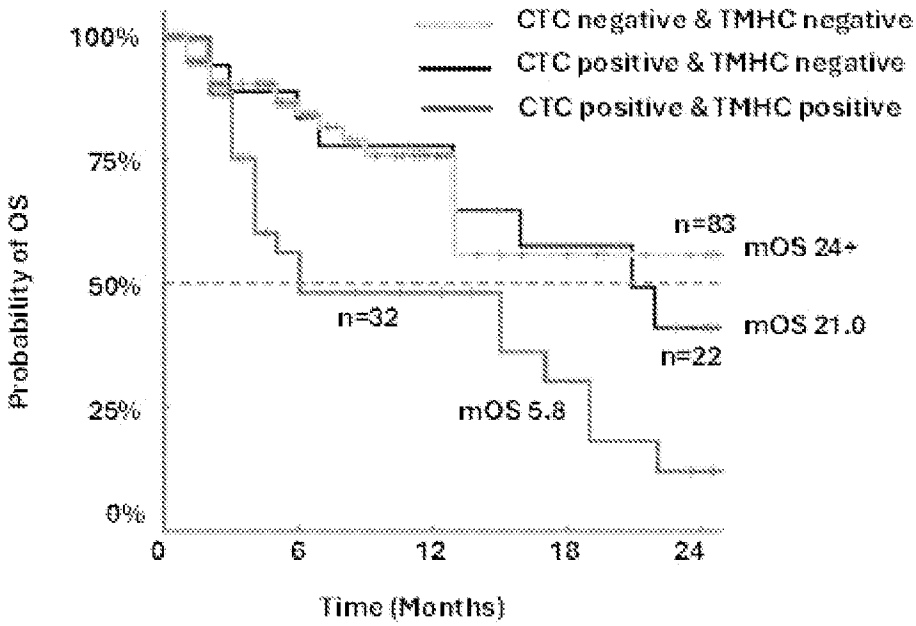

100%
75%
50%
25%
0%
Probability of OS
CTC negative & TMHC negative
CTC positive & TMHC negative
CTC positive & TMHC positive
n=83
mOS 24+
n=32
mOS 21.0
n=22
mOS 5.8
0
6
12
18
24
Time (Months)

FIG. 5

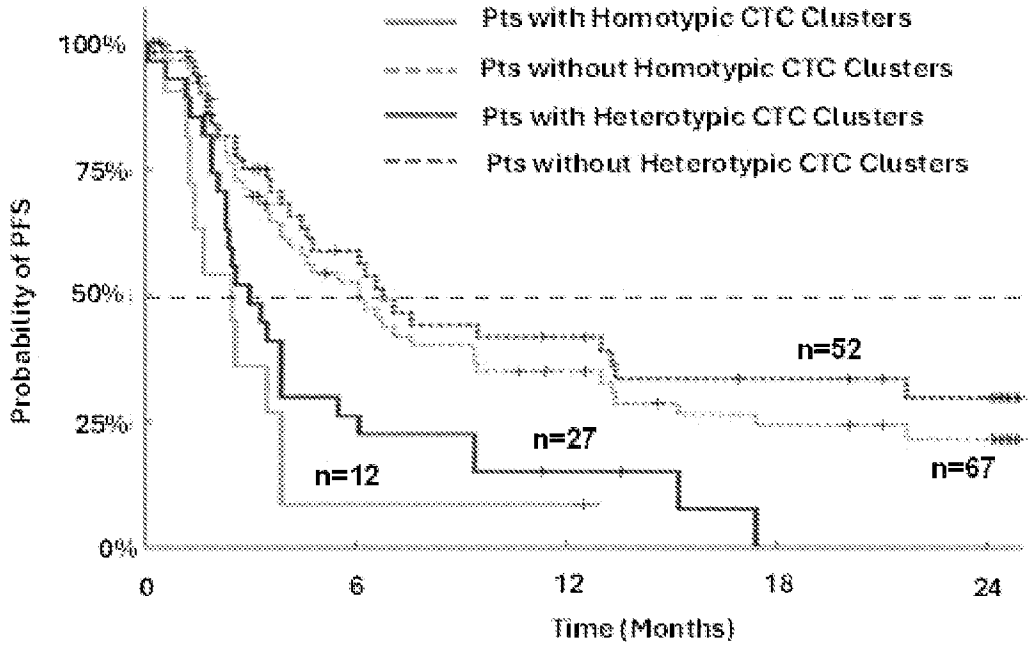
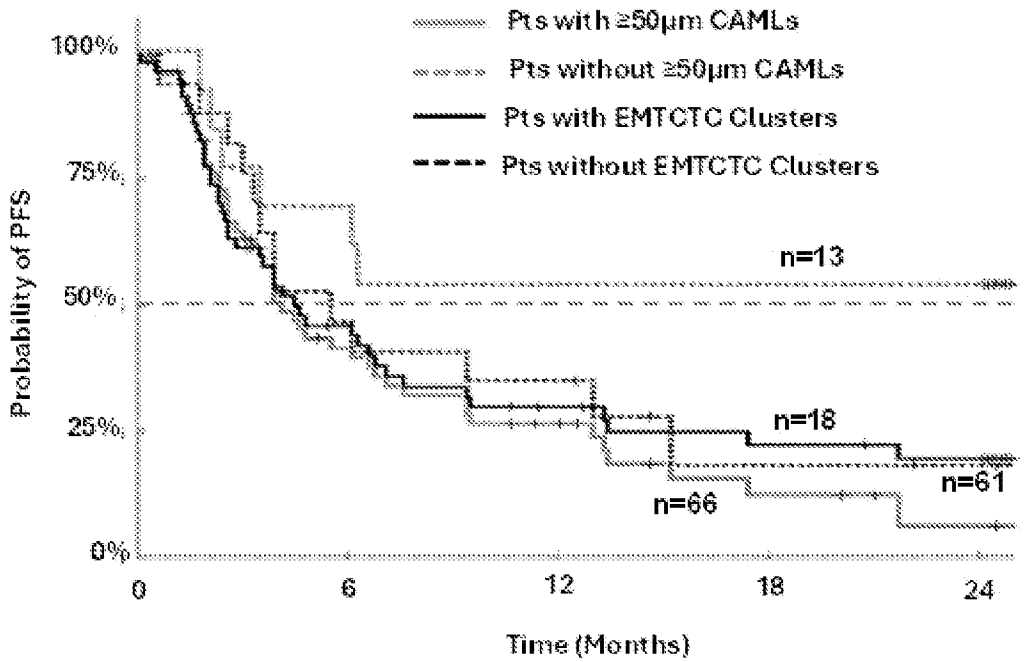

Pts with Homotypic CTC Clusters
Pts without Homotypic CTC Clusters
Pts with Heterotypic CTC Clusters
Pts without Heterotypic CTC Clusters
100%
75%
50%
25%
0%
Probability of PFS
n=52
n=27
n=12
n=67
0
6
12
18
24
Time (Months)

FIG. 6


Pts with ≥50µm CAMLs
Pts without ≥50µm CAMLs
Pts with EMTCTC Clusters
Pts without EMTCTC Clusters
100%
75%
50%
25%
0%
Probability of PFS
n=13
n=18
n=61
n=66
0
6
12
18
24
Time (Months)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/032257

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***G01N 33/49*** (2024.01); ***A61P 35/00*** (2024.01); *A61P 43/00* (2024.01); *B01D 21/00* (2024.01); *B01D 37/00* (2024.01); *B01D 43/00* (2024.01)

CPC: ***G01N 33/4915; A61P 35/00;*** *G01N 2800/52; A61P 43/00; B01D 21/00; B01D 37/00; B01D 43/00*

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)

See Search History Document

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

See Search History Document

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

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	WO 2019/178226 A1 (CREATV MICROTECH INC.) 19 September 2019 (19.09.2019)	
X	entire document	1, 6-14, 16-18
Y	entire document	5
	US 2019/0128892 A1 (CREATV MICROTECH INC.) 02 May 2019 (02.05.2019)	
Y	entire document	5
	US 2020/0049713 A1 (CREATV MICROTECH INC.) 13 February 2020 (13.02.2020)	
A	entire document	1, 5-14, 16-18

☐ 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
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"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)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"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
"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
"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
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 July 2024 (28.07.2024)	**27 September 2024 (27.09.2024)**

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US **Commissioner for Patents** **P.O. Box 1450, Alexandria, VA 22313-1450**	**MATOS** **TAINA**
Facsimile No. **571-273-8300**	Telephone No. **571-272-4300**

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:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I: claims 1, 6, 7, and also claims 5, 8-14 and 16-18 (in part) are drawn to methods for predicting overall survival (OS) and/or progression free survival (PFS) of a subject having cancer.

Group II: claims 2, 3 and also claims 5, 8-14, and 16-18 (in part) are drawn to methods for predicting presence of metastatic spread and/or metastatic progression in a subject having cancer and methods for predicting cancer progression in a subject having cancer.

Group III: claims 4, 15 and also claims 5, 8-14, and 16-18 (in part) are drawn to methods for predicting response to treatment in a subject having cancer.

The inventions listed in Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The special technical features of Group I, methods for predicting overall survival (OS) and/or progression free survival (PFS) of a subject having cancer, are not present in Groups II and III; the special technical features of Group II, methods for predicting presence of metastatic spread and/or metastatic progression in a subject having cancer and methods for predicting cancer progression in a subject having cancer, are not present in Groups I and III; and the special technical features of Group III, methods for predicting response to treatment in a subject having cancer, are not present in Groups I and II.

Additionally, even if Groups I-III were considered to share the technical features of a method for predicting cancer, comprising determining the presence of Tumor Macrophage Hybrid Cells (TMHCs) in a biological sample from a subject having cancer, these shared technical features do not represent a contribution over the prior art as disclosed by WO 2019/178226 to Creatv MicroTech, Inc (hereinafter, "Creatv").

Creatv teaches a method for predicting cancer (Methods for monitoring treatment response and disease progression in subjects using circulating cells, Title; methods for predicting disease progression and/or treatment response in subjects having cancer, such as a solid tumor cancer, Para. [0008]) comprising determining the presence of Tumor Macrophage Hybrid Cells (TMHCs) in a biological sample from a subject having cancer (the methods of the invention comprise determining the number of circulating cells, such as CAMLs, in two or more biological samples obtained from a subject having cancer over time, Para. [0009], See instant disclosure, Fig. 2, which lists CAMLs as an alias for Tumor Macrophage Hybrid Cells).

The inventions listed in Groups I-III therefore lack unity under Rule 13 because they do not share a same or corresponding special technical feature.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/032257

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

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 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.: **1, 5-14, 16-18**

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.