(54) **Methods for Predicting Treatment Outcome and/or for Selecting a Subject Suitable for Immune Checkpoint Therapy.**

(57) The present invention relates to the field of biomarker development for cancer immunotherapy with PD-1 inhibitor compounds and/or PD-L1 inhibitor compounds. Provided are assays for quantifying PD-1 expression (i.e. immunostaining intensity) in cells present in a tumor sample (i.e. intratumoral cells), which are advantageously used to identify a unique sub-population of intratumoral cells referred to herein as PD-1^T cells, which serves as a biomarker for cancer immunotherapy with PD-1 inhibitor compounds and/or PD-L1 inhibitor compounds alone or in combination with other therapeutic agents (e.g. CTLA-4 inhibitor compound, e.g. ipilimumab). The present invention also provides methods of selecting a human subject diagnosed with cancer (e.g. non-small cell lung cancer (NSCLC)) suitable for immune checkpoint therapy with agents such as PD-1 inhibitors (e.g. nivolumab) and/or PD-L1 inhibitors (e.g. atezolizumab) alone or in combination with other therapeutic agents (e.g. CTLA-4 inhibitor compound, e.g. ipilimumab), methods for predicting responsiveness to immune checkpoint therapy with agents such as PD-1 inhibitors and/or PD-L1 inhibitors, and method of treatment of a human subject diagnosed with cancer using PD-1 inhibitors and/or PD-L1 inhibitors alone or in combination with other therapeutic agents (e.g. CTLA-4 inhibitor compound, e.g. ipilimumab) (//dc).

Title: **Methods for Predicting Treatment Outcome and/or for Selecting a Subject Suitable for Immune Checkpoint Therapy.**

FIELD OF THE INVENTION

The present invention relates to the field of cancer, immunotherapy and immune checkpoint molecules such as programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1), and conditions or diseases involving PD-1/PD-L1 axis signaling, e.g. cancer. More particularly, the present invention relates to the field of biomarker development for cancer immunotherapy with PD-1 inhibitor compounds and/or PD-L1 inhibitor compounds. Provided are assays for quantifying PD-1 expression (i.e. immunostaining intensity) in cells present in a tumor sample (i.e. intratumoral cells), which are advantageously used to identify a unique sub-population of intratumoral cells referred to herein as PD-1^T cells, which serves as a biomarker for cancer immunotherapy with PD-1 inhibitor compounds and/or PD-L1 inhibitor compounds alone or in combination with other therapeutic agents (e.g. CTLA-4 inhibitor compound, e.g. ipilimumab). The present invention also provides methods of selecting a human subject diagnosed with cancer (e.g. non-small cell lung cancer (NSCLC)) suitable for immune checkpoint therapy with agents such as PD-1 inhibitors (e.g. nivolumab) and/or PD-L1 inhibitors (e.g. atezolizumab) alone or in combination with other therapeutic agents (e.g. CTLA-4 inhibitor compound, e.g. ipilimumab), methods for predicting responsiveness to immune checkpoint therapy with agents such as PD-1 inhibitors and/or PD-L1 inhibitors, and method of treatment of a human subject diagnosed with cancer using PD-1 inhibitors and/or PD-L1 inhibitors alone or in combination with other therapeutic agents (e.g. CTLA-4 inhibitor compound, e.g. ipilimumab). Collectively, the assays and methods of the present invention can be advantageously used to reliably guide patient selection, predict clinical response to PD-1 and/or PD-L1 inhibitor compounds alone or in combination with other therapeutic agents (e.g. CTLA-4 inhibitor compound, e.g. ipilimumab) prior to initiating treatment or early during treatment, and to determine the best treatment strategy per cancer patient.

BACKGROUND

Cancer is a leading cause of death worldwide, accounting for more than 8.8 million deaths in 2015. Cancer can be generally defined as a group of diseases involving

abnormal cell growth. Generally, when cancer develops, normal cells become progressively abnormal over time as they acquire mutations which allow them to escape immune surveillance, survive, grow uncontrollably, and spread through the body (Lazebnik, Y., (2010), Nature Reviews Cancer, Vol. 10, pages 232-233; Bekele and Brindley (2012), Clinical Lipidology, Vol.7, pages 313-328).

Several studies have shown that tumors or tumor cells have the ability to suppress the host's adaptive immunity by, e.g., suppressing immune responses mediated by effector T cells (also known as T lymphocytes) through inhibiting effector T cell function or activity (e.g. secretion of cytokines and cytotoxic molecules, ability to migrate, etc.) and/or reducing or blocking proliferation of effector T cells. One way by which tumors or tumor cells suppress the host's adaptive immunity (e.g. affect T cell functions) is through expression of so-called inhibitory "immune checkpoint ligands" (Romano and Romero (2015), Journal for immunotherapy, Vol 3: 15).

Immune checkpoints are molecules in the immune system that either turn up or turn down a signal in immune cells (e.g. secretion of cytokines from effector T cells) so as to reduce immune responses and mitigate collateral tissue damage. An example of an immune checkpoint molecule is the programmed cell death protein 1 (PD-1) that provides an inhibitory signal to cells upon encounter of one of its immune checkpoint ligands, programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2). Another example of an immune checkpoint molecule is the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which is a protein receptor that downregulates immune responses (e.g. downregulates T cell functions (Postow et al (2015) J. Clinical oncology, Vol. 33, pages 1974-1983; Śledzińska et al (2015), Mol Oncol. Vol 9(10), pages 936-965).

PD-L1 and PD-1 are often referred to as the "PD-1/PD-L1 axis" or "PD-1/PD-L1 pathway". The PD-1/PD-L1 pathway is also referred to as a "negative immune checkpoint" or 'inhibitory immune checkpoint' because it reduces or turns down immune signals (e.g. affects T cell functions such as secretion of cytokines by effector T cells). Normally, inhibitory immune checkpoint pathways, such as for instance the "PD-1/PD-L1 pathway, serve as safeguard mechanisms aimed at keeping the immune

system from overreacting to a stimulus or mistaking a component of the body for a dangerous invader.

In the context of cancer, tumor cells are believed to shield themselves from the host immune system or escape host immune surveillance (e.g. cancer cells displaying tumor antigens should normally be recognized and destroyed by effector T cells) by inhibiting or interfering with effector T cell function or activity (e.g. cytokine production or effector T cell proliferation) through signaling via the PD-1/PD-L1 pathway (Taku Okazaki and Tasuku Honjo (2007), International Immunology, Vol: 19, pages 813-824; Iwai Y et al (2002), PNAS Vol: 99, pages 12293). For instance, it was shown that the expression of PD-L1 is often up-regulated (i.e. a higher expression of the protein) at the external surface (cell surface) of cancer cells (Taku Okazaki and Tasuku Honjo (2007), International Immunology, Vol: 19, pages 813-824). In this context, the interaction between the PD-L1 on the cancer cell surface and the PD-1 receptor on an immune cell (e.g. T-cell) is promoted or increased. This leads to decreased or reduced immune cell (e.g. effector T cell) function or activity, e.g. decreased or reduced secretion of cytokines and/or decreased or reduced proliferation of T cells, which in turn prevents or hinders the host's immune system from attacking the tumor cells. PD-L1 can also be expressed by non-cancerous cells within the tumor micro-environment, with the same deleterious effects on the host's immune cell function. Globally, this represents one way by which cancer cells may escape detection by the host immune system.

These results prompted the development of new cancer therapies, also referred to as "immune checkpoint therapies", which are aimed at inhibiting or blocking the PD-1 molecule and/or PD-L1 molecule or signaling via the PD-1/PD-L1 pathway. Another immune checkpoint therapy consists of blocking CTLA-4 function.

Non-limiting examples of immune checkpoint inhibitor agents targeting PD-L1 or PD-1, which are FDA approved or being tested in clinical trials for the treatment of cancer include, for instance, PD-L1 antibodies (e.g. durvalumab, atezolizumab, avelumab, and others), as well as anti-PD-1 antibodies (e.g. nivolumab, pembrolizumab, BGB-A317, and others) (Meng et al (2015), Cancer Treatment Review, Vol. 41, pages 868-876; Brahmer et al (2010) J Clin Oncol 28:3167-75; Brahmer et al (2012) N. Engl. J.

Med. Vol: 366, pages 2455-65; Flies et al (2011) Yale J. Biol. Med. Vol.84, pages 409-21; Topalian et al. (2012b) N. Engl. J. Med. Vol. 366, pages 2443-54; Diggs et al (2017), Biomarker Research, Vol.5:12, pages 1-6). Non-limiting examples of immune checkpoint inhibitor agents targeting CTLA-4 include ipilimumab (Bristol-Myers Squibb, Princeton, NJ, FDA-approved) and tremelimumab (formerly Pfizer, currently MedImmune/AstraZeneca, Wilmington, DE, in clinical development), (Postow et al (2015) J. Clinical oncology, Vol. 33, pages 1974-1983).

Although immune checkpoint therapy (e.g. using PD-1 inhibitors or PD-L1 inhibitors) shows impressive results in the clinic for various types of cancers (e.g. non-small cell lung cancer (NSCLC), melanoma, kidney cancer, bladder cancer, hepatocellular cancer, gastrointestinal tract (GI) cancers such as stomach cancer, Hodgkin's lymphoma, and others), a considerable fraction of cancer patients (up to 70-90% in certain patient populations) failed to respond to such therapy (Vareki et al (2017) Critical Reviews in Oncology/Hematology, Vol. 116, pages 116-124). In addition, there are a number of tumor types, including prevalent tumor types such as breast cancer, ovarian cancer, colorectal cancer, and prostate cancer, in which only a small fraction of patient responds. Hence, it is highly desirable to have better ways (methods) to predict treatment outcome and/or better ways to select suitable patients for such therapy in these specific patient populations (e.g. breast cancer, ovarian cancer, colorectal cancer, and prostate cancer patient populations).

Currently, the outcome of immune checkpoint therapy (e.g. response or resistance) is mostly assessed after the treatment has been initiated (i.e. in the early phase of the treatment). This is disadvantageous for cancer patients who turn out to be *non-responders* (or resistant to therapy) because immune checkpoint therapy is often associated with autoimmune adverse effects, toxicity. Furthermore, it delays the start with another, potentially more suited, treatment. Finally, immune checkpoint therapy is costly. Therefore, having a better way(s) (e.g. methods) to select patients or to predict which patients will respond to immune checkpoint therapy, would allow medical practitioners to reliably select patients who would benefit from immune checkpoint therapy (e.g. using PD-1/PD-L1 inhibitors), before initiating such treatment. This is crucial to avoid the autoimmune adverse effects, toxicity, and high costs of such agents when given to a cancer patient who turns out to be a non-responder (Xiangjiao

et al (2015), Cancer treatment reviews, Vol. 41, pages 868-876). In addition, having such methods would allow timely start of other treatments in patients who would not respond to checkpoint therapy, including treatments that may subsequently render the same patients responsive to checkpoint blockade.

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One way to improve the selection of patients or to predict treatment outcome is by using so-called "predictive biomarkers". Substantial efforts have been devoted to find (and validate) such predictive biomarker(s) using various strategies, e.g. by exploiting the characteristics of tumor cells *per se* (e.g. expression of immune checkpoint ligands

10 such as PD-L1, tumor-specific SNP/mutations, expression profile of inflammatory genes, etc.) or by exploiting the characteristics of cells from the host's immune system (e.g. characteristics/functions of tumor infiltrating T cell (TILS), blood biomarkers (e.g. tumor antigen-specific antibodies), mutations/SNP in immune cells, etc.) (Vareki et al (2017), Critical Review in Oncology/Hematology, Vol.116, pages 116-124).

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For instance, PD-L1 is one of the most currently studied biomarkers for predicting responsiveness to immune checkpoint therapy, using agents targeting or blocking PD-1 and/or PD-L1.

20 There are currently four FDA-approved PD-L1 immunohistochemistry (IHC) assays (carried out on tumor tissue sections), which are to be used in combination with a particular immune checkpoint inhibitor, with varying cut-off marks. The FDA-approved PD-L1 IHC assays include Roche Ventana SP263 (cut-off 25%), Roche Ventana SP142 (cut-off 1% or 50%, depending on cancer type), Dako 22C3 (cut-off 1% or 50%,

25 depending on cancer type), and Dako 28-8 (cut-off 1% or 5%, depending on cancer type) (Diggs and Hsueh (2017), Biomarker Research, Vol. 5:12, pages 1-6). Detection of PD-L1 in tumor cells or other intratumoral cells using these FDA-approved immunohistochemistry (IHC) assays is currently used in the clinic for predicting patient response to anti-PD-1/PD-L1 therapy before treatment has been started (Patel and

30 Kurzrock (2015), Mol. Cancer Therapy, Vol. 14, pages 847–856; Vareki et al (2017), Critical Review in Oncology/Hematology, Vol.116, pages 116-124).

Although some success has been achieved in predicting responsiveness (or resistance) to immune checkpoint therapy using the PD-L1-based IHC assays,

accurate prediction is not always achieved. This is reflected in the finding that PD-1 inhibitors and PD-L1 inhibitors have activity in a subset of individuals who do not meet the PD-L1 IHC bioassay cut-off. In addition, also part of the patients that do meet the PD-L1 IHC bioassay cut-off do not experience clinical benefit from PD-1 inhibitors and PD-L1 inhibitors. The reasons for this suboptimal predictive capacity are not well known. In addition, recent studies suggested that several additional factors could be involved in the response to immune checkpoint inhibitors (e.g. anti-PD-1/PD-L1 antibodies) after treatment has been initiated, which may account for variability in predictive scores. Taken together, although PD-L1 is considered as a useful biomarker, there are concerns that, as a single biomarker, it may not be sufficient for accurate prediction (Vareki et al (2017), Critical Review in Oncology/Hematology, Vol.116, pages 116-124; Diggs and Hsueh (2017), Biomarker Research, Vol. 5:12, pages 1-6).

Other strategies for achieving better patient selection or predicting treatment outcome using biomarkers other than PD-L1 have been developed in parallel and include for instance: 1) immune cell phenotyping using flow cytometry for different immune cells (e.g. peripheral blood mononuclear cells (PBMNC) or TILS) using a combination of markers (e.g. such as CD3, CD4, CD8, Ki67, CTLA-4, PD-1, LAG-3, TIM-3, ICOS for activated T cells; CD3, CD4, CD25, FOXP3, CD127, Ki67, CD45RA for regulatory T cells; CD45RO for memory T cells, etc.). The purpose of phenotyping is to quantitate the effect of therapy (e.g. determining proportion of certain cell types) on specific subtypes of immune cells, 2) assessing the expression of IFN-gamma *per se* or IFN-gamma-inducible genes such as indoleamine 2, 3-dioxygenase (IDO) in tumors, 3) examining the mutational load of the tumor or gene expression (e.g. inflammatory genes (such as IFN-gamma related genes) to establish genetic signatures, and others (Vareki et al (2017), Critical Reviews in Oncology/Hematology, Vol. 116, pages 116-124), and other strategies.

Although promising, the strategies or biomarkers described above are not optimal and still require further optimization before they can be reliably used in the clinic (Ma et al (2016), J. Hematology & Oncology, Vol. 9:47, pages 1-21; Vareki et al (2017), Critical Reviews in Oncology/Hematology, Vol. 116, pages 116-124).

Based on the above, it can be concluded that: 1) the quest for finding reliable predictive biomarker(s) yielded several candidates, which individually show some potential but are still not optimal, e.g. a single biomarker is likely not sufficient for accurate prediction, 2) some of the biomarkers identified until now are biomarkers suitable for predicting whether a patient will respond or will be resistant to immune checkpoint therapy (early on) during therapy, i.e. the biomarker-based prediction is made after therapy has been initiated, and 3) despite progress in identifying various biomarkers, there is still a great lack of reliable biomarker(s) that can be used by clinicians to select or exclude patients for treatment with immune checkpoint inhibitors (e.g. PD-1 and/or PD-L1 inhibitors), prior to treatment, i.e. the biomarker-based prediction needs to be made before initiating treatment.

Therefore, there is a need for new or alternative or improved biomarker(s), and methods using them to reliably select cancer patients suitable for immune checkpoint therapy (e.g. with PD-1 inhibitors (e.g. nivolumab) and/or PD-L1 inhibitors (e.g. atezolizumab) and to reliably predict responsiveness to immune checkpoint therapy (e.g. with PD-1 and/or PD-L1 inhibitors) prior to initiating said immune checkpoint therapy or early on (early phase) during treatment.

It is an objective of the present invention to provide such predictive biomarker(s) and related method(s) to better predict or to better select cancer patients who will be likely to benefit (or not) from immune checkpoint therapy with PD-1 inhibitors (e.g. nivolumab) and/or PD-L1 inhibitors (e.g. atezolizumab), prior to initiating therapy or early on during treatment, in order to spare patients from unnecessary risks (e.g. occurrence of undesirable side-effects, toxicity, costly treatment, etc.), and/or to avoid depriving patients from a better-suited therapy.

SUMMARY
The present inventors have uncovered a way to reliably guide patient selection as well as to predict clinical response to immune checkpoint therapy with PD-1 inhibitors (e.g. nivolumab) and/or PD-L1 inhibitors (e.g. atezolizumab), prior to initiating treatment with said agents or early on during treatment.

More specifically, the present inventors have devised reliable methods for: 1) selecting a human subject diagnosed with cancer (e.g. NSCLC) suitable for immune checkpoint therapy with PD-1 inhibitors (e.g. nivolumab, pembrolizumab, BGB-A317, and others) and/or PD-L1 inhibitors (e.g. durvalumab, atezolizumab, avelumab, and others), 2) predicting responsiveness to immune checkpoint therapy with PD-1 inhibitors and/or PD-L1 inhibitors, prior to initiating therapy with said agents or early on during treatment, and 3) treating a human subject diagnosed with cancer using PD-1 inhibitors and/or PD-L1 inhibitors, alone or in combination with another therapeutic agent (e.g. CTLA-4 inhibitor compounds such as ipilimumab). More particularly, the methods of the invention rely on the use of a biomarker consisting of using the density (number of cells per mm^2) or percent of a specific subpopulation of cells present in a tumor sample (i.e. intratumoral cells), which is referred to herein as "PD-1^T cell population" to make a treatment outcome prediction and/or for selecting a patient suitable for therapy with a PD-1 inhibitor compound and/or a PD-L1 inhibitor compound, alone or in combination with another therapeutic agent (e.g. CTLA-4 inhibitor compound such as ipilimumab). The present inventors also devised new assays for reliably quantifying the expression of PD-1 (i.e. immunostaining intensity) in cells in a tumor sample (i.e. intratumoral cells), which are used to reliably identify the PD-1^T cell population (i.e. density or %) in a tumor sample (e.g. a single cell suspension or tumor tissue sections) as well as methods for establishing a pre-determined reference value 1 (REF1) of intensity of immunostaining for PD-1 (for use in the cytometric assay as taught herein) and a pre-determined reference value 2 (REF2) of intensity of immunostaining for PD-1 (for use in the IHC assay as taught herein).

The treatment outcome prediction / patient selection methods of the invention, PD-1 expression quantifying assays (i.e. cytometric and IHC) (using REF 1 and REF2 as taught herein) and biomarker (i.e. percent (%) or density of PD-1^T cells), as taught in more details herein, represent an improvement compared to the prediction methods, quantification assays and biomarkers used in the field of immune checkpoint therapy with PD-1 inhibitor compounds and/or PD-L1 inhibitor compounds for cancer treatment, prior the filing date of the present invention.

One main improvement consists of the unique assays used herein for quantifying PD-1 expression (i.e. immunostaining intensity) in intratumoral cells (i.e. cells present in the tumor). Specifically, the present inventors have devised and calibrated two different assays for quantifying PD-1 expression (i.e. immunostaining intensity). The first assay (referred to herein as *cytometric assay*) is suitable for cytometric measurements (e.g. measurements made per individual cell in suspension in a fluid, for instance using flow cytometry techniques). The second assay (referred to herein as *immunohistochemistry (IHC) assay*) is suitable for tissue measurements (e.g. measurements made per individual cell in a tissue section such as FFPES or FFFS, for example using immunohistochemistry techniques). The unique characteristics of the two PD-1 expression quantifying assays as taught herein, as well as their applications in treatment outcome prediction / patient selection methods as well as treatment methods, are further described below.

DETAILED DESCRIPTION

Definitions
A portion of this disclosure contains material that is subject to copyright protection (such as, but not limited to, diagrams, device photographs, or any other aspects of this submission for which copyright protection is or may be available in any jurisdiction). The copyright owner has no objection to the facsimile reproduction by anyone of the patent document or patent disclosure, as it appears in the Patent Office patent file or records, but otherwise reserves all copyright rights whatsoever.

Various terms relating to the methods, compositions, uses and other aspects of the present invention are used throughout the specification and claims. Such terms are to be given their ordinary meaning in the art to which the invention pertains, unless otherwise indicated. Other specifically defined terms are to be construed in a manner consistent with the definition provided herein. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred materials and methods are described herein. For purposes of the present invention, the following terms are defined below.

As used herein, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. For example, a method for administrating a drug includes the administrating of a plurality of molecules (e.g. 10's, 100's, 1000's, 10's of thousands, 100's of thousands, millions, or more molecules).

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As used herein, the term "and/or" indicates that one or more of the stated cases may occur, alone or in combination with at least one of the stated cases, up to with all of the stated cases.

10　As used herein, the term "at least" a particular value means that particular value or more. For example, "at least 2" is understood to be the same as "2 or more" i.e., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, ..., etc.

The term "to comprise" and its conjugations as used herein is used in its non-limiting 15　sense to mean that items following the word are included, but items not specifically mentioned are not excluded. It also encompasses the more limiting "to consist of"."

The terms "cancer" and "tumor" (used interchangeably), as used herein, refer to or describe the physiological condition in humans that is typically characterized by 20　unregulated cell growth. The terms "cancer" and "tumor" also refer to cells that have undergone a malignant transformation that makes them pathological to the host organism. Cancer cells can be distinguished from non-cancerous cells by techniques known to the skilled person.

25　The term "tumor tissue sample" or "tumor biopsy sample" or "tumor sample" as used herein, refers to piece(s) or slice(s) of tissue that has/have been removed from a tumor, including following a surgical tumor resection. The tumor tissue sample can be subjected to a variety of well-known post-collection preparative and storage techniques (e.g., fixation, storage of fixed tissue, freezing, homogenization, etc.) prior 30　performing immunostaining or prior to determining the cell densities, cell phenotype, gene expression profile, gene markers, or, etc.). In the present invention, the tumor tissue sample may be fixed in formalin and embedded in a rigid fixative, such as paraffin (wax) or epoxy (referred to herein as "formalin fixed paraffin embedded" (FFPE)) tissue, which is placed in a mould and later hardened to produce a block which

is readily cut (Bancroft's Theory and Practice of Histological Techniques, 7th Edition; Authors: Kim Suvarna Kim Suvarna Christopher Layton John Bancroft; Imprint: Churchill Livingstone; Publication Date: 26th October 2012; ISBN: 9780702042263).

5   Alternatively, the tumor tissue sample may be a (fresh) frozen tissue sample (FFFS). During the (fresh) frozen section procedure, the surgeon removes a portion of the tissue mass. This biopsy is then given to a pathologist (a doctor who examines tissues and uses laboratory tests to make a diagnosis). The pathologist freezes the tissue in a cryostat machine, cuts it with a microtome, and then fixes it (e.g. , the fresh frozen

10   sections are fixed, e.g. in formalin, after thawing), and stains it with various dyes and/or subject it to immunohistochemistry procedure (e.g. for PD-1 staining as taught herein) so that it can be examined under the microscope (A Practical Guide to Frozen Section Technique. Editor: Stephen R. Peters; Publisher: Springer; 2010 edition (19 Jan. 2010); ISBN: 9781441912336; Frozen section of lung specimens. Sienko A, Allen TC,

15   Zander DS, Cagle PT. Arch Pathol Lab Med. 2005 Dec;129(12):1602-9. Review. PMID: 16329732; Frozen Section Biopsy; Erin Brender, MD; Alison Burke, MA; Richard M. Glass, MD JAMA. 2005;294(24):3200. doi:10.1001/jama.294.24.3200"). The procedure usually takes only minutes. It is understood that thin slices of FFPE tissue sample or FFFS sample can be prepared using a microtome, placed on a glass slide

20   and submitted e.g. to immunohistochemistry (IHC) procedures or immunostaining procedures. In the present invention, either FFPE sample or FFFS sample can be equally used in the method of the invention without affecting the reliability or quality of the results. In a preferred embodiment of the invention, the tumor tissue sample is in the form of a FFPE sample.

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In other situations, the tumor tissue sample may be processed into a single cell tumor suspension using standard methods for use in flow cytometry assays or may be processed for use in DNA or RNA microarrays, etc.

30   The term "immune checkpoint molecule " as used herein refers to a protein that is expressed by T cells or other immune cells that either turn up a signal (also known as "stimulatory checkpoint molecules") or turn down a signal (also known as "inhibitory checkpoint molecules"). Five stimulatory checkpoint molecules on T cells are members of the tumor necrosis factor (TNF) receptor superfamily - CD27, CD40, OX40, GITR

and CD137. Another two stimulatory checkpoint molecules belong to the B7-CD28 superfamily - CD28 itself and ICOS. Inhibitory checkpoint molecules have been increasingly considered as new targets for cancer immunotherapy due to their potential for use in multiple types of cancers. Currently approved checkpoint inhibitors block CTLA-4 and PD-1, and also one of the ligands of PD-1, PD-L1. In the context of the present invention, PD-1 and PD-L1 are particularly preferred, alone or in combination with other immune checkpoint therapy agents such as CTLA-4 inhibitor compounds.

The term "Programmed Death-1 (PD-1)" receptor, as used herein, refers to an immune-inhibitory receptor belonging to the CD28 family. In humans, PD-1 is encoded by the PDCD1 gene. PD-1 is expressed predominantly on previously activated T cells in vivo, and binds to two ligands, PD-L1 and PD-L2. The term "PD-1" as used herein includes human PD-1 (hPD-1), variants, isoforms, and species homologs of hPD-1, and analogues having at least one common epitope with hPD-1. The complete hPD-1 sequence can be found under GENBANK Accession No. U64863) PD-1 is expressed on immune cells such as activated T cells (including effector T cells), B cells, myeloid cells, thymocytes, and natural killer (NK) cells (Suya Dai et al (2014) Cellular Immunology, Vol:290, pages 72-79; Gianchecchi et al (2013), Autoimmun. Rev. 12 (2013) 1091–1100).

The term "Programmed Death Ligand-1 (PD-L1)", as used herein, refers to one of two cell surface glycoprotein ligands for PD-1 (the other being PD-L2) that down-regulate immune cell activation and cytokine secretion upon binding to PD-1. PD-L1 is also known as cluster of differentiation 274 (CD274) or B7 homolog 1 (B7-H1). In humans, PD-L1 is encoded by the CD274 gene. The term "PD-L1" as used herein includes human PD-L1 (hPD-L1), variants, isoforms, and species homologs of hPD-L1, and analogues having at least one common epitope with hPD-L1. The complete hPD-L1 sequence can be found under GENBANK Accession No. Q9NZQ7. PD-L1 is expressed on a variety of cells including cells of hematopoietic lineage such as activated T cells, B cells, monocytes, dendritic cells (DCs), mast cells, and macrophages. PD-L1 is also expressed on peripheral non-hematopoietic tissue such as heart cells, skeletal muscle cells, pancreatic islet cells, placenta cells, lung cells, hepatocytes, epithelium cells,

kidney cells, mesenchymal stem cells, liver cells, and others (Suya Dai et al (2014) Cellular Immunology, Vol:290, pages 72-79).

In more detail, PD-L1 is expressed on T and B cells, myeloid cells (e.g. dendritic cells,
5 macrophages, neutrophils), mesenchymal stem cells, and bone marrow-derived mast cells. PD-L1 is also expressed on a wide range of non-hematopoietic cells (e.g., cornea, lung, vascular epithelium, liver non-parenchymal cells, mesenchymal stem cells, pancreatic islets, placental syncytiotrophoblasts, keratinocytes, brown adipose tissue, etc.), and is upregulated on a number of cell types after activation. Both type I
10 and type II interferons (IFNs) and hypoxia upregulate PD-L1. PD-L1 is expressed in many cancers. The expression of PD-L1 is further up-regulated (i.e. increased compared to resting conditions) on cells by various immune stimuli including for instance anti-IgM antibody, LPS and anti-CD40 antibody for B cells, anti-CD3 antibody for T cells, anti-CD40 antibody, LPS, IFN gamma and granulocyte macrophage colony
15 stimulating factor for macrophages and anti-CD40 antibody, IFN gamma, IL-4, IL-12 and GM-CSF for dendritic cells (Taku Okazaki and Tasuku Honjo (2007), International Immunology, Vol: 19, pages 813-824).

The term "PD-1/PD-L1 axis" as used herein consists of the PD-1 receptor and its ligand
20 PD-L1. The term "PD-1/PD-L1 axis signaling" is a way of communication between cells (cell signaling), for instance between a first cell expressing PD-1 and a second cell expressing PD-L1, and which involves the release of a biochemical signal (e.g. release of proteins, lipids, ions, neurotransmitters, enzymes, gases, etc.), which in turn causes an effect (e.g. inhibition, activation, blockade, etc.) on one or both cells. The term "cell
25 signaling" in general refers to the biochemical relationship between a variety of signal transduction molecules that play a role in the transmission of a signal from one portion of a cell to another portion of the cell. A "cell surface receptor" includes, for example, molecules and complexes of molecules that are located on the surface of a cell and are capable of receiving a signal and transmitting such a signal across the plasma
30 membrane of a cell. An example of a cell surface receptor of the present invention is the PD-1 receptor, which is, for example, located on the surface of activated B cells, activated T cells and myeloid cells. In the context of the present invention, an example of "PD-1/PD-L1 axis signaling" is when PD-L1 expressed at the cell surface of a first cell (e.g. a cancer cell or a cancer-infiltrating immune cell) binds to its receptor PD-1

expressed at the cell surface of a second cell (e.g. a T cell, such as an effector T cell). The binding of PD-L1 to its receptor PD-1 transmits an inhibitory signal to the T-cell which results in a decrease in T cell proliferation (e.g. effector T cells) as well as T cell activity (e.g. secretion of cytokines and chemokines as discussed herein; Wei F et al

5　(2013) PNAS; Vol: 110, E2480-2489). Thus, one possible end result of PD-1/PD-L1 axis signaling is the dampening or inhibition of immune activity or function mediated by T cells (e.g. effector T cells). Such a situation may be detrimental in the context of cancer (e.g. lung cancer, bladder cancer, GI tract cancer, melanoma, etc.), as discussed herein. Another example of "PD-1/PD-L1 axis signaling" is when PD-L1

10　expressed at the cell surface of a first cell (e.g. pancreatic cell) binds to its receptor PD-1 expressed at the cell surface of a second cell (e.g. a T cell, such as an effector T cell). The binding of PD-L1 to its receptor PD-1 transmits an inhibitory signal to the T-cell which ultimately causes a reduction or inhibition of T-mediated secretion of cytokines (e.g. Interferon gamma, TNF alpha, and others) and chemokines (e.g.

15　CXCL9, CXCL10) as well as reduced T cell (e.g. effector T cell) proliferation (Wei F et al (2013) PNAS; Vol: 110, E2480-2489). Thus, one possible end result of PD-1/PD-L1 axis signaling is the dampening or inhibition of immune activity or function mediated by T cells (E.g. effector T cells). This may be advantageous in the context of autoimmune diseases (e.g. diabetes type 1, rheumatoid arthritis, systemic lupus

20　erythematosus, etc.), where dampening of an overly active immune system (e.g. T-cell mediated effects) is desired, as discussed herein. Other examples of end results of PD-1/PD-L1 axis signaling are described in the scenarios above.

The term "cytotoxic T-lymphocyte-associated protein 4" (abbreviated "CTLA-4" and

25　also known as cluster of differentiation 152 (CD152)), as used herein, refers to a protein receptor that functions as an immune checkpoint. More specifically CTLA-4 downregulates immune responses (e.g. downregulates T cell functions). CTLA-4 is a member of the immunoglobulin superfamily that is expressed by activated T cells and transmits an inhibitory signal to T cells. CTLA-4 is homologous to the T-cell co-

30　stimulatory protein, CD28, and both molecules bind to CD80 and CD86, also called B7-1 and B7-2 respectively, on antigen-presenting cells. The CTLA-4 protein is encoded by the CTLA-4 gene in humans (Ensembl ref: ENSG00000163599). Normally, after T-cell activation, CTLA-4 is upregulated on the plasma membrane where it functions to downregulate T-cell function through a variety of mechanisms, including

preventing co-stimulation by outcompeting CD28 for its ligand, B7, and also by inducing T-cell cycle arrest Postow et al (2015) J. Clinical oncology, Vol. 33, pages 1974-1983; Pardoll, D. et al (2012).

5   In the field of cancer, there is growing interest in finding or developing CTLA-4 inhibitor compounds or antagonists of CTLA-4 to increase immune activity (i.e. to increase host's adaptive immunity against cancer cells). Non-limiting examples of CTLA-4 inhibitor compounds currently considered for clinical use in the treatment of cancer (e.g. melanoma) include antagonistic antibodies against CTLA-4 such as ipilimumab

10   ((Yervoy®, MDX-010, Bristol-Myers Squibb, FDA approved for melanoma in 2011) as a means of inhibiting immune system tolerance to tumours and thereby providing a potentially useful immunotherapy strategy for patients with cancer. A further example of CTLA-4 inhibitor compounds (not yet approved) is tremelimumab (CP-675206, Pfizer) (Postow et al (2015) J. Clinical oncology, Vol. 33, pages 1974-1983; Pardoll,

15   D. et al (2012), Nature Reviews Cancer, Vol. 12, pages 252-264).

The term "immune checkpoint inhibitor(s)" as used herein refers to a compound(s) or pharmaceutical agent(s) or drug(s) or candidate drug(s) (e.g. antibodies, fusion proteins, small molecule drugs (natural or synthetic), interfering RNA (e.g. siRNA) that

20   totally or partially reduces, inhibits, interferes with or modulates one or more immune checkpoint proteins or their ligands, particularly inhibitory immune checkpoint molecules such as PD-1 or CTLA-4 and/or the PD-1 ligand PD-L1.

Non-limiting examples of PD-1 inhibitor compounds include PD-1 antibodies such as

25   nivolumab (Opdivo®, Bristol-Myers Squibb), pembrolizumab (Keytruda®, Merck), BGB-A317, and others such as PDR001 (Novartis). Further PD-1 inhibitors also include any anti-PD-1 antibody described in US8008449, US7521051 and US8354509. Also contemplated are fusion proteins that bind to PD-1 (e.g. anti-PD-1 fusion proteins AMP-224 (MedImmune) and AMP-514 (MedImmune)).

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Non-limiting examples of PD-L1 inhibitor compounds include anti-PD-L1 antibodies such as durvalumab (MEDI4736, Imfinzi®, MedImmune), atezolizumab (Tecentriq®, Roche), avelumab (Bavencio®, Merck), and others such as BMS-936559 (BMS) (Meng et al (2015), Cancer Treatment Review, Vol. 41, pages 868-876; Brahmer et al (2010)

J Clin Oncol 28:3167-75; Brahmer et al (2012) N. Engl. J. Med. Vol: 366, pages 2455-65; Flies et al (2011) Yale J. Biol. Med. Vol.84, pages 409-21; Topalian et al. (2012b) N. Engl. J. Med. Vol. 366, pages 2443-54; Diggs et al (2017), Biomarker Research, Vol.5:12, pages 1-6). Further PD-L1 inhibitors include any anti-PD-L1 antibody described in US8383796. Also contemplated are fusion proteins that bind to PD-L1.

Non-limiting examples of CTLA-4 inhibitor compounds include ipilimumab ((Yervoy®, MDX-010, Bristol-Myers Squibb, FDA approved for melanoma in 2011) and (not yet approved) is tremelimumab (CP-675206, Pfizer) (Postow et al (2015) J. Clinical oncology, Vol. 33, pages 1974-1983; Pardoll, D. et al (2012), Nature Reviews Cancer, Vol. 12, pages 252-264).

The term "formalin-fixed paraffin embedded tissue section(s) (abbreviated "FFPE")", as used herein, refers to a single part or piece of a tissue sample, e.g., a thin slice of tumor tissue cut from a tumor sample (e.g. biopsy) or from a normal tissue. It is understood that multiple sections of a given thickness (e.g. 4 or 5 micrometre, etc.) of a single tissue sample (e.g. tumor sample) may be prepared according to standard histology procedures (Bancroft's Theory and Practice of Histological Techniques, 7th Edition; Authors: Kim Suvarna Kim Suvarna Christopher Layton John Bancroft; Imprint: Churchill Livingstone; Publication Date: 26th October 2012; ISBN: 9780702042263) and processed (e.g. antibody staining for instance with anti-PD-1 antibody as taught herein) and analyzed in accordance with the methods of the present invention.

The term "(fresh) frozen tissue section(s) (FFFS)" as used herein refers to a single part or piece of a tissue sample, e.g., a thin slice of tumor tissue freshly cut from a tumor sample (e.g. biopsy). It is understood that multiple sections of a given thickness (e.g. 4 or 5 micrometre, etc.) of a single tissue sample (e.g. tumor sample) may be prepared according to standard histology procedures (A Practical Guide to Frozen Section Technique. Editor: Stephen R. Peters; Publisher: Springer; 2010 edition (19 Jan. 2010); ISBN: 9781441912336; Frozen section of lung specimens. Sienko A, Allen TC, Zander DS, Cagle PT. Arch Pathol Lab Med. 2005 Dec;129(12):1602-9. Review. PMID: 16329732; Frozen Section Biopsy; Erin Brender, MD; Alison Burke, MA; Richard M. Glass, MD JAMA. 2005;294(24):3200. doi:10.1001/jama.294.24.3200) and processed (e.g. antibody staining for instance with anti-PD-1 antibody as taught

herein) and analyzed in accordance with the methods of the present invention. For instance, during the (fresh) frozen section procedure, the surgeon removes a (fresh) portion of the tumor tissue mass. This tumor biopsy is then given to a pathologist (a doctor who examines tissues and uses laboratory tests to make a diagnosis). The pathologist freezes the tissue in a cryostat machine, cuts it with a microtome, and then fixes it (e.g. with formalin) and stains it with various dyes (e.g. nuclear stains to label nuclei) and/or subject it to immunohistochemistry procedures such as taught herein (e.g. for PD-1 staining) so that it can be examined under the microscope for further analyses as taught herein. The skilled person is well-acquainted with methods and protocols to perform or obtain (fresh) frozen tissue suitable for the method as taught herein. Further, the use of (fresh) frozen section biopsy is advantageous if more tissue is needed to make further analyses, because the surgeon can obtain an additional sample, avoiding a second operation.

The term "tumor single cell suspension" (abbreviated "TSS") as used herein refers to a situation where the tumor sample is processed or broken up so that single cells are floating or in suspension within a liquid or fluid. This is different from a situation where cells are part of a clump of cells or tissue attached to each other by extracellular matrix. Single cell suspensions (e.g. TSS) can be prepared from a solid tissue (e.g. solid tumor tissue or biopsy) using various standard methods (e.g. Thommen et al (2015), Cancer Immunol Res. Vol.3(12), pages 1344-55). Single-cell suspensions are typically used in a situation or in experiments requiring cell separation, cell analysis (e.g. flow cytometry analysis) and cell culture.

The terms "immunohistochemistry assay (abbreviated as "IHC assay") and "immunostaining assay" (used interchangeably) as used herein refer to terms that are well-known in the art. IHC assay refers to a procedure for selectively imaging or visualizing or detecting antigens (proteins) in cells of a tissue section (e.g. FFPES or FFFS), or in cells in a cell suspension, by exploiting the principle of antibodies binding specifically to antigens in biological tissues or cells. Typically, an IHC assay begins with antigen retrieval, which may vary in terms of reagents and methods. The antigen retrieval process may involve pressure cooking, protease treatment, microwaving, or heating histologic sections in baths of appropriate buffers, with the standard goal of unmasking antigens hidden by formalin crosslinks or other fixation, and others. See,

e.g., Leong et al. Appl. Immunohistochem. 4(3):201 (1996). Imaging or visualizing or detecting an antibody-antigen interaction (e.g. anti-PD-1 antibody binding to human PD-1 protein in a cell) can be accomplished in a number of ways using standard techniques. In the most common instance, an antibody is conjugated to an enzyme,

5   such as peroxidase, that can catalyse a colour-producing reaction (see immunoperoxidase staining). Alternatively, the antibody can also be tagged to a fluorophore, such as fluorescein or rhodamine, and the like.

In the context of the invention, the term "prior to initiating treatment" is used to refer to

10   a situation where treatment with a PD-1 inhibitor compound and/or a PD-L1 inhibitor compound, alone or in combination with another immune checkpoint therapy agent such as CTLA-4 inhibitor compound, has not been started yet, i.e. the cancer subject has not received such therapy yet or is naïve to such therapy. It is understood that, prior to initiating treatment with the PD-1 inhibitor compound and/or the PD-L1 inhibitor

15   compound, alone or in in combination with another immune checkpoint therapy agent such as a CTLA-4 inhibitor compound, the cancer subject may have been naïve to any cancer treatment or may have been previously treated with one or more different cancer therapeutic agents such as chemotherapy agents, hormone therapy (e.g. tamoxifen), immunotherapy etc.

20

In the context of the present invention, the term "early on during treatment", as used herein, refers to a situation where treatment with a PD-1 inhibitor compound and/or a PD-L1 inhibitor compound, alone or in in combination with another immune checkpoint therapy agent such as CTLA-4 inhibitor compound, has just been started, i.e. the

25   cancer subject has been receiving such therapy for a short period, e.g. 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, or 8 weeks. It is understood that, once the treatment with the PD-1 inhibitor compound and/or the PD-L1 inhibitor compound has been initiated, alone or in in combination with another immune checkpoint therapy agent such as CTLA-4 inhibitor compound, the cancer subject may

30   have been naïve to any cancer treatment or may have been previously treated with one or more different cancer therapeutic agents such as chemotherapy agents, hormone therapy (e.g. tamoxifen), immunotherapy etc.

The term "subject" or "patient" (used interchangeably) as used herein refers to a human subject male or female, adult, child or infant, suffering from a cancer (e.g. NSCLC), regardless of the stage or state of the cancer.

5 The terms "treat," treating", "treatment", "therapy" and the like as used herein refer to reducing or ameliorating a disorder (e.g. cancer) and/or symptoms associated therewith. It is appreciated that treating a disorder or condition (e.g. cancer such as lung cancer) does not require that the disorder, condition or symptoms associated therewith be completely eliminated. It is further understood that the terms "treat,"

10 treating", "treatment", "therapy" and as used herein may be a first or first line of treatment (i.e. patient is naïve to any cancer treatment) or a second or third line treatment and so on (i.e. the first treatment or second treatment and so on was not effective or has failed).

15 <u>Cytometric assay</u>

The present inventors have devised a unique assay for quantifying the expression of PD-1 (intensity of immunostaining for PD-1) in cells in a tumor sample, which is referred to herein as the *cytometric assay*. The cytometric assay as taught herein differs from existing assays in that it makes use of an external reference value for PD-

20 1 expression (i.e. immunostaining intensity) (referred to here as "reference value 1" (REF1) as taught in more details below), which is derived from peripheral T lymphocytes obtained from a blood sample from at least one healthy human donor (cancer-free).

25 Because the expression of PD-1 in peripheral T lymphocytes (e.g. CD3 positive and PD-1 positive cells (CD3+/PD-1+) peripheral T lymphocytes) is steady (does not substantially vary across healthy donors (cancer free), PD-1 expression (i.e. intensity of immunostaining for PD-1) detected in these cells can serve as a reliable reference value (does not substantially vary across healthy (cancer-free) subjects to establish a

30 threshold (cut off-mark) of immunostaining intensity for PD-1 in other cells (e.g. intratumoral cells). Said threshold value (REF1) can then be used to segregate PD-1 positive cells (PD-1+ cells such as intratumoral cells) into two groups, i.e. PD-1+ intratumoral cells having an intensity of immunostaining for PD-1 equal to or above REF1 vs the remaining PD-1+ intratumoral cells having an immunostaining intensity

for PD-1 below the threshold REF1. In the present invention, intratumoral cells having the highest intensity of immunostaining for PD-1, as determined using the REF1 threshold as explained above, are referred to as $PD\text{-}1^T$ cells or PD-1^T intratumoral cells, as taught in more details below.

5

One advantage of using an external reference REF1 (more stable reference value as explained above) derived from peripheral T lymphocytes obtained from blood of a healthy human donor (cancer-free) is that once established for one individual (healthy donor) of a given population, the same REF1 (i.e. value obtained) can be used each

10   time a new tumor sample is processed without having to establish REF1 anew with a new healthy donor subject. A further advantage is that the cytometric assays as taught herein using REF1 can be standardized across laboratories, rendering the methods of the invention reliable. For example, a limitation of the prior art methods, e.g. flow cytometry methods, is that an internal reference (as cut-off mark) is used which is

15   based on specific gating strategies selected by the scorer. However, gating strategies/criteria are not standardized and vary greatly across users, and thus such methods are difficult to reproduce or to standardize across institutions or scores (e.g. clinician, pathologists, etc.). The cytometric assay of the invention offers a solution to this problem, as presented below:

20

In a first aspect, the present invention relates to a (cytometric) assay for quantifying PD-1 expressing cells (PD-1^T) in a tumor sample obtained from a human subject, said method comprising the steps of:

1a.   Providing a solid tumor sample from said human subject, wherein the sample

25   is in the form a single cell suspension (TSS);

1b.   Incubating the sample of step (a) under conditions allowing specific antigen-antibody binding with an (labeled) antibody directed to a human T lymphocyte marker (M), and an (labeled) antibody directed to human PD-1 to allow detection of cells positive for M (M+ cells), PD-1 (PD-1+ cells) and both M and PD-1 (M+/PD-1+ cells)

30   in said sample;

1c.   Measuring the intensity of immunostaining of PD-1 for M+/PD-1+ cell; and

1d.   Quantifying the number of PD-1^T cells, wherein the PD-1^T cells are the M+/PD-1+ cells of step (c) having an intensity of immunostaining for PD-1 that is equal or higher than a pre-determined reference value 1 (REF1), relative to the total number of

M+ cells in the tumor sample to obtain the percent (%) of PD-1^T cells in said tumor sample, wherein the pre-determined REF1 was obtained by a method as taught herein.

In step 1b, it is understood that antibody must be detectable and thus the primary antibody directed to a human T lymphocyte marker (M, e.g. CD3+) or to human PD-1 may be labeled (e.g. with a fluorescent tag) or the primary antibody may be unconjugated and then recognized/visualized by a labeled secondary antibody. The skilled person knows how to select a primary antibody and/or secondary antibody to detecting specific antigen-antibody binding.

In an embodiment, the human T lymphocyte marker (M) is selected from CD3, CD4 and CD8, or combinations thereof. In a preferred embodiment, the human T lymphocyte marker (M) is CD3. It is understood that cells which are positive (as detected by flow cytometry methods) for a T lymphocyte marker (M, e.g. CD3) can be referred to as M+ cells, e.g. CD3+ cells. The same principle applies for cells which are positive for CD3 and PD-1, i.e. can be referred to as CD3+/PD-1+ cells.

The term "PD-1^T cells in a tumor sample" or "PD-1^T intratumoral cells" (used interchangeably), as used herein, refers to a population of cells or cells (any cell type, preferably infiltrating T lymphocytes (TILS), which express PD-1 (as detected by immunohistochemistry or flow cytometry techniques using an anti-PD-1 antibody as taught herein), which display or have an intensity of immunostaining for PD-1 which is at or above a pre-determined threshold value or reference value such as REF 1 and/or REF 2 as taught herein. It is understood that "PD-1T" is the same as "PD-1^T". In the context of the present invention, specific percentages (i.e. specific cut-off marks, as taught herein) of PD-1^T cells in a tumor sample are used as a biomarker in the methods as taught herein for reliably predicting response to immune checkpoint therapy with a PD-1 inhibitor compound and/or a PD-L1 inhibitor compound, alone or in combination with another immune checkpoint therapy agent such as a CTLA-4 inhibitor compound and/or for reliably selecting a cancer subject suitable for immune checkpoint therapy with a PD-1 inhibitor compound and/or a PD-L1 inhibitor compound, alone or in combination with another immune checkpoint therapy agent such as CTLA-4 inhibitor compound (e.g. ipilimumab) and/or for reliably selecting or deciding on a suitable treatment for a given cancer subject, according to the methods as taught herein.

The term "antibody directed to human PD-1" or "labeled antibody directed to human PD-1" (used interchangeably), as used herein refer to an anti-PD-1 antibody (primary antibody) that binds specifically to human PD-1. It is understood that the anti-PD-1 antibody may be labeled (e.g. fluorescent label or other type of label) to facilitate its detection or visualization, e.g. in PD-1 immunostained tissue slices (e.g. FFPE sections) or in flow cytometry procedures. The primary antibody may be purchased with the label already attached (e.g. EH12.1, BD-Biosciences, cat. No 561272 is an example of that) or may be purchased without the label (e.g. Roche Ventana benchmark ultra, NAT105, Roche Diagnostics, cat. no. 760-4895, Ventana/DAB detection is an example of that) and the label may be present on the secondary antibody used to detect the primary antibody, etc. The skilled person knows how to select for or make a primary antibody detectable using labels. Non-limiting examples of anti-PD-1 antibodies include mouse anti-PD-1 PE-Cy7 (EH12.1, BD-Biosciences, cat. No 561272, e.g. particularly useful for flow cytometry analysis), and mouse monoclonal anti-PD-1 (Roche Ventana benchmark ultra, NAT105, Roche Diagnostics, cat. no. 760-4895, Ventana/DAB detection, "ready to use concentration", e.g. particularly useful for detection of the PD-1 protein in formalin-fixed, paraffin-embedded (FFPE) tissue). Other examples include anti-PD-1 (MIH4), anti-PD-1 (EH12.2H7), anti-PD-1 (J116), anti-PD-1 (eBioJ105).

In an embodiment, the (labeled) antibody (primary antibody) directed to human PD-1 is selected from the group of anti-PD-1 (EH12.1, BD-Biosciences, cat. No 561272), anti-PD-1 (MIH4), anti-PD-1 (EH12.2H7), anti-PD-1 (NAT105, Roche Ventana benchmark ultra, Roche Diagnostics, cat. no. 760-4895, Ventana/DAB detection, "ready to use concentration"), anti-PD-1 (J116), anti-PD-1 (eBioJ105). In a preferred embodiment, the (labeled) antibody directed to human PD-1 is EH12.1, BD-Biosciences, cat. No 561272. It was found that such antibody is particularly useful (works well in cytometry analysis or assays such as taught herein).

In an embodiment, the (labeled) antibody (primary antibody) directed to human PD-1 used for establishing REF1 in the method as taught herein and the labeled antibody (primary antibody) directed to human PD-1 (in step 1b) are the same, e.g. EH12.1, BD-Biosciences, cat. No 561272. This is particularly advantageous to prevent

experimental errors such as e.g. to avoid non-specific background due to variation in antibody specificity, binding, etc.

The skilled person is well-acquainted with flow cytometry methods and knows how to set or establish conditions allowing specific antigen-antibody binding as specified in step 1b, and knows how to carry out staining of cells in a reproducible manner. Likewise, in step 1c, the skilled person knows how to measure the intensity of immunostaining for PD-1 per cell in a flow cytometry assay using standard apparatus and techniques and softwares, etc. in a reproducible manner, e.g. using a BD LSR Fortessa Cell analyzer (BD LSR Fortessa Cell analyzer (BD Bioscience)) for flow cytometry analysis and for instance using the BD FACS Diva Software version 7 and FlowJo v10.0.6 (Tree Star Inc.), for data analysis etc.

In step 1d, the term "relative to the total number of M+ cells in the tumor sample" refers to the total of cells, which are positive for M+. For instance, if M is CD3, than the total number of CD3+ cells in the tumor sample include CD3+ cells, CD3+/PD-1+, and CD3+/PD-1- cells.

The term "reference value" or "threshold value" as used herein refers to a predetermined value that is obtained by the method of the invention. Specifically, the reference value REF1 is established based on the intensity of immunostaining for PD-1, which is measured in peripheral T lymphocytes obtained from a blood sample from at least one healthy human donor (a human subject not suffering from cancer), as explained herein. Because PD-1 immunostaining intensity for PD-1 in peripheral T lymphocytes is substantially stable (does not vary much) across healthy individuals, it can serve as a reliable (stable) basis to establish a reference value for quantifying PD-1 expression (i.e. immunostaining intensity from PD-1) in cells present in a tumor sample (intratumoral cells). In the method of the present invention, peripheral T lymphocytes having the highest immunostaining intensity from PD-1 are used to set a reference value 1 (i.e. REF1). REF1 serves as a threshold (cut-off mark) to identify cells having the "highest immunostaining intensity of PD-1" in a tumor sample.

It is understood that the methods and assays as taught herein may be performed in an automated manner or semi-automated manner, e.g. which includes some human interventions, e.g. setting the equipment, processing samples etc.

5 The IHC assay

A further aspect of the present invention is the following. The present inventors have devised a further unique assay for quantifying the expression of PD-1 (intensity of immunostaining for PD-1) in cells in a tumor sample (e.g. tumor tissue section (FFPES or FFFS), which is referred to herein as the *IHC assay*. Specifically, the IHC assay is

10 devised for tumor tissue sections (such as formalin-fixed-paraffin- embedded (FFPE) tissue sections or slices or fresh fixed frozen section (FFFS). The IHC assay as taught herein differs from existing assays in that it is a calibrated assay making use of an external reference value for PD-1 expression (i.e. immunostaining intensity) (referred to herein as "reference value 2" (REF2), obtainable by the method as taught herein).

15

When first established (determined) using the method as taught herein, the reference value REF2 represents a calibrated value derived from a separate sample taken from the same tumor, which is in the form of a single cell suspension (e.g. TSS). The single cell suspension sample is processed in the cytometric assay as described herein using

20 REF1 to determine the % of PD-1^T intratumoral cells in said sample. The rationale for running a second sample in parallel in the cytometric assay as taught herein is to ensure that the same amount (e.g. 5%) of PD-1^T cells are found in the tumor tissue sections (i.e. the two samples are from the same tumor and thus should contain approximately the same percent (%) of PD-1^T cells, e.g. 5%). Once the amount (e.g.

25 5%) of PD-1^T cells present in the tumor (cytometric) sample is known, the corresponding population of PD-1^T cells (e.g. 5%) can be identified in the tissue sections (FFPE sections). By doing so, one can be sure that the population of PD-1^T cells (e.g. 5%) identified in the tissue sections (FFPE sections) represents substantially the same cells (or population of cells) having the highest intensity of

30 immunostaining for PD-1 as detected by cytometry. Once these cells have been identified, REF2 can be determined by taking the minimal intensity of immunostaining for PD-1 in this group or population of cells.

It is understood that, once REF2 has been established once by the method as taught herein, one need not to establish REF2 again when running a subsequent tumor sample (although possible but not needed or not essential). This means that the same REF2 value can be used again (without having to run the method for establishing REF2 as taught herein) as a pre-determined cut-off mark for any subsequent tumor samples (e.g. from the same patient or different patients) to segregate PD-1 positive cells in tumor samples (e.g. FFPES or FFFS samples) sections) into two groups, i.e. PD-1+ intratumoral cells having the highest intensity of immunostaining for PD-1 vs the remaining PD-1+ intratumoral cells having an immunostaining intensity for PD-1 below the threshold REF2. In the present invention, intratumoral cells present in tissue section (e.g. FFPS or FFFS) having the highest intensity of immunostaining for PD-1, as determined using the REF2 threshold as explained above, are also referred to herein as PD-1^T cells or PD-1^T intratumoral cells.

Therefore, the advantage of using an external value REF2, as described herein, is that it allows determining the intensity of immunostaining for PD-1 (which is needed for identifying PD-1^T cells) in an unbiased manner, e.g. without bias due to experimental variations, e.g. background, use of different antibodies, different immunostaining protocols, etc. Another advantage, is that it can be standardized across laboratories, rendering the methods of the invention reliable. Another advantage of the use of an external reference REF2 is that it allows determining the intensity of immunostaining for PD-1 in an unbiased manner without a need for a simultaneous analysis of single cell suspension from the same tumor, as such material is generally not available in clinical settings, as explained above. A further advantage is that the use of an external reference REF2 to identify PD-1^T cells in tissue sections (e.g. FFPES or FFFS) avoids the need of observer-dependent criteria based on a visual grading system established by a pathologist or scorer. For instance, in the context of tumor tissue sections (e.g. FFPE sections), in combination with a visual grading system (e.g. 0 = no staining, 1 = weak, 2 = intermediate staining, and 3 = strong staining) to establish a threshold of immunostaining intensity. A main disadvantage of such methods is that the internal reference used as well as the grading system may vary across scorers. Methods using such a (subjective) visual grading system are not only time consuming, but they are less reliable because they cannot be standardized and replicated across different

laboratories and by individual scorers. The IHC assay of the invention offers a solution to this problem as presented below:

In a further aspect, the present invention relates to an immunohistochemical (IHC)
5   assay for quantifying PD-1 expressing cells (PD-1^T cells) in a tumor sample obtained from human subject diagnosed with a cancer, said method comprising the step of:

5a.   Providing a solid tumor sample from said subject, wherein said tumor sample is in the form of one or more formalin-fixed paraffin embedded tissue section(s) (FFPES) or one or more freshly fixed frozen section (FFFS);

10   5b.   Incubating the FFPES sample or FFFS sample of step (5a) under conditions allowing specific antigen-antibody binding with an antibody directed to human PD-1 to allow detection of PD-1+ cells in said FFPES sample or FFFS sample;

5c.   Providing one or more digital image(s) of the FFPES sample or FFFS sample of step (5b);

15   5d.   Subjecting the one or more digital image(s) of step (5c) to an (automated) image analysis system to measure the intensity of immunostaining of PD-1 for PD-1+ cells; and

5e.   Quantifying the number of PD-1^T cells relative to the total number of nuclei in the FFPES sample or FFFS sample thereby obtaining the percent (%) of PD-1^T cells
20   or quantifying the absolute number (or density) of PD-1^T cells per mm^2 in the FFPES sample or FFFS sample, wherein the PD-1^T cells are the PD-1+ cells having an intensity of immunostaining for PD-1 that is equal or higher than a pre-determined REF2, wherein the pre-determined REF2 was obtained by a method as taught herein.

25   The term "solid tumor" as used herein is a well-known term in the art referring to an abnormal mass of tissue that usually does not contain cysts or liquid areas. Solid tumors may be benign (not cancer), or malignant (cancer). Different types of solid tumors are named for the type of cells that form them. Examples of solid tumors are sarcomas, carcinomas, and lymphomas. Leukemias (cancers of the blood) generally
30   do not form solid tumors. In the present invention, non-limiting examples of solid tumors include tumors from lung tissue, including non-small cell lung cancer (NSCLC) and small cell lung cancer; breast cancer; melanoma; urogenital cancers, including kidney cancer, ovarian cancer and bladder cancer; gastrointestinal tract (GI) cancers, including colorectal cancer and hepatocellular cancer; head and neck cancer; skin

cancer, including Merkel cell carcinoma. In a preferred embodiment, the solid tumor sample is from a NSCLC or is a solid NSCLC.

In an embodiment, the (primary) antibody directed to human PD-1 is selected from
5   anti-PD-1 (EH12.1, BD, cat. No 561272), anti-PD-1 (MIH4), anti-PD-1 (EH12.2H7), anti-PD-1 (NAT105, Roche Diagnostics, cat. no. 760-4895, Ventana/DAB detection, "ready to use concentration"), anti-PD-1 (J116), anti-PD-1 (eBioJ105).

In a preferred embodiment, the (primary) antibody directed to human PD-1 is the anti-
10   PD-1 (NAT105, Roche Diagnostics, cat. no. 760-4895, Ventana/DAB detection, "ready to use concentration"). It was found that particularly good results (works well) are obtained when using this antibody on FFPE sections.

In an embodiment, the (primary) antibody directed to human PD-1 used in step 5b (e.g.
15   NAT105, Roche Diagnostics, cat. no. 760-4895, Ventana/DAB detection, "ready to use concentration") may be combined with a second, labeled (primary) antibody directed against markers for different immune cells (e.g. such as CD3, CD4, CD8, Ki67, CTLA-4, PD-1, LAG-3, TIM-3, ICOS, T-bet for activated T cells; CD3, CD4, CD25, FOXP3, CD127, Ki67, CD45RA for regulatory T cells; CD45RO for memory T cells, etc.) for
20   quantification of PD-1 expression on relevant target populations.

In step 5c, one or more digital images of the FFPES sample or FFFS sample can be provided using standard techniques and apparatus, in an automated or semi-automated manner, e.g. using a CE-IVD certified Philips Ultra Fast Scanner 300
25   (Philips Digital Pathology Solutions). Measurement of the intensity of immunostaining for PD-1 can also be performed using standard techniques and equipment/software, such as HALO™ image analysis software, v.2.0.1145.19 (Indica Labs). In an embodiment, a stand-alone software application or an algorithm (e.g. a custom-made algorithm) may be used to perform the image analysis (e.g. to detect intensity of
30   immunostaining for PD-1 by applying a pre-determined reference value, REF2 as determined by the method as taught herein).

In step 5e, the term "relative to the total number of nuclei in the FFPES sample or FFFS samle" refers to all nuclei or all nuclei in selected areas present in the FFPES

sample or FFFS sample as identified by a nuclear stain (e.g. eosin stain or haematoxylin stain, and the like using standard protocols). The total number of nuclei in the FFPES sample or FFFS sample determined either manually by a scorer or automatically via the use of a computer program or e.g. using the equipment's,

5   apparatus, software etc., as described above.

In a preferred embodiment, the tumor sample is a FFPES sample.

Methods for predicting treatment outcome and for selecting patients

In a further aspect of the claimed invention, the present inventors have found that

10   accurate prediction for therapy outcome (responsiveness or resistance) with PD-1 inhibitors and/or PD-L1 inhibitors, alone or in combination with another therapeutic agent such as a CTLA-4 inhibitor compound (e.g. ipilimumab) and/or accurate selection of a cancer subject suitable for therapy with said PD-1 inhibitors and/or PD-L1 inhibitors, alone or in combination with another therapeutic agent such as a CTLA-

15   4 inhibitor compound (e.g. ipilimumab), can be reliably made, prior initiating therapy or early on during therapy, by determining the proportion (or %) of PD-1^T cells in a tumor samples either in the form of a single cell suspension or tumor tissue sections (e.g. FFPE sections) by using the cytometric assay and the IHC assay, respectively, for quantifying PD-1 expression (i.e. immunostaining intensity), as taught herein.

20

Specifically, the present inventors have uncovered that specific proportions (or percent (%)) or densities of PD-1^T cells (i.e. number of PD-1^T cells per mm^2) in a tumor sample (e.g. FFPES or FFFS) can be used as thresholds (cut-off marks) to accurately predict therapy outcome (responsiveness) with PD-1 inhibitors and/or PD-L1 inhibitors, alone

25   or in combination with another therapeutic agent such as a CTLA-4 inhibitor compound (e.g. ipilimumab), and/or accurately select a cancer subject suitable for therapy with said PD-1 inhibitors and/or PD-L1 inhibitors, alone or in combination with another therapeutic agent such as a CTLA-4 inhibitor compound (e.g. ipilimumab), prior initiating treatment or early on during treatment. In other words, said threshold values

30   for the percent (%) or proportion of PD-1^T cells in a tumor sample (tumor single cell suspension or tissue sections such as FFPE sections) can be used to accurately stratify patients, prior initiating treatment or early on during treatment, into responders (e.g. patients with a meaningful clinical response, e.g. reduction in tumor burden, radiological response defined by RECIST 1.1 criteria) or non-responders (e.g. e.g.

patients with no meaningful clinical response, e.g. no reduction in tumor burden, radiological response defined by RECIST 1.1 criteria) to therapy with PD-1 inhibitors and/or PD-L1 inhibitors. Said threshold values for percent (%) or density of PD-1^T cells in a tumor sample (tumor single cell suspension or tissue sections such as FFPES or

5   FFFS) can also be used to accurately stratify patients, prior initiating treatment or early on during treatment, into patient suitable for or patient not suitable for therapy with PD-1 inhibitors and/or PD-L1 inhibitors alone.

Overall, the assays (cytometric and IHC) and methods of the invention (treatment

10   outcome prediction and patient selection) represent technological advancements in the field of immune checkpoint therapy since prior the filing date of the present invention, there was a paucity of methods (and predictive biomarkers) for accurately or reliably predicting therapy outcome as well as for selecting patient suitable for immune checkpoint therapy with PD-1 inhibitors and/or PD-L1 inhibitors, particularly

15   for use before treatment started but also early on during treatment. The present inventors have found a solution to this problem by providing a predictive biomarker, i.e. specific proportion or prevent (%) of PD-1^T cells in a tumor sample (e.g. single cell suspension or tissue section, e.g. FFPES or FFFS) as taught herein, which can be used as a single biomarker for accurately or reliably predicting therapy outcome as

20   well as for selecting patients suitable for immune checkpoint therapy with PD-1 inhibitors and/or PD-L1 inhibitors alone or in combination with another therapeutic agent such as CTLA-4 inhibitor compound, prior initiating therapy or early on during treatment.

25   Furthermore, the assays and methods of the invention as taught herein contribute to spare patients from unnecessary risks (e.g. occurrence of undesirable side-effects, toxicity, costly treatment, etc.), and/or avoid depriving patients from a better-suited therapy. The details of the methods and related advantages will be further described below:

30

In a further aspect, the present invention relates to a method for predicting if a human subject diagnosed with a cancer is likely to respond to treatment with a PD-1 inhibitor compound and/or PD-L1 inhibitor compound prior initiating treatment with said

compound(s) or early after initiating treatment with said compound(s), the method comprising the steps of:

7a.   Providing a solid tumor sample from said human subject, wherein the sample is in the form of one or more FFPES or one or more FFFS;

7b.   Incubating the FFPES sample or FFFS sample of step (7a) under conditions allowing specific antigen-antibody binding with a (labeled) antibody directed to human PD-1 to allow detection of PD-1+ cells in said sample;

7c.   Providing digital images of the FFPES sample or FFFS sample of step (7b);

7d.   Subjecting the digital images of step (7c) to an (automated) image analysis system to measure the intensity of immunostaining of PD-1 per PD-1+ cells;

7e.   Quantifying the number of PD-1^T cells relative to the total number of nuclei thereby obtaining the percent (%) of PD-1^T cells in the FFPES sample or FFFS sample or quantifying the absolute number (density) of PD-1^T cells per mm^2 in the FFPES sample or FFFS sample, wherein the PD-1^T cells are the PD-1+ cells of step (7d) having an intensity of immunostaining for PD-1 that is equal or higher than a pre-determined REF2, wherein the REF2 is determined according to the method as taught herein; and wherein

7f.   (i) if there is at least 0.60% to at least 1.5% of PD-1^T cells, preferably at least 0.90 % of PD-1^T cells relative to the total number of nuclei in the FFPES sample; or if there is at least 20 to at least 70 PD-1^T cells per mm^2, preferably at least 35 PD-1^T cells per mm^2 in the FFPES sample or FFFS sample, it indicates that the human subject diagnosed with a cancer is likely to respond to treatment with the PD-1 inhibitor compound and/or the PD-L1 inhibitor compound alone ; or

(ii) if there is less than 0.60% to less than 1.5 %, preferably less than 0.90 % of PD-1^T cells relative to the total number of nuclei in the FFPES sample or FFFS sample; or if there is less than 20 to less than 70 PD-1^T cells per mm^2, preferably less than 35 PD-1^T cells per mm^2 in the FFPES sample or FFFS sample, it indicates that the human subject diagnosed with a cancer is not likely to respond to treatment with the PD-1 inhibitor compound and/or the PD-L1 inhibitor compound alone; and

7g.   Optionally, repeating steps (7a) to (7f) for one or more subsequent FFPES or FFFS from one or more subsequent human subjects diagnosed with cancer.

The steps of the method (steps 7a – 7e) may be performed as taught herein.

In step 7f(i), "at least 0.60% to at least 1.5 % of PD-1^T cells" includes, for instance, at least 0.60%, at least 0.65%, at least 0.70%, at least 0.75%, at least 0.80%, at least 0.85%, at least 0.90%, at least 0.95%, at least 1.00%, at least 1.10%, at least 1.20%, at least 1.30%, at least 1.40%, or for instance at least 1.50% PD-1^T cells. It was found that particularly good prediction (accurate prediction) about the likelihood that a cancer subject will respond to treatment with a PD-1 inhibitor compound and/or PD-L1 inhibitor compound (alone) prior initiating treatment with said compound(s) or early after initiating treatment with said compound(s) if there is at least 0.90% of PD-1^T cells in the tumor sample (e.g. FFPES or FFFS).

In step 7f(i), "at least 20 to at least 70 PD-1^T cells per mm^2" includes, for instance, at least 20 PD-1^T cells per mm^2, at least 25 PD-1^T cells per mm^2, at least 30 PD-1^T cells per mm^2, at least 35 PD-1^T cells per mm^2, at least 40 PD-1^T cells per mm^2, at least 45 PD-1^T cells per mm^2, at least 50 PD-1^T cells per mm^2, at least 55 PD-1^T cells per mm^2, at least 60 PD-1^T cells per mm^2, at least 65 PD-1^T cells per mm^2, or for instance at least 70 PD-1^T cells per mm^2. It was found that particularly good prediction (accurate prediction) about the likelihood that a cancer subject will respond to treatment with a PD-1 inhibitor compound and/or PD-L1 inhibitor compound (alone) prior initiating treatment with said compound(s) or early after initiating treatment with said compound(s) if there is at least 35 PD-1^T cells per mm^2 in the tumor sample (e.g. FFPES or FFFS).

In step 7f(ii), "less than 0.60% to less than 1.5%" includes, for instance, less than 1.50%, less than 1.45%, less than 1.40%, less than 1.35%, less than 1.30%, less than 1.25%, less than 1.20%, less than 1. 15%, less than 1.10%, less than 1.00%, less than 0.95%, less than 0.90%, less than 0.85%, less than 0.80%, less than 0.75%, less than 0.70%, less than 0.65%, or for instance less than 0.60%. It was found that particularly good prediction (accurate prediction) about the likelihood that a cancer subject will not respond to treatment with a PD-1 inhibitor compound and/or PD-L1 inhibitor compound alone prior initiating treatment with said compound(s) or early after initiating treatment with said compound(s) if there is less than 0.90% of PD-1^T cells in the tumor sample (e.g. FFPES or FFFS).

In step 7f(ii), "less than 20 to less than 70 PD-1^T cells per mm^2" includes, for instance, less than 70 PD-1^T cells per mm^2, less than 65 PD-1^T cells per mm^2, less than 60 PD-1^T cells per mm^2, less than 55 PD-1^T cells per mm^2, less than 50 PD-1^T cells per mm^2, less than 45 PD-1^T cells per mm^2, less than 40 PD-1^T cells per mm^2, less than 35 PD-1^T cells per mm^2, less than 30 PD-1^T cells per mm^2, less than 25 PD-1^T cells per mm^2, or for instance less than 20 PD-1^T cells per mm^2. It was found that particularly good prediction (accurate prediction) about the likelihood that a cancer subject will not respond to treatment with a PD-1 inhibitor compound and/or PD-L1 inhibitor compound alone prior initiating treatment with said compound(s) or early after initiating treatment with said compound(s) if there is less than 35 PD-1^T cells per mm^2 in the tumor sample (e.g. FFPES of FFFS).

The term "not responding to treatment with a PD-1 inhibitor compound and/or PD-L1 inhibitor compound alone" as used herein refers to a situation where the cancer subject will not benefit (e.g. no increased survival, no cancer reduction etc.) from treatment consisting of the PD-1 inhibitor compound and/or PD-L1 inhibitor compound *per se*, regardless of what other compounds or medication said patient may be using in parallel, e.g. vitamin C, aspirin, other cancer therapeutics, etc.

In a further aspect, the present invention relates to a method for selecting a human subject diagnosed with a cancer suitable for treatment with a PD-1 inhibitor compound and/or PD-L1 inhibitor compound prior initiating treatment with said compound(s) or early after initiating treatment with said compound(s), the method comprising the steps of:

8a.   Providing a (solid) tumor sample from said human subject, wherein the sample is in the form of one or more FFPES or one or more FFFS;

8b.   Incubating the FFPES sample or the FFFS of step (8a) under conditions allowing specific antigen-antibody binding with an (labeled) antibody directed to human PD-1 to allow detection of PD-1+ cells in said sample;

8c.   Providing digital images of the FFPES sample or FFFS sample of step (8b);

8d.   Subjecting the digital images of step (8c) to an (automated) image analysis system to measure the intensity of immunostaining of PD-1 per PD-1+ cells;

8e.   Quantifying the number of PD-1^T cells relative to the total number of nuclei thereby obtaining the percent (%) of PD-1^T cells in the FFPES sample or FFFS sample

or quantifying the absolute number of PD-1^T cells per mm^2 in the FFPES sample or FFFS sample, wherein the PD-1^T cells are the PD-1+ cells of step (8d) having an intensity of immunostaining for PD-1 that is equal or higher than a pre-determined REF2, wherein the REF2 is determined according to the method as taught herein; and wherein

8f.   (i) if there is at least 0.60% to 1.5%, preferably at least 0.90 % of PD-1^T cells relative to the total number of nuclei in the FFPES sample or FFFS sample; or if there is at least 20-70 PD-1^T cells per mm^2, preferably least 35 PD-1^T cells per mm^2 in the FFPES sample or FFFS sample, it indicates that the human subject diagnosed with a cancer is suitable for treatment with the PD-1 inhibitor compound and/or the PD-L1 inhibitor compound alone; or

(ii) if there is less than 0.60% to less than 1.5% of PD-1^T cells, preferably less than 0.90% PD-1^T cells relative to the total number of nuclei in the FFPES sample or FFFS sample; or if there is less than 20 to less than 70 PD-1^T cells per mm^2, preferably less than 35 PD-1^T cells per mm^2 in the FFPES sample or FFFS sample, it indicates that the human subject diagnosed with a cancer is not suitable for treatment with the PD-1 inhibitor compound and/or the PD-L1 inhibitor compound alone; and

8g.   Optionally, repeating steps (8a) to (8f) for one or more subsequent FFPES or one or more FFFS from one or more subsequent human subjects diagnosed with cancer.

The steps of the method (steps 8a – 8e) may be performed as taught herein.

In step 8f(i), "at least 0.60% to 1.5 % of PD-1T cells" includes, for instance, at least 0.60%, at least 0.65%, at least 0.70%, at least 0.75%, at least 0.80%, at least 0.85%, least 0.90%, at least 0.95%, at least 1.00%, at least 1.10%, at least 1.20%, at least 1.30%, at least 1.40%, or for instance at least 1.50% PD-1^T cells. It was found that particularly good prediction (accurate prediction) about the likelihood that a cancer subject is suitable for treatment with a PD-1 inhibitor compound and/or PD-L1 inhibitor compound prior initiating treatment with said compound(s) or early after initiating treatment with said compound(s), if there is at least 0.90% of PD-1^T cells in the tumor sample (e.g. FFPES or FFFS).

In step 8f(i), "at least 20 to 70 PD-1^T cells per mm^2" includes, for instance, at least 20 PD-1^T per mm^2, at least 25 PD-1^T per mm^2, at least 30 PD-1^T per mm^2, at least 35 PD-1^T per mm^2, at least 40 PD-1^T per mm^2, at least 45 PD-1^T per mm^2, at least 50 PD-1^T per mm^2, at least 55 PD-1^T per mm^2, at least 60 PD-1^T per mm^2, at least 65 PD-1^T per mm^2, or for instance at least 70 PD-1^T per mm^2. It was found that particularly good prediction (accurate prediction) about the likelihood that a cancer subject is suitable for treatment with a PD-1 inhibitor compound and/or PD-L1 inhibitor compound prior initiating treatment with said compound(s) or early after initiating treatment with said compound(s), if there is at least 35 PD-1T per mm^2 in the tumor sample (e.g. FFPES or FFFS).

In step 8f(ii), "less than 0.60% to less than 1.5%" includes for, instance, less than 1.50%, less than 1.45%, less than 1.40%, less than 1.35%, less than 1.30%, less than 1.25%, less than 1.20%, less than 1.15%, less than 1.10%, less than 1.00%, less than 0.95%, less than 0.90%, less than 0.85%, less than 0.80%, less than 0.75%, less than 0.70%, less than 0.65%, or for example less than 0.60%. It was found that particularly good prediction (accurate prediction) about the likelihood that a cancer subject is not suitable for treatment with a PD-1 inhibitor compound and/or PD-L1 inhibitor compound prior initiating treatment with said compound(s) or early after initiating treatment with said compound(s), if there is less than 0.90% of PD-1^T cells in the tumor sample (e.g. FFPES or FFFS).

In step 8f(ii), "less than 20 to less than 70 PD-1^T cells per mm^2" includes, for instance, less than 70 PD-1^T cells per mm^2, less than 65 PD-1^T cells per mm^2, less than 60 PD-1^T cells per mm^2, less than 55 PD-1^T cells per mm^2, less than 50 PD-1^T cells per mm^2, less than 45 PD-1^T cells per mm^2, less than 40 PD-1^T cells per mm^2, less than 35 PD-1^T cells per mm^2, less than 30 PD-1^T cells per mm^2, less than 25 PD-1^T cells per mm^2, or for instance less than 20 PD-1^T cells per mm^2. It was found that particularly good prediction (accurate prediction) about the likelihood that a cancer subject will not respond to treatment with a PD-1 inhibitor compound and/or PD-L1 inhibitor compound alone prior initiating treatment with said compound(s) or early after initiating treatment with said compound(s) if there is less than 35 PD-1^T cells per mm^2 in the tumor sample (e.g. FFPES of FFFS).

The term "not suitable for treatment with a PD-1 inhibitor compound and/or PD-L1 inhibitor compound alone" as used herein refers to a situation where the cancer subject will not benefit (e.g. no increased survival, no cancer reduction, no response according to standardized criteria, e.g. RECIST 1, etc.) from treatment consisting of the PD-1 inhibitor compound and/or PD-L1 inhibitor compound *per se*, i.e. what was given to the patient, regardless of what other compounds or medication said patient may be using in parallel, e.g. vitamin C, aspirin, other cancer therapeutics, etc.

In embodiment relating to the methods as taught herein, the cancer may be selected from the group of lung cancer, non-small cell lung cancer (NSCLC), small cell lung cancer; breast cancer; melanoma; urogenital cancer, including kidney cancer, ovarian cancer, bladder cancer; gastrointestinal tract (GI) cancer, colorectal cancer, hepatocellular cancer; head and neck cancer, skin cancer, Merkel cell carcinoma. In a preferred embodiment, the cancer is NSCLC.

In an embodiment relating to the methods as taught herein, the PD-1 inhibitor compound may be selected from nivolumab, pembrolizumab, PDR001, AMP-224, and AMP-514. In a preferred embodiment, the PD-1 inhibitor compound is nivolumab.

In an embodiment relating to the methods as taught herein, the PD-L1 inhibitor compound may be selected from avelumab, atezolizumab, durvalumab, and BMS-936559. In a preferred embodiment, the PD-L1 inhibitor compound is atezolizumab.

In a preferred embodiment relating to the methods as taught herein, the human subject diagnosed with a cancer has not been treated with a PD-1 inhibitor compound and/or a PD-L1 inhibitor compound. This means that the cancer subject is naïve to any PD-1 inhibitor compound and/or a PD-L1 inhibitor compound, such as those disclosed herein.

Medical uses

In a further aspect, the present invention relates to a PD-1 inhibitor compound or PD-L1 inhibitor compound for use in a method for treating a human subject diagnosed with a cancer, said method comprising the steps of:

9a.   Predicting that the human subject diagnosed with a cancer is likely to respond to treatment with the PD-1 inhibitor compound and/or the PD-L1 inhibitor compound according to the method as taught herein above or determining that the human subject diagnosed with a cancer is suitable for treatment with the PD-1 inhibitor compound

5   and/or the PD-L1 inhibitor compound according to the method as taught herein; and wherein:

9b.   (i) if it is determined in step (9a) that said human subject is suitable for treatment with the PD-1 inhibitor compound and/or the PD-L1 inhibitor compound alone, it indicates that the subject can be treated with the PD-1 inhibitor compound and/or the

10   PD-L1 inhibitor compound alone or if it is determined in step (9a) that said human subject is likely to respond to treatment with the PD-1 inhibitor compound and/or the PD-L1 inhibitor compound alone, it indicates that the subject can be treated with the PD-1 inhibitor compound and/or the PD-L1 inhibitor compound alone;

(ii) if it is determined in step (9a) that said human subject is not suitable for

15   treatment with the PD-1 inhibitor compound and/or the PD-L1 inhibitor compound alone, it indicates that the subject can be treated with a therapy that does not consist of a treatment with the PD-1 inhibitor compound and/or the PD-L1 inhibitor compound alone or if it is determined in step (9a) that said human subject is not likely to respond to treatment with the PD-1 inhibitor compound and/or the PD-L1 inhibitor compound

20   alone, it indicates that the subject can be treated with a therapy that does not consist of a treatment with the PD-1 inhibitor compound and/or the PD-L1 inhibitor compound alone.

In step 9(ii), if it is determined that a cancer patient can be treated with a therapy that

25   does not consist of a treatment with the PD-1 inhibitor compound and/or the PD-L1 inhibitor compound alone, such cancer patient may benefit, for instance, from treatment which consist of one or more therapeutic compounds which are not PD-1 inhibitor compounds or PD-L1 inhibitor compounds (e.g. chemotherapeutic agents, hormonal agents, etc.). Alternatively, said patient may receive a treatment consisting

30   of a PD-1 inhibitor compounds and/or PD-L1 inhibitor compound in combination with a compound that is capable of increasing the amount or proportion of PD-1^T in the tumor per se. Such compound capable of increasing the amount or proportion of PD-1^T in the tumor, can for example, be any compounds identified by the screening methods as taught herein. Alternatively, the patient may receive a first treatment

consisting of a compound capable of increasing the amount or proportion of PD-1^T in the tumor (i.e. aim of the treatment is to increase the proportion or amount of PD-1^T cells in the tumor in the subject to a level so that the subject can meet the cut-off mark to be considered as a cancer subject suitable for therapy with a PD-1 inhibitor

5   compounds and/or a PD-L1 inhibitor compound), followed by therapy with a PD-1 inhibitor compounds and/or PD-L1 inhibitor compound.

In an embodiment relating to the methods as taught herein, the cancer may be selected from the group of lung cancers, including non-small cell lung cancer (NSCLC) and

10   small cell lung cancer; breast cancer; melanoma; urogenital cancers, including kidney cancer, ovarian cancer and bladder cancer; gastrointestinal tract (GI) cancers, colorectal cancer and hepatocellular cancer; head and neck cancer; skin cancer, including Merkel cell carcinoma. In a preferred embodiment, the cancer is NSCLC.

15   In an embodiment relating to the methods as taught herein, the PD-1 inhibitor compound may be selected from nivolumab, pembrolizumab, PDR001, AMP-224, and AMP-514. In a preferred embodiment, the PD-1 inhibitor compound is nivolumab.

In an embodiment relating to the methods as taught herein, the PD-L1 inhibitor

20   compound may be selected from avelumab, atezolizumab, durvalumab, and BMS-936559. In a preferred embodiment, the PD-L1 inhibitor compound is atezolizumab.

In an embodiment relating to the methods as taught herein, the human subject diagnosed with a cancer has not been treated with a PD-1 inhibitor compound and/or

25   a PD-L1 inhibitor compound as defined above.

In an embodiment relating to the methods as taught herein, the tumor tissue sample is preferably a FFPES sample.

30   <u>Screening Methods</u>

In a further aspect, the present invention relates to a method for screening a test compound capable of increasing the percent (%) of PD-1^T cells in a tumor in a subject suffering from cancer, said method comprising the steps of:

14a. Providing a first tumor sample from said patient, wherein said first tumor sample is a TSS sample and obtained at a time point before administering the test compound to said subject;

14b. Providing a second tumor sample form said patient, wherein said second tumor sample is a TSS sample obtained at a time point after administering the test compound to said subject;

14c. Quantifying the percent (%) of PD-1^T cells in the first TSS sample and second TSS sample according to the method of claim 1; and

14d.   (i) Determining that the test compound is capable of increasing the percent (%) of PD-1^T cells in the tumor from the subject suffering from cancer if the percent (%) of PD-1^T cells in the second TSS sample is higher than the percent (%) of PD-1^T cells in the first TSS sample, or (ii) Determining that the test compound is not capable of increasing the percent (%) of PD-1^T cells in the tumor from the subject suffering from cancer if the percent (%) of PD-1^T cells in the second TSS sample is equal or lower than the percent (%) of PD-1^T cells in the first TSS sample.

In a further aspect, the present invention relates to a method for screening a test compound capable of increasing the percent (%) or density (per mm^2) of PD-1^T cells in a tumor in a subject suffering from cancer, said method comprising the steps of:

15a. Providing a first tumor sample from said patient, wherein said first tumor sample is a FFPES sample or FFFS sample and obtained at a time point before administering the test compound to said subject;

15b. Providing a second tumor sample form said patient, wherein said second tumor sample is a FFPES sample or a FFFS obtained at a time point after administering the test compound to said subject;

15c. Quantifying the percent (%) or density (per mm^2) of PD-1^T cells in the first FFPES sample or FFFS and second FFPES sample or FFFS according to the IHC assay as taught herein; and

15d.   (i) Determining that the test compound is capable of increasing the percent (%) or density (per mm^2) of PD-1^T cells in the tumor from the subject suffering from cancer if the percent (%) or density (per mm^2) of PD-1^T cells in the second FFPES sample or FFFS sample is higher than the percent (%) or density (per mm^2) of PD-1^T cells in the first FFPES sample or FFFS sample, or

(ii) Determining that the test compound is not capable of increasing the percent (%) or density (per mm^2) of PD-1^T cells in the tumor from the subject suffering from cancer if the percent (%) or density (per mm^2) of PD-1^T cells in the second FFPES sample or FFFS sample is equal or lower than the percent (%) or density (per mm^2) of

5 PD-1^T cells in the first FFPES sample or FFFS sample, or

(iii) Determining that the test compound is capable of increasing the percent (%) or density of PD-1^T cells in the tumor from the subject suffering from cancer if the second FFPES sample or FFFS sample comprises preferably at least 0.1% of PD-1^T cells, for instance 0.60% to at least 1.5% of PD-1^T cells, or for instance at least 0.90%

10 of PD-1^T cells relative to the total number of nuclei in the second FFPES sample or FFFS sample; or comprises preferably at least 20 to at least 70 PD-1^T cells per mm^2, for instance at least 35 PD-1^T cells per mm^2 in the second FFPES sample or FFFS sample, or

(iv) Determining that the test compound is not capable of increasing the percent

15 (%) or density of PD-1^T cells in the tumor from the subject suffering from cancer if the second FFPES sample of FFFS sample comprises preferably less than 0.1% of PD-1^T cells, for instance less than 0.60% to less than 1.5 %, or for instance less than 0.90% of PD-1^T cells relative to the total number of nuclei in the second FFPES sample or FFFS sample; or if there is preferably less than 20 to less than 70 PD-1^T cells per mm^2,

20 for instance less than 35 PD-1^T cells per mm^2 in the second FFPES or FFFS sample.

In a preferred embodiment the tumor sample is a FFPES.

Medical uses (combination therapy)

25 In a further aspect, the present invention relates to a CTLA-4 inhibitor compound for treating a subject suffering from cancer, wherein said subject is not suitable for treatment with a PD-1 inhibitor compound and/or a PD-L1 inhibitor compound alone as determined according to the method as taught herein or is not responsive to treatment with a PD-1 inhibitor compound and/or a PD-L1 inhibitor compound alone

30 as determined according to the method as taught herein, wherein the treatment is in combination with a PD-1 inhibitor compound and/or PD-L1 inhibitor compound.

In a further aspect, the present invention relates to a PD-1 inhibitor compound and/or PD-L1 inhibitor compound for treating a subject suffering from cancer, wherein said

subject is not suitable for treatment with a PD-1 inhibitor compound and/or a PD-L1 inhibitor compound alone as determined according to the method as taught herein or is not responsive to treatment with a PD-1 inhibitor compound and/or a PD-L1 inhibitor compound alone as determined according to the method as taught herein, wherein the treatment is in combination with a CTLA-4 inhibitor compound.

In a further aspect, the present invention relates to a PD-1 inhibitor compound and/or PD-L1 inhibitor compound in combination with a CTLA-4 inhibitor compound for use in a method for treating a human subject diagnosed with a cancer, said method comprising the steps of:

18a. Predicting that the human subject diagnosed with a cancer is likely to respond to treatment with the PD-1 inhibitor compound and/or the PD-L1 inhibitor compound according to the method as taught herein or determining that the human subject diagnosed with a cancer is suitable for treatment with the PD-1 inhibitor compound and/or the PD-L1 inhibitor compound according to the method as taught herein; and wherein

18b. if it is determined in step (18a) that said human subject is not suitable for treatment with the PD-1 inhibitor compound and/or the PD-L1 inhibitor compound alone, it indicates that the subject can be treated with a PD-1 inhibitor compound and/or a PD-L1 inhibitor compound in combination with the CTLA-4 inhibitor compound or if it is determined in step (18a) that said human subject is not likely to respond to treatment with the PD-1 inhibitor compound and/or the PD-L1 inhibitor compound alone, it indicates that the subject can be treated with a PD-1 inhibitor compound and/or a PD-L1 inhibitor compound in combination with the CTLA-4 inhibitor compound.

In an embodiment, the CTLA-4 inhibitor compound is ipilimumab, and the PD-1 inhibitor compound may be selected from nivolumab, pembrolizumab, PDR001, AMP-224, and AMP-514, and the PD-L1 inhibitor compound may be selected from avelumab, atezolizumab, durvalumab, and BMS-936559.

In a preferred embodiment, the CTLA-4 inhibitor compound is ipilimumab, the PD-1 inhibitor compound is nivolumab, and the PD-L1 inhibitor compound is atezolizumab.

Methods for establishing a reference value (i.e. REF 1 and REF2)

In a further aspect, the present invention relates to a method for establishing a pre-determined reference value 1 (REF1) of intensity of immunostaining for PD-1, said method comprising the steps of:

5   (i)   Providing at least one blood sample from a healthy human donor;

         (ii)   Isolating peripheral blood mononuclear cells (PBMC) from said blood sample;

         (iii)   Incubating the PBMC sample of step (ii) under conditions allowing specific antigen-antibody binding with an (e.g. labeled) antibody directed to human

10   T lymphocyte marker (M) and with a (labeled) antibody directed to human PD-1 to allow detection of M+ cells, PD-1+ cells and M+/PD-1+ cells in said PBMC sample;

         (iv)   Measuring the intensity of immunostaining of PD-1 per M+/PD-1+ cells of step (iii);

15   (v)   Selecting the upper 0.50% to upper 1.5%, preferably the upper 1% of the M+/PD-1+ cells of step (iv) having the highest intensity of immunostaining for PD-1; and

         (vi)   Determining the minimal intensity of immunostaining for PD-1 in the upper 0.50% to upper 1.5%, preferably in the upper 1.0% of the M+/PD-1+ cells

20   of step (v) thereby establishing the pre-determined REF1.

In step (i), at least one blood sample means that it can be obtained from the one healthy donor or from multiple (different) healthy donors.

25   In step (ii), isolating peripheral blood mononuclear cells (PBMC) can be performed according to standard protocols.

In steps (v) and (vi), the upper 0.50% to 1.5% of M+ /PD-1+ cells (e.g. CD3+/PD-1+ cells) include e.g. taking for instance the upper 0.50%, the upper 0.55%, the upper

30   0.60%, the upper 0.65%, the upper 0.70%, the upper 0.75%, the upper 0.80%, the upper 0.85%, the upper 0.90%, the upper 0.95%, the upper 1.00%, the upper 1.05%, the upper 1.10%, the upper 1.15%, the upper 1.20% the upper 1.25%, the upper 1.30%, the upper 1.35%, the upper 1.40%, the upper 1.45%, or for instance the upper

1.5% of M+ /PD-1+ cells (e.g. CD3+/PD-1+ cells) in a tumor sample. Particularly good results have been obtained by taking the upper 1.00% of M+ /PD-1+ cells (e.g. CD3+/PD-1+ cells) in a tumor sample for establishing the REF1 threshold value, and for subsequent analyses.

5

In step (vi), the term "minimal intensity of immunostaining for PD-1" means that once, for instance, the upper 0.50% M+/PD-1+ cells (e.g. CD3+/PD-1+) cells having the highest intensity of immunostaining for PD-1 have been identified (in step (v)), the intensity of immunostaining for PD-1 (value) that is actually taken to establish REF1 is

10 the minimal minimal intensity of immunostaining for PD-1 in that group (i.e. the upper 0.50% group of cells). In other words, the minimal intensity of immunostaining for PD-1 in the upper 0.50% group is basically the intensity of immunostaining for PD-1 (value) measured in the cell having the lowest intensity of immunostaining for PD-1 within that group of cell.

15

In an embodiment, the human T lymphocyte marker (M) is selected from CD3, CD4 and CD8, or combinations thereof. In a preferred embodiment, the human T lymphocyte marker (M) is CD3.

20 In a further aspect, the present invention relates to a method for establishing a pre-determined reference value 2 (REF2) of intensity of immunostaining for PD-1, said method comprising the steps of:

    a) Providing a solid tumor sample from a subject diagnosed with a cancer, wherein said tumor sample is divided in two parts, wherein the first part is processed to

25   be in the form of one or more formalin-fixed paraffin embedded tissue section(s) (FFPES) or one or more freshly fixed frozen section (FFFS) and wherein the second part is processed to be in the form of a single cell suspension (TSS) sample;

    b) Quantifying the percent (%) of PD-1^T cells in the TSS sample of step (a)

30   according to the method of claim 1;

    c) Incubating the FFPES sample of step (a) under conditions allowing specific antigen-antibody binding with an (e.g. labeled) antibody directed to human PD-1 to allow detection of PD-1+ cells in said FFPES sample or FFFS sample;

d) Providing one or more digital image(s) of the FFPES sample or FFFS sample of step (c);

e) Subjecting the one or more digital image(s) of step (d) to an (automated) image analysis system to measure the intensity of immunostaining of PD-1 per PD-1+ T lymphocytes (TILS);

f) Selecting the upper percent (%) P of PD-1+ cells from step (e) having the highest intensity of immunostaining for PD-1, wherein the upper percent (%) P of PD-1+ T lymphocytes (TILS) is equal to the percent (%) of PD-1^T cells in the TSS sample of step (b); and

g) Determining the minimal intensity of immunostaining for PD-1 in the upper percent (%) P of PD-1+ T lymphocytes (TILS) of step (f) thereby establishing a pre-determined reference value 2 (REF2).

In step 5a, it is understood that the first part and second part of the tumor need not be the same size, although it is preferable that the first part and second part of the tumor are substantially the same size.

In an embodiment, the (labeled) (primary) antibody directed to human PD-1 used in step b (referring to the cytometric assay as taught herein, e.g. using EH12.1, BD, cat. No 561272) and the labeled (primary) antibody directed to human PD-1 used in step c (e.g. NAT105, Roche Diagnostics, cat. no. 760-4895, Ventana/DAB detection, "ready to use concentration") are different, i.e. not the same antibody, without affecting the accuracy of the data (i.e. obtaining the percent (%) of PD-1T cells in the FFPES).

In an embodiment, T lymphocytes (TILS) may be identified based on their specific morphology, staining characteristics or imaging metrics (cell shape, nuclear size, nucleus to cytoplasm ratio, cytoplasm and chromatin characteristics etc.) or based on the use of TILS markers including for instance CD3, CD4, CD8, T-bet, and the like using standard protocols).

In a preferred embodiment, the tumor sample is a FFPES sample.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Definition of the PD-1^T cell subset. Peripheral blood mononuclear cells (PBMC) from healthy human donors and tumor digests from cancer subject were immunostained for CD3 and PD-1. PD-1 positive cells were defined using Full Minus One (FMO) and matching isotype controls. PD-1^T cells were defined as the population of peripheral blood T cells with the highest intensity of immunostaining for PD-1 (Top 1%), corresponding to max. 1% percent of total CD3+ cells. The reference value 1 (REF1) was defined as the minimal intensity of immunostaining (fluorescence) for PD-1 in the PD-1^T subset.

Figure 2. Figure 2 depicts variability of the reference value REF1 that is associated with different cut-offs for the PD-1^T population. Six peripheral blood samples were tested. The gating strategy used was the same as that used in Figure 1. For this analysis, the gate for PD-1^T was set individually in each sample to result in a defined percentage of cells corresponding to either 1%, 0.75%, 0.5% or 0.25% of total CD3+ cells. Then, the intensity of immunostaining (fluorescence) for PD-1 for the REF1 was calculated for each cut-off.

Figure 3. Figure 3 depicts the receiver operating characteristic (ROC) curve, which was used to define the optimal cut-off of PD-1^T cells in the IHC method for predicting responsiveness to immune checkpoint therapy with anti-PD-1 and/or anti-PD-L1 agents. Panel (a) depicts the percentage (%) of PD-1^T cells relative to the total cells. Panel (b) depicts the number of PD-1^T cells per mm^2 of tumor area. The percentage (%) of PD-1^T cells and the number of PD-1^T cells per mm^2 of tumor area were quantified and correlated with the clinical response data. ROC curves were calculated and distinct cut-off values were analysed to identify the value with the optimal sensitivity and specificity for predicting responsiveness to immune checkpoint therapy with anti-PD-1 and/or anti-PD-L1 agents.

Figure 4. Figure 4 depicts the results for the quantification of PD-1^T T cells by IHC in responding and non-responding non-small cell lung cancer (NSCLC) patients. PD-1^T T cells were quantified as described herein and correlated with the clinical response data. Panel (a) shows the percentage of PD-1^T T cells per total cells comprised in the tumor sample while panel (b) shows the number of PD-1^T T cells per mm^2 of tumor area.

EXAMPLES

Example 1: Method to determine reference value 1 (REF1) in peripheral blood mononuclear cells.

5   Buffy coats were collected from healthy human blood donors (cancer-free) and peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Histopaque-1077 (Sigma, Cat. No. 10771) according to standard procedures. Samples were washed twice with PBS, re-suspended in freezing medium consisting of 90% fetal bovine serum and 10% dimethyl sulfoxide, and stored in liquid
10   nitrogen (for cryopreservation) until further use.

Cryopreserved PBMC samples were thawed and washed with PBS. For immunostaining, cells (PBMC) were re-suspended in 50 µl PBS and blocked with Fc receptor blocking agent (eBioscience, cat. No 14-9161-73) for 20 min at 4°C. Cells
15   were stained with live/dead Zombie UV assay (Invitrogen, cat. No. 423107) to assess live versus dead status, washed, re-suspended in 50 µl of staining buffer (PBS, 2mM EDTA, 0.1% NaN3, 2% fetal calf serum) containing antibodies for surface staining and incubated for 20 min at 4°C. Corresponding isotype antibodies or full minus one (FMO) antibody stainings were used as a control. The following antibodies were used for
20   surface staining: Anti-CD45 PerCP-Cy5.5 (2D1, eBioscience, cat. no 45-9459-42), anti-CD3 APC-eFluor780 (SK7, eBioscience, Nr. 47-0036-42), anti-PD-1 PE-Cy7 (EH12.1, BD, cat. No 561272), and anti-CD8 BV605 (RPA-T8, Biolegend, cat. no 301040). The following isotype control was used: mouse IgG1 κ PE-Cy7 (MOPC-21, BD, cat. no 557646).
25

After washing twice in staining buffer, cells (PBMC) were resuspended in 200 µl IC Fixation buffer (eBioscience). Cell (PBMC) samples were subjected to a BD LSR Fortessa Cell analyzer ((BD LSR Fortessa Cell analyzer (BD Bioscience))) for flow cytometry analysis. All samples were washed twice with staining buffer before
30   acquisition in the flow cytometer. Data were collected using the BD FACS Diva Software version 7 and further analyzed with FlowJo v10.0.6 (Tree Star Inc.).

In order to identify the peripheral T lymphocytes within the PBMC samples, gating on CD45+CD3+ cells after exclusion of doublets and dead cells was performed. The

intensities of the immunostaining for PD-1 and CD3 were plotted against each other and the population of cells with the highest intensity of immunostaining for PD-1 within total CD3+ T lymphocytes were identified (i.e. this corresponds to the top 1% of CD3+/PD-1+ T lymphocytes with the highest intensity of immunostaining for PD-1=

5   max. 1% of total CD3+ T cells) (see Figure 1).

The average minimal fluorescence intensity value of this subset from six healthy donor samples was then defined as the reference value 1 (REF1). In addition, also REF1 values using the top 0.75%, top 0.5% and top 0.25% cut-offs of cells with the highest

10   intensity of immunostaining for PD-1 within total CD3+ T lymphocytes were determined. Note that cut-offs based on the top 1%, top 0.75%, and top 0.5% all result in a minimal fluorescence intensity value that shows a high consistency between donors, defining these cut-offs as unambiguous (see Figure 2). In experiments described below, a REF1 that identifies the top 1% of cells with the highest intensity

15   of immunostaining for PD-1 within total CD3+ T lymphocytes was utilized.

Example 2: Method to determine the percentage of PD-1^T in single cell tumor suspensions.

Fresh tumor samples were collected from ten (human) individuals diagnosed with non-

20   small cell lung cancer (NSCLC) while undergoing surgical treatment. Solid tumor lesions were cut in half and one half of the tumor was mechanically dissociated and enzymatically digested using accutase (PAA), collagenase IV (Worthington), hyaluronidase (Sigma) and DNAse type IV (Sigma) for 1h at 37°C, directly after excision (Thommen et al (2015), Cancer Immunol. Res., Vol. 3(12), pages 1344-55).

25   The other half was in the assay of example 3 below. Single cell suspensions were prepared from each tumor sample and all samples were cryopreserved until further use.

Cryopreserved tumor digests were thawed and immunostaining was performed as

30   described for PBMC in example 1. Flow cytometry was performed as described in example 1. T lymphocytes were identified using the gating strategy as described in example 1. The percentage of tumor-specific PD-1^T T lymphocytes (termed PD-1^T) for each tumor were identified by quantifying all cells in the tumor suspension with a PD-1 fluorescence intensity (i.e. intensity of immunostaining for PD-1) that was equal or

larger than REF1 (see example 1), normalized to the number of CD3+ T lymphocytes (% PD-1^T of CD3+) (See Figure 1).

Example 3: Method to establish reference value 2 (REF2) to determine PD-1^T

5   **lymphocytes in formalin-fixed paraffin embedded samples.**

From the other half of the ten tumor specimens used in example 2 for flow cytometry formalin-fixed paraffin embedded (FFPE) samples were prepared according to standard pathology guidelines. For analysis, strict serial 4 μm sections were cut.

10   Deparaffinization was performed according to standard protocols. Immunohistochemistry staining was performed within 48 hrs to avoid unspecific background staining. For staining, water was carefully pipetted onto the slide to cover the FFPE sections. The slide was then transferred onto a heating plate (set to 50°C) and incubated for 7-10 seconds. The water was discarded. The slides were dried in

15   the oven at 70°C for 30 min and were then stained for PD-1 using the Roche Ventana benchmark ultra (NAT105, Roche Diagnostics, cat. no. 760-4895, Ventana/DAB detection, "ready to use concentration") on a Leica-Bond™ III/max autostainer.

Slides were scanned at high resolution on a CD-IVD certified Philips Ultra Fast

20   Scanner 300 (Philips Digital Pathology Solutions) and digital image analysis was carried out using the HALO™ image analysis software, v.2.0.1145.19 (Indica Labs).

The percentage of PD-1^T lymphocytes in FFPE sections reflects all T lymphocytes (TILS) with a PD-1 staining intensity above the reference value 2 (REF2) normalized

25   to the total cell count (identified by morphology, using a built-in cell segmentation algorithm of the HALO software designed for this purpose). REF2 was set to obtain an optimal match between the percentage of PD-1^T cells in the FFPE sample and in the matched tumor suspension (as defined in example 2) for a collection of ten tumor samples. An image analysis algorithm incorporating REF2 was generated for

30   automated analysis of PD-1^T lymphocytes of subsequent FFPE samples.

Example 4: Method to quantify PD-1^T lymphocytes for prediction of treatment response in pretreatment biopsies of patients undergoing immune checkpoint therapy with anti-PD-1 and/or anti-PD-L1 inhibitor agents.

Biopsies obtained for diagnostic or for research purposes from patients planned to undergo immunotherapy with PD-1/PD-L1 targeting agents were collected and FFPE samples were prepared according to standard pathology guidelines. Samples were

5 processed, stained and scanned as described in example 3. The number of PD-1^T T lymphocytes (TILS) was quantified as described in example 3 using the image analysis algorithm containing REF2. For prediction of therapy response, the percentage of PD-1^T T lymphocytes (TILS) per total cells (identified by total nuclei count) was determined. Alternatively, the area of the biopsy was measured and the number of PD-

10 1^T lymphocytes per mm^2 area was determined. Response to anti-PD-1 therapy with nivolumab (Opdivo®, Bristol-Myers Squibb) was defined as radiologic complete or partial response (determined by CT scan or equal) at week 6-12. To define the optimal threshold values for prediction of response to anti-PD-1/-PD-L1 therapy, receiver operating characteristic (ROC) curves were calculated for the percentage of PD-1^T

15 lymphocytes per total cells and the number of PD-1^T lymphocytes per mm^2 area, indicating that the most preferable threshold values would be the percentage of PD-1^T lymphocytes per total cells > 0.9% PD-1^T per total cells and the number of PD-1^T lymphocytes per mm^2 area > 35 PD-1^T per mm2 (see Figures 3 and 4). Note that performance of the predictive assay deteriorates when moving towards thresholds that

20 either yield a lower or higher number of PD-1^T cells, emphasizing the value of a calibrated cut-off to define these cells.

25

CONCLUSIES

1.   Een (cytometrische) test om PD-1 tot expressie brengende cellen
(PD-1^T) in een tumormonster verkregen uit een menselijke patiënt te kwantificeren,
5   welke werkwijze de stappen omvat:

a)   Het verschaffen van een vaste tumor monster van de menselijke
patiënt, waarbij het monster in de vorm van een suspensie met losse cellen
(TSS) is;

10   b)   Incubatie van het monster van stap a) onder omstandigheden die
specifieke antigeen-antilichaambinding mogelijk maken met een (gemerkt)
antilichaam gericht tegen een menselijke T-lymfocytmarker (M), en een
(gelabeld) antilichaam gericht tegen menselijk PD-1 om detectie van cellen
positief voor M (M+ cellen), PD-1(PD-1+ cellen) en zowel M als PD-1(M+ /
15   PD-1+ cellen) mogelijk te maken in het monster;

c)   Meten van de intensiteit van immuun kleuring van PD-1voor M+/PD-
1+ cellen; en

20   d)   Kwantificeren van het aantal PD-1^T cellen, waarbij de PD-1^T cellen
de M+ / PD-1+ cellen van stap c) zijn met een intensiteit van immuun-kleuring
voor PD-1die gelijk is aan of hoger is dan een vooraf bepaalde
referentiewaarde 1(REF1), ten opzichte van het totale aantal M+ cellen in het
tumormonster om het percentage (%) PD-1^T cellen in het tumormonster te
25   verkrijgen, waarbij de vooraf bepaalde REF1werd verkregen door een
werkwijze volgens conclusie 21.

2.   Werkwijze volgens conclusie 1, waarbij de menselijke T-lymfocyt-
merker (M) wordt gekozen uit CD3, CD4 en CD8, of combinaties daarvan.
30

3.   Werkwijze volgens conclusie 1of 2, waarbij de menselijke T-
lymfocytmarker (M) CD3 is.

4.   Werkwijze volgens een van de voorgaande conclusies, waarbij het (gemerkte) antilichaam gericht tegen menselijk PD-1is gekozen uit de groep bestaande uit anti-PD-1(EH12.1), anti-PD-1(MIH4), anti-PD-1(EH12.2H7), anti-PD-1(NAT105), anti-PD-1(J116), anti-PD-1(eBioJ105), bij voorkeur anti-PD-1(EH12.1).

5

5.   Een Immunohistochemische (IHC) test voor het kwantificeren van PD-1 tot expressie brengende cellen (PD-1^T cellen) in een tumormonster verkregen uit een menselijke patiënt gediagnosticeerd met een kanker, waarbij de werkwijze de stap omvat:

10   a)   Verschaffen van een solide tumor monster van genoemd patiënt, waarbij genoemd tumor monster in de vorm is van één of meer met formaline gefixeerde in paraffine ingebed weefsel sectie (s) (FFPES) of één of meer sectie (vers) ingevroren weefsel (s) (FFFS);

          b)   Incubatie van het FFPES-monster of FFFS-monster van stap a)

15   onder omstandigheden die specifieke antigeen-antilichaambinding mogelijk maken met een antilichaam gericht tegen menselijk PD-1om detectie van PD-1+ cellen in genoemd FFPES-monster of FFFS-monster mogelijk te maken;

          c)   Verschaffen van één of meer digitale afbeelding (en) van het

20   FFPES-monster of FFFS-monster van stap b);

          d)   Het onderwerpen van de een of meer digitale afbeelding (en) van stap c) aan een (geautomatiseerd) beeldanalysesysteem om de intensiteit van immuun kleuring van PD-1voor PD-1+ cellen te meten; en

          e)   Kwantificeren van het aantal PD-1^T cellen ten opzichte van het totale

25   aantal kernen in het FFPES-monster of FFFS-monster waardoor het percentage (%) PD-1^T cellen wordt verkregen of kwantificeren van het absolute aantal (of de dichtheid) van PD-1^T cellen per mm^2 in de FFPES monster of FFFS monster, waarbij de PD-1^T cellen de PD-1+ cellen zijn met een intensiteit van immuun kleuring voor PD-1die gelijk of groter is dan een

30   vooraf bepaald REF2, waarbij de vooraf bepaalde REF 2 werd verkregen door een werkwijze volgens conclusie 22.

6.   De werkwijze volgens conclusie 5, waarbij het (gemerkte) antilichaam gericht tegen humaan PD-1wordt gekozen uit een nti-PD-1(EH12.1),

anti-PD-1(MIH4), anti-PD-1(EH12.2H7), anti-PD-1(NAT105), anti-PD-1(J116), anti-PD-1(eBioJ105), bij voorkeur anti-PD-1(NAT105).

7.   Een methode om te voorspellen of een met kanker gediagnosticeerde

5   menselijke patiënt verwacht kan worden te reageren op de behandeling met een PD-1-remmende verbinding en/of PD-L1-remmende verbinding voorafgaand aan het initiëren van behandeling met genoemde verbinding (en) of kort na het initiëren van de behandeling met genoemde verbinding (en), waarbij de werkwijze de stappen omvat van:

10   a)   Het verschaffen van een solide tumor monster van genoemde menselijke patiënt, waarbij het monster de vorm heeft van één of meer FFPES of één of meer FFFS;

b)   Incuberen van he FFPES monster of FFFS monster van stap a) onder omstandigheden die antigeen-antilichaambinding met een (gemerkt)

15   antilichaam gericht tegen humaan PD-1om detectie van PD-1+ cellen in genoemd monster mogelijk maken;

c)   Verstrekken van digitale beelden van het FFPES- monster of FFFS-monster van stap b);

d)   Het onderwerpen van de digitale beelden van stap c) aan een

20   (geautomatiseerd) beeldanalysesysteem om de intensiteit van immuun kleuring voor PD-1per PD-1+ cel te meten;

e)   Kwantificeren van het aantal PD-1^T cellen betrokken op het totale aantal kernen waardoor het percentage (%) van PD-1^T cellen in het FFPES-monster of FFFS-monster wordt verkregen of kwantificeren van het absolute

25   aantal (of dichtheid) van PD-1^T cellen per mm^2 in de FFPES monster of FFFS monster, waarbij de PD-1^T cellen de PD-1+ cellen van stap d) zijn met een intensiteit van de immuun kleuring voor PD-1die gelijk of groter is dan een vooraf bepaald REF2, waarbij de REF2 wordt bepaald met een werkwijze volgens conclusie 22; en waarin

30   f)   (i) indien er ten minste 0,60% tot ten minste 1,5% PD-1^T cellen is, bij voorkeur ten minste 0,90% van PD-1^T cellen ten opzichte van het totale aantal kernen in het FFPES-monster of FFFS-monster; of als er ten minste 20 tot ten minste 70 PD-1^T cellen zijn per mm^2, bij voorkeur ten minste 35 PD-1^T cellen per mm^2 in de FFPES monster of FFFS monster zijn, betekent dit dat de

menselijke patiënt gediagnosticeerd met een kanker waarschijnlijk zal reageren op behandeling met de PD-1 remmende verbinding en/of PD-L1remmende verbinding alleen; of

(ii) indien er minder dan 0,60% tot minder dan 1,5%, bij voorkeur

5   minder dan 0,90% van de PD-1^T cellen ten opzichte van het totale aantal kernen in het monster of FFPES FFFS monster zijn; of als er minder dan 20 tot minder dan 70 PD-1^T cellen per mm^2, bij voorkeur minder dan 35 PD-1^T cellen per mm^2 in de FFPES of FFFS monster zijn, dit betekent dit dat de menselijke patiënt gediagnosticeerd met een kanker waarschijnlijk niet

10   reageert op behandeling met de PD-1-remmende verbinding en/of de PD-L1-remmende verbinding alleen; en

g)   Eventueel herhalen van stappen a) tot en met f) met één of meer volgende FFPES of FFFS monster van één of meer volgende menselijke patiënten gediagnosticeerd met kanker.

15

8.   Een methode voor het selecteren van een menselijke patiënt met de diagnose kanker geschikt voor behandeling met een PD-1-remmende verbinding en/of PD-L1- remmende verbinding voorafgaand aan het initiëren van behandeling met genoemde verbinding (en) of kort na het initiëren van behandeling met

20 genoemde verbinding (en), waarbij de werkwijze de stappen omvat van:

a)   Verschaffen van een (solide) tumormonster van genoemde menselijke patiënt, waarbij het monster in de vorm is van één of meer FFPES of één of meer FFFS;

b)   Incubatie van het FFPES of FFFS-monster van stap a) onder

25   omstandigheden die specifieke antigeen-antilichaambinding mogelijk maken met een (bijvoorbeeld gelabeld) antilichaam gericht tegen menselijk PD-1om detectie van PD-1+ -cellen in genoemd monster mogelijk te maken;

c)   Verstrekken van digitale beelden van het FFPES- monster of FFFS-monster van stap b);

30   d)   Het onderwerpen van de digitale beelden van stap c) aan een (geautomatiseerd) beeldanalysesysteem om de intensiteit van de immuun kleuring voor PD-1per PD-1+ cel te meten;

e)   Kwantificeren van het aantal PD-1^T cellen betrokken op het totale aantal kernen waardoor het percentage (%) van PD-1^T cellen in het FFPES-

voorbeeld of FFFS-monster wordt verkregen of kwantificering van het absolute (of dichtheid) aantal PD-1-^{T} cellen per mm^2 in de FFPES monster of FFFS monster, waarbij de PD-1^T cellen de PD-1+ cellen van stap d) zijn met een intensiteit van immuun kleuring voor PD-1die gelijk of groter is dan een

5   vooraf bepaald REF2, waarbij de REF2 werd bepaald met een werkwijze volgens conclusie 22; en waarin

f)   (i) als er ten minste 0,60% tot ten minste 1,5% is, bij voorkeur ten minste 0,90% van PD-1^T cellen zijn ten opzichte van het totale aantal kernen in het FFPES-monster of FFFS monster; of als er ten minste 20 tot ten minste

10   70 PD-1-^{T} cellen zijn per mm^{2,} bij voorkeur ten minste 35 PD-1^T cellen per mm^2 in de FFPES monster of FFFS monster zijn, dit betekent dat de menselijke patiënt gediagnosticeerd met een kanker geschikt is voor behandeling met de PD-1remmende verbinding en/of PD-L1 remmende verbinding alleen; of

15   (ii) als er minder dan 0,60% tot minder dan 1,5% PD-1^T cellen is, bij voorkeur minder dan 0,90% PD-1^T cellen ten opzichte van het totale aantal kernen in het FFPES-monster of FFFS-monster; of als er minder dan 20 tot minder dan 70 is PD-1^{T}cellen per mm^{2,} bij voorkeur minder dan 35 PD-1^T cellen per mm^2 in de FFPES of FFFS monster, dit betekent dat de menselijke

20   patiënt gediagnosticeerd met een kanker niet geschikt is voor behandeling met de PD-1remmende verbinding en/of PD-L1remmende verbinding alleen; en

g)   Eventueel herhalen van stappen a) tot en met f) voor één of meer volgende FFPES of FFFS monsters van één of meer volgende menselijke

25   patiënten gediagnosticeerd met kanker.

9.   Een PD-1-remmende verbinding of PD-L1-remmende verbinding voor gebruik bij een werkwijze voor het behandelen van een menselijke patiënt gediagnosticeerd met een kanker, waarbij de werkwijze de stappen omvat van:

30   a).   Voorspellen dat de menselijke patiënt die een diagnose van een kanker heeft waarschijnlijk reageert op behandeling met de PD-1- remmende verbinding en/of de PD-L1- remmende verbinding volgens de werkwijzen van conclusie 7 of het bepalen dat de menselijke patiënt die is gediagnosticeerd met een kanker geschikt is voor behandeling met de PD-1-remmende

verbinding en/of de PD-L1-remmende verbinding volgens de werkwijzen van conclusie 8; en waarin

b)   (i) als in stap a) wordt bepaald dat genoemde menselijke patiënt geschikt is voor behandeling met de PD-1- remmende verbinding en/of de PD-L1- remmende verbinding alleen, dit aangeeft dat de patiënt kan worden behandeld met de PD-1remmende verbinding en/of PD-L1remmende verbinding alleen of als het bepaald is in stap a) dat de genoemde humane patiënt waarschijnlijk zal reageren op behandeling met de PD-1remmende verbinding en/of PD-L1remmende verbinding alleen het aangeeft dat de patiënt kan worden behandeld met de PD-1-remmende verbinding en/of de PD-L1-remmende verbinding alleen;

(ii) wanneer wordt bepaald in stap a) dat de genoemde humane patiënt niet geschikt is voor behandeling met de PD-1remmende verbinding en/of PD-L1remmende verbinding alleen, dit betekent dat de patiënt kan worden behandeld met een therapie die niet bestaat uit een behandeling met de PD-1remmende verbinding en/of PD-L1remmende verbinding alleen of indien wordt vastgesteld in stap a) dat de genoemde humane patiënt waarschijnlijk niet zal reageren op de behandeling met de PD-1 remmende verbinding en/of de PD-L1-remmende verbinding alleen, dit aangeeft dat de patiënt kan worden behandeld met een therapie die niet bestaat uit een behandeling met de PD-1-remmende verbinding en/of de PD-L1-remmende verbinding alleen.

10.   Werkwijze volgens een van de voorgaande conclusies, waarbij de kanker wordt gekozen uit de groep van longkankers, waaronder niet-kleincellige longkanker (NSCLC) en kleincellige longkanker; borstkanker; melanoom; urogenitale kankers, waaronder nierkanker, eierstokkanker en blaaskanker; gastro-intestinale tractus (GI) kanker, waaronder colorectale kanker en hepatocellulaire kanker; hoofd-halskanker; huidkanker, waaronder Merkel-cel carcinoom; bij voorkeur NSCLC.

11.   Methoden volgens conclusies 7-10, waarbij de PD-1-remmer-verbinding wordt gekozen uit nivolumab, pembrolizumab, PDR001, AMP-224, AMP-514, bij voorkeur nivolumab.

12.   Werkwijze volgens conclusies 7-11, waarbij de PD-L1-remmende verbinding wordt gekozen uit avelumab, atezolizumab, durvalumab, BMS-936559, bij voorkeur atezolizumab.

5   13.   Werkwijze volgens conclusies 7-12, waarbij de humane patiënt gediagnosticeerd met een kanker niet is behandeld met een PD-1-remmerverbinding en/of een PD-L1-remmende verbinding.

14.   Werkwijze voor het screenen van een testverbinding dat in staat is

10   het percentage (%) van PD-1^T cellen toe te laten nemen in een tumor bij een patiënt die lijdt aan kanker, waarbij de werkwijze de stappen omvat:

a)   Het verschaffen van een eerste tumormonster van de patiënt, waarbij het eerste tumormonster een TSS-monster is en wordt verkregen op een tijdstip voorafgaand aan het toedienen van de testverbinding aan het

15   subject;

b)   Het verschaffen van een tweede tumormonster van de patiënt, waarbij het tweede tumormonster een TSS-monster is verkregen op een tijdstip na het toedienen van de testverbinding aan het subject;

c).   Kwantificeren van het percentage (%) PD-1-^{T} cellen in het eerste

20   TSS-monster en tweede TSS-monster volgens de werkwijze van conclusie 1; en

d)   (i) Bepalen dat de testverbinding in staat is om het percentage (%) PD-1^T cellen in de tumor te verhogen van de patiënt die lijdt aan kanker als het percentage (%) PD-1^T cellen in het tweede TSS-monster hoger is dan het

25   percentage (%) PD-1-^{T} cellen in het eerste TSS-monster, of (ii) bepalen dat de testverbinding niet in staat is om het percentage (%) PD-1-^{T} cellen in de tumor van het subject te verhogen als het percentage (%) PD-1-^{T} cellen in het tweede TSS-monster gelijk is aan of lager dan het percentage (%) PD-1-^{T} cellen in het eerste TSS-monster.

30

15.   Werkwijze voor het screenen van een testverbinding die in staat is tot het verhogen van het percentage (%) of dichtheid van PD-1^T cellen in een tumor bij een patiënt die lijdt aan kanker, waarbij de werkwijze de stappen omvat van:

a) Het verschaffen van een eerste tumormonster van genoemde patiënt, waarbij genoemd eerste tumormonster een FFPES-monster of een FFFS-monster is en wordt verkregen op een tijdstip voorafgaand aan het toedienen van de testverbinding aan genoemde patiënt;

5 b) Het verschaffen van een tweede tumormonster van de patiënt, waarbij het tweede tumormonster een FFPES-monster of een FFFS-monster is verkregen op een tijdstip na toediening van de testverbinding aan de patiënt;

c) Kwantificeren van het percentage (%) of dichtheid (per mm$^{2)}$ van

10 PD-1^T cellen in de eerste FFPES of FFFS monster en tweede FFPES monster of FFFS monster volgens de werkwijze van conclusie 5; en

d) (i) Bepaling of de testverbinding in staat is het percentage (%) of dichtheid van PD-1^{T}cellen toe te laten nemen in de tumor van de patiënt die lijdt aan kanker als het percentage (%) of dichtheid van PD-1^{T}cellen in de

15 tweede FFPES-monster of FFFS-monster hoger is dan het percentage (%) of dichtheid van PD-1-^{T} cellen in het eerste FFPES-monster of FFFS-monster, of

(ii) Bepalen dat de testverbinding niet in staat is om het percentage (%) of dichtheid van PD-1^T cellen in de tumor van de persoon die lijdt aan

20 kanker te verhogen als het percentage (%) of dichtheid van PD-1^T cellen in het tweede FFPES-monster of FFFS-monster gelijk is aan of kleiner is dan het percentage (%) of dichtheid van PD-1-^{T} cellen in het eerste FFPES-monster of FFFS-monster, of

(iii) Bepalen dat de testverbinding in staat is om het percentage (%)

25 of de dichtheid van PD-1^T cellen in de tumor te verhogen van de patiënt die lijdt aan kanker als het tweede FFPES-monster of FFFS-monster bij voorkeur ten minste 0,1% PD-1^T cellen omvat, bijvoorbeeld 0,60% tot ten minste 1,5% PD-1^T cellen, of bijvoorbeeld ten minste 0,90% van PD-1^T cellen ten opzichte van het totale aantal kernen in het tweede FFPES-monster of FFFS-monster;

30 of omvat bij voorkeur ten minste 20 tot ten minste 70 PD-1^T cellen per mm$^{2.}$ bijvoorbeeld ten minste 35 PD-1^T cellen per mm^2 in het tweede FFPES-monster of FFFS-monster, of

(iv) Bepalen dat de testverbinding niet in staat is om het percentage (%) of dichtheid van PD-1^T cellen in de tumor te verhogen van de patiënt die

lijdt aan kanker als het tweede FFPES-monster of FFFS-monster bij voorkeur minder dan 0,1% van de tumor omvat PD-1^T cellen, bijvoorbeeld minder dan 0,60% tot minder dan 1,5%, of bijvoorbeeld minder dan 0,90% PD-1^T cellen ten opzichte van het totale aantal kernen in het tweede FFPES-monster of

5 FFFS-monster omvat; of als er bij voorkeur minder dan 20 tot minder dan 70 PD-1^T cellen per mm^2, bijvoorbeeld minder dan 35 PD-1^T cellen per mm^2 in de tweede FFPES of FFFS monster.

16. Een CTLA-4-remmende verbinding voor de behandeling van een
10 patiënt die lijdt aan kanker, waarbij genoemde patiënt niet geschikt is voor behandeling met een PD-1-remmende verbinding en/of een PD-L1-remmende verbinding alleen zoals bepaald volgens conclusie 8 of niet reageert op behandeling met een PD-1-remmende verbinding en/of een PD-L1-remmende verbinding alleen, zoals bepaald volgens conclusie 7, waarbij de behandeling in combinatie is met een
15 PD-1-remmende verbinding en/of PD-L1-remmende verbinding.

17. PD-1-remmende verbinding en/of PD-L1-remmende verbinding voor het behandelen van een patiënt die lijdt aan kanker, waarbij genoemde patiënt niet geschikt is voor behandeling met een PD-1-remmende verbinding en/of een PD-L1-
20 remmende verbinding alleen, zoals bepaald volgens conclusie 8 of niet reageert op behandeling met een PD-1-remmende verbinding en/of een PD-L1-remmende verbinding alleen zoals bepaald volgens conclusie 7, waarbij de behandeling in combinatie is met een CTL4A-remmerverbinding.

25 18. Een PD-1-remmende verbinding en/of PD-L1-remmende verbinding in combinatie met een CTLA-4-remmende verbinding voor gebruik in een werkwijze voor het behandelen van een menselijke patiënt gediagnosticeerd met een kanker, waarbij de werkwijze de stappen omvat van:
a) Voorspellen dat de menselijke patiënt die de diagnose van een
30 kanker heeft waarschijnlijk reageert op behandeling met de PD-1- remmende verbinding en/of de PD-L1- remmende verbinding volgens de werkwijze van conclusie 7 of het bepalen dat de menselijke patiënt die is gediagnosticeerd met een kanker geschikt is voor behandeling met de PD-1-remmende

verbinding en/of de PD-L1-remmende verbinding volgens de werkwijze van conclusie 8; en waarin

b) Als in stap a) wordt bepaald dat genoemde menselijke patiënt niet geschikt is voor behandeling met de PD-1- remmende verbinding en/of de PD-L1- remmende verbinding alleen, dit aangeeft dat de patiënt kan worden behandeld met een PD-1-remmende verbinding en/of PD-L1inhibitor verbinding in combinatie met CTLA-4 inhibitor verbinding of indien wordt vastgesteld in stap a) dat de genoemde humane patiënt waarschijnlijk niet zal reageren op de behandeling met de PD-1remmende verbinding en/of de PD-L1- remmende alleen, dit aangeeft dat de patiënt kan worden behandeld met een PD-1-remmende verbinding en/of een PD-L1-remmende verbinding in combinatie met de CTLA-4-remmende verbinding.

19. Verbinding voor gebruik volgens conclusies 16-18, waarbij de CTLA-4- remmende verbinding ipilimumab is en waarbij de PD-1-remmende verbinding is gekozen uit nivolumab, pembrolizumab, PDR001, AMP-224 en AMP-514, en waarbij de PD-L1-remmerverbinding wordt gekozen uit avelumab, atezolizumab, durvalumab en BMS-936559.

20. Verbinding voor gebruik volgens conclusies 16-18, waarbij de CTLA-4- remmende verbinding ipilimumab is, en waarbij de PD-1-remmende verbinding nivolumab is, en waarbij de PD-L1-remmende verbinding atezolizumab is.

21. Werkwijze voor het vaststellen van een vooraf bepaalde referentiewaarde 1(REF1) van intensiteit van immuun kleuring voor PD-1, waarbij de werkwijze de stappen omvat van:

i. Het verstrekken van ten minste één bloed monster van een gezonde menselijke donor;

ii. Isoleren van perifere mononucleaire bloedcellen (PBMC) uit genoemde bloedmonster;

iii. Incubatie van het PBMC monster van stap (ii) onder omstandigheden die specifieke antigeen-antilichaambinding mogelijk maken met een (bijvoorbeeld gemerkt) antilichaam gericht tegen humaan T-lymfocytmarker (M) en met een gemerkt antilichaam gericht tegen menselijk

PD-1om detectie van M+ -cellen, PD-1+ cellen en M+ / PD-1+ -cellen in genoemd PBMC-monster mogelijk te maken;

iv.   Het meten van de intensiteit van immuun kleuring van PD-1 voor M+ / PD-1+ cellen van stap (iii);

5   v.   Het selecteren van de bovenste 0,50% tot de bovenste 1,5%, bij voorkeur de bovenste 1% van de M+ / PD-1+ cellen van stap (iv) met de hoogste intensiteit van immuun kleuring voor PD-1; en

vi.   Bepaling van de minimale intensiteit van immuun kleuring voor PD-1in de bovenste 0,50% tot bovenste 1,5%, bij voorkeur in de bovenste 1,0%

10   van de M+ / PD-1+ cellen van stap (v) waardoor de vooraf bepaalde REF1wordt vastgesteld.

22.   Werkwijze voor het vaststellen van een vooraf bepaalde referentiewaarde 2 (REF2) van intensiteit van immuun kleuring voor PD-1, waarbij de

15   werkwijze de stappen omvat van:

a)   Het verschaffen van een solide tumormonster van een patiënt gediagnosticeerd met een kanker, waarbij het tumormonster is verdeeld in twee delen, waarbij het eerste deel wordt verwerkt om in de vorm te zijn van één of meer met formaline gefixeerde in paraffine ingebedde weefselsectie (s)

20   (FFPES)) of één of meer vers gefixeerde bevroren sectie (FFFS) en waarbij het tweede deel wordt verwerkt in de vorm van een monster met een enkele cel suspensie (TSS);

b)   Kwantificering van het percentage (%) PD-1-^{T} cellen in het TSS-monster van stap a) volgens de werkwijze van conclusie 1;

25   c)   Incubatie van het FFPES-monster van stap a) onder omstandigheden die specifieke antigeen-antilichaambinding mogelijk maken met een (bijvoorbeeld gemerkt) antilichaam gericht tegen menselijk PD-1om detectie van PD-1+ -cellen in genoemd FFPES-monster of FFFS-monster mogelijk te maken;

30   d)   Het verschaffen van één of meer digitale afbeelding (en) van het FFPES-monster of FFFS-monster van stap c);

e)   Het onderwerpen van de één of meer digitale afbeelding (en) van stap (d) aan een (geautomatiseerd) beeldanalysesysteem om de intensiteit van immuun kleuring van PD-1voor PD-1+ T-lymfocyten (TILS) te meten;

f)   Selecteren van het bovenste percentage (%) P van PD-1+ cellen uit stap e) met de hoogste intensiteit van immuun kleuring voor PD-1, waarbij het bovenste percentage (%) P van PD-1+ T-lymfocyten (TILS) gelijk is tot het percentage (%) PD-1- ^{T} cellen in het TSS-monster van stap b); en

5   g)   Bepaling van de minimale intensiteit van immuun kleuring voor PD-1in het bovenste percentage (%) P van PD-1+ T-lymfocyten (TILS) van stap f) waardoor een vooraf bepaalde referentiewaarde 2 (REF2) wordt vastgesteld.

10

Fig. 1
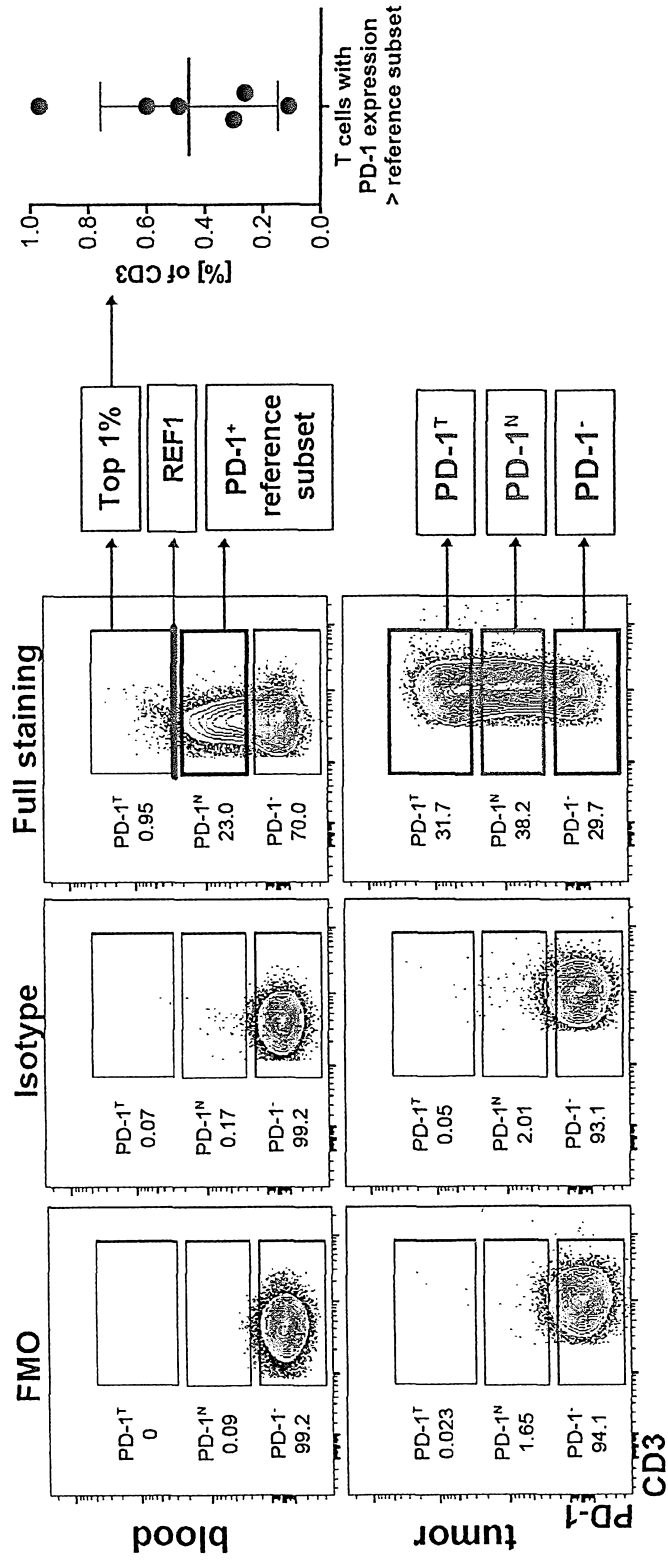
Fig. 2

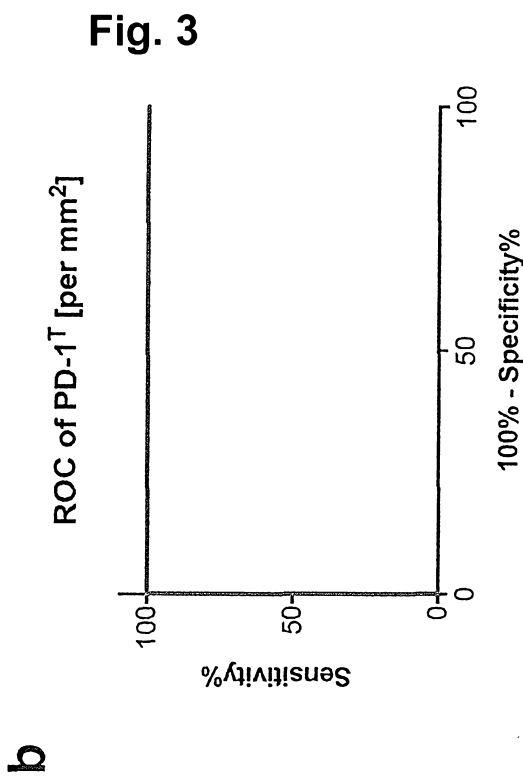Variability of REF1 fluorescence intensity with different PD-1$^{\mathrm{T}}$ cut-offs

Fig. 3

b

ROC of PD-1^T [per mm^2]

(Sensitivity% vs 100% − Specificity% plot)

	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 0.4315	100	59.04% to 100%	8.333	0.2108% to 38.48%	1.091
> 2.087	100	59.04% to 100%	16.67	2.086% to 48.41%	1.2
> 4.334	100	59.04% to 100%	25	5.486% to 57.19%	1.333
> 6.664	100	59.04% to 100%	33.33	9.925% to 65.11%	1.5
> 9.148	100	59.04% to 100%	41.67	15.17% to 72.33%	1.714
> 10.47	100	59.04% to 100%	50	21.09% to 78.91%	2
> 11.45	100	59.04% to 100%	58.33	27.67% to 84.83%	2.4
> 12.45	100	59.04% to 100%	66.67	34.89% to 90.08%	3
> 13.91	100	59.04% to 100%	75	42.81% to 94.51%	4
> 14.82	100	59.04% to 100%	83.33	51.59% to 97.91%	6
> 21.89	100	59.04% to 100%	91.67	61.52% to 99.79%	12
> 35.01	100	59.04% to 100%	100	73.54% to 100%	
> 59.28	85.71	42.13% to 99.64%	100	73.54% to 100%	
> 78.46	71.43	29.04% to 96.33%	100	73.54% to 100%	
> 80.4	57.14	18.41% to 90.1%	100	73.54% to 100%	
> 91.9	42.86	9.899% to 81.59%	100	73.54% to 100%	
> 112	28.57	3.669% to 70.96%	100	73.54% to 100%	
> 129.8	14.29	0.361% to 57.87%	100	73.54% to 100%	

a

ROC of PD-1^T [% of total]

(Sensitivity% vs 100% − Specificity% plot)

	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 0.0175	100	59.04% to 100%	7.692	0.1946% to 36.03%	1.083
> 0.031	100	59.04% to 100%	15.38	1.921% to 45.45%	1.182
> 0.086	100	59.04% to 100%	23.08	5.038% to 53.81%	1.3
> 0.16	100	59.04% to 100%	30.77	9.092% to 61.43%	1.444
> 0.1945	100	59.04% to 100%	38.46	13.86% to 68.42%	1.625
> 0.2525	100	59.04% to 100%	46.15	19.22% to 74.87%	1.857
> 0.303	100	59.04% to 100%	53.85	25.13% to 80.78%	2.167
> 0.3315	100	59.04% to 100%	61.54	31.58% to 86.14%	2.6
> 0.378	100	59.04% to 100%	69.23	38.57% to 90.91%	3.25
> 0.4605	100	59.04% to 100%	76.92	46.19% to 94.98%	4.333
> 0.5385	100	59.04% to 100%	84.62	54.55% to 98.08%	6.5
> 0.6415	100	59.04% to 100%	92.31	63.97% to 99.81%	13
> 0.901	100	59.04% to 100%	100	75.29% to 100%	
> 1.42	85.71	42.13% to 99.64%	100	75.29% to 100%	
> 2.014	71.43	29.04% to 96.33%	100	75.29% to 100%	
> 2.27	57.14	18.41% to 90.1%	100	75.29% to 100%	
> 2.387	42.86	9.899% to 81.59%	100	75.29% to 100%	
> 2.553	28.57	3.669% to 70.96%	100	75.29% to 100%	
> 2.63	14.29	0.361% to 57.87%	100	75.29% to 100%	

Fig. 4

A B S T R A C T

The present invention relates to the field of biomarker development for cancer immunotherapy with PD-1 inhibitor compounds and/or PD-L1 inhibitor compounds. Provided are assays for quantifying PD-1 expression (i.e. immunostaining intensity) in cells present in a tumor sample (i.e. intratumoral cells), which are advantageously used to identify a unique sub-population of intratumoral cells referred to herein as PD-1^T cells, which serves as a biomarker for cancer immunotherapy with PD-1 inhibitor compounds and/or PD-L1 inhibitor compounds alone or in combination with other therapeutic agents (e.g. CTLA-4 inhibitor compound, e.g. ipilimumab). The present invention also provides methods of selecting a human subject diagnosed with cancer (e.g. non-small cell lung cancer (NSCLC)) suitable for immune checkpoint therapy with agents such as PD-1 inhibitors (e.g. nivolumab) and/or PD-L1 inhibitors (e.g. atezolizumab) alone or in combination with other therapeutic agents (e.g. CTLA-4 inhibitor compound, e.g. ipilimumab), methods for predicting responsiveness to immune checkpoint therapy with agents such as PD-1 inhibitors and/or PD-L1 inhibitors, and method of treatment of a human subject diagnosed with cancer using PD-1 inhibitors and/or PD-L1 inhibitors alone or in combination with other therapeutic agents (e.g. CTLA-4 inhibitor compound, e.g. ipilimumab) (//dc).

SAMENWERKINGSVERDRAG (PCT)

RAPPORT BETREFFENDE NIEUWHEIDSONDERZOEK VAN INTERNATIONAAL TYPE

IDENTIFICATIE VAN DE NATIONALE AANVRAGE	KENMERK VAN DE AANVRAGER OF VAN DE GEMACHTIGDE **73455NL**
Nederlands aanvraag nr. **2020422**	Indieningsdatum **12-02-2018**
	Ingeroepen voorrangsdatum

Aanvrager (Naam)	
Stichting Het Nederlands Kanker Instituut- Antoni van Leeuwenhoek Ziekenhuis, et al	
Datum van het verzoek voor een onderzoek van internationaal type **28-04-2018**	Door de Instantie voor Internationaal Onderzoek aan het verzoek voor een onderzoek van internationaal type toegekend nr. **SN71178**

I. CLASSIFICATIE VAN HET ONDERWERP (bij toepassing van verschillende classificaties, alle classificatiesymbolen opgeven)

Volgens de internationale classificatie (IPC)

G01N33/574

II. ONDERZOCHTE GEBIEDEN VAN DE TECHNIEK

Onderzochte minimumdocumentatie

Classificatiesysteem	Classificatiesymbolen
IPC	**G01N**

Onderzochte andere documentatie dan de minimum documentatie, voor zover dergelijke documenten in de onderzochte gebieden zijn opgenomen

III.	GEEN ONDERZOEK MOGELIJK VOOR BEPAALDE CONCLUSIES	(opmerkingen op aanvullingsblad)
IV.	GEBREK AAN EENHEID VAN UITVINDING	(opmerkingen op aanvullingsblad)

Form PCT/ISA 201 A (11/2000)

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

A. CLASSIFICATIE VAN HET ONDERWERP

INV. G01N33/574
ADD.

Volgens de Internationale Classificatie van octrooien (IPC) of zowel volgens de nationale classificatie als volgens de IPC

B. ONDERZOCHTE GEBIEDEN VAN DE TECHNIEK

Onderzochte minimum documentatie (classificatie gevolgd door classificatie symbolen)

G01N

Onderzochte andere documentatie dan de minimum documentatie, voor dergelijke documenten, voor zover dergelijke documenten in de onderzochte gebieden zijn opgenomen

Tijdens het onderzoek geraadpleegde elektronische gegevensbestanden (naam van de gegevensbestanden en, waar uitvoerbaar, gebruikte trefwoorden)

EPO-Internal

C. VAN BELANG GEACHTE DOCUMENTEN

Categorie °	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
X	OSCAR ARRIETA ET AL: "Expression of PD-1/PD-L1 and PD-L2 in peripheral T-cells from non-small cell lung cancer patients", ONCOTARGET, deel 8, nr. 60, 24 november 2017 (2017-11-24), XP55496203, DOI: 10.18632/oncotarget.22025	21
Y	* het gehele document * * In particular: Title; Abstract; Figures 1 and 2; Tables 1-3 *	1-20,22

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[X] Verdere documenten worden vermeld in het vervolg van vak C.

[ ] Leden van dezelfde octrooifamilie zijn vermeld in een bijlage

Datum waarop het onderzoek naar de stand van de techniek van internationaal type werd voltooid	Verzenddatum van het rapport van het onderzoek naar de stand van de techniek van internationaal type
1 augustus 2018	

ONDERZOEKSRAPPORT BETREFFENDE HET
RESULTAAT VAN HET ONDERZOEK NAAR DE STAND
VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Nummer van het verzoek om een onderzoek naar
de stand van de techniek

NL 2020422

C.(Vervolg). VAN BELANG GEACHTE DOCUMENTEN

Categorie *	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
Y	SI-PEI WU ET AL: "Stromal PD-L1-Positive Regulatory T cells and PD-1-Positive CD8-Positive T cells Define the Response of Different Subsets of Non-Small Cell Lung Cancer to PD-1/PD-L1 Blockade Immunotherapy", JOURNAL OF THORACIC ONCOLOGY, deel 13, nr. 4, 18 december 2017 (2017-12-18), bladzijden 521-532, XP055496261, Philadelphia, PA, US ISSN: 1556-0864, DOI: 10.1016/j.jtho.2017.11.132 * het gehele document * * In particular: Title; Abstract; Materials and methods section; Figures 2-4; Discussion. * -----	1-20,22
Y	HONG ZHENG ET AL: "Expression of PD-1 on CD4+ T cells in peripheral blood associates with poor clinical outcome in non-small cell lung cancer", ONCOTARGET, deel 7, nr. 35, 30 augustus 2016 (2016-08-30), XP55496200, DOI: 10.18632/oncotarget.9316 * het gehele document * * In particular: Title; Abstract; First paragraph of the Results section. * -----	1-22
Y	T MENIAWY ET AL: "Up regulation of PD-L1 on peripheral blood CD3+T cells predicts poor prognosis in patients with non-small cell lung cancer (NSCLC) treated with epidermal growth factor receptor (EGFR) inhibitors", EUROPEAN JOURNAL OF CANCER, deel 51, nr. Suppl. 3, 1 september 2015 (2015-09-01), bladzijde s47, XP55496360, * het gehele document * -----	1-22

1

File No. SN71178	Filing date *(day/month/year)* 12.02.2018	Priority date *(day/month/year)*	Application No. NL2020422

International Patent Classification (IPC)
INV. G01N33/574

Applicant
Stichting Het Nederlands Kanker Instituut-Antoni van Leeuwenhoek Ziekenhuis, et al

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This opinion contains indications relating to the following items:

☒ Box No. I   Basis of the opinion

☐ Box No. II   Priority

☐ Box No. III   Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

☐ Box No. IV   Lack of unity of invention

☒ Box No. V   Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

☐ Box No. VI   Certain documents cited

☐ Box No. VII   Certain defects in the application

☒ Box No. VIII   Certain observations on the application

	Examiner C.F. Angioni

WRITTEN OPINION

Box No. I   Basis of this opinion

1. This opinion has been established on the basis of the latest set of claims filed before the start of the search.

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the application and necessary to the claimed invention, this opinion has been established on the basis of:

    a. type of material:

    ☐ a sequence listing

    ☐ table(s) related to the sequence listing

    b. format of material:

    ☐ on paper

    ☐ in electronic form

    c. time of filing/furnishing:

    ☐ contained in the application as filed.

    ☐ filed together with the application in electronic form.

    ☐ furnished subsequently for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

Box No. V   Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty	Yes:	Claims	1-20, 22
	No:	Claims	21
Inventive step	Yes:	Claims	
	No:	Claims	1-22
Industrial applicability	Yes:	Claims	1-22
	No:	Claims	

2. Citations and explanations

see separate sheet

Box No. VIII   Certain observations on the application

see separate sheet

1   Reference is made to the following documents:

D1   OSCAR ARRIETA ET AL: "Expression of PD-1/PD-L1 and PD-L2 in peripheral T-cells from non-small cell lung cancer patients", ONCOTARGET, deel 8, nr. 60, 24 november 2017 (2017-11-24), XP55496203

D2   SI-PEI WU ET AL: "Stromal PD-L1-Positive Regulatory T cells and PD-1-Positive CD8-Positive T cells Define the Response of Different Subsets of Non-Small Cell Lung Cancer to PD-1/PD-L1 Blockade Immunotherapy", JOURNAL OF THORACIC ONCOLOGY, deel 13, nr. 4, 18 december 2017 (2017-12-18), bladzijden 521-532, XP055496261

D3   HONG ZHENG ET AL: "Expression of PD-1 on CD4 sup+ /supT cells in peripheral blood associates with poor clinical outcome in non-small cell lung cancer", ONCOTARGET, deel 7, nr. 35, 30 augustus 2016 (2016-08-30), XP55496200

D4   T MENIAWY ET AL: "Up regulation of PD-L1 on peripheral blood CD3+T cells predicts poor prognosis in patients with non-small cell lung cancer (NSCLC) treated with epidermal growth factor receptor (EGFR) inhibitors", EUROPEAN JOURNAL OF CANCER, deel 51, nr. Suppl. 3, 1 september 2015 (2015-09-01), bladzijde s47, XP55496360

Re Item V.

2   Novelty

The subject-matter of claim 21 is not new.

Document D1 discloses (see relevant passages as cited in the search report): A method for establishing a reference value for T-lymphocytes in PBMC samples taken from healthy and NSCLC individuals. The reference value is the value of T-lymphocytes in healthy individuals (cut off point) and is found to be around 1.1% in PD-1+/CD3+ T-lymphocytes.

3   Inventive step

The subject matter of claims 1-20 and 22 does not appear to involve an inventive step in the sense.

3.1   Document D2, which is considered to represent the most relevant state of the art to the subject matter of claim 1, discloses (see relevant passages as cited in the search report):

A cytometryic assay for quantifying PD-1 expressing cells in tumor infiltrating lymphocytes (TIL) isolated from a tumor sample taken from an NSCLC patient.

3.2   The subject-matter of independent claim 1 differs from D2 in that the TILs of the tumor sample are compared to a predetermined reference value (REF1) obtained in a method as defined in claim 21. No technical effect is derivable from this difference.

3.3   The problem to be solved by the present invention may therefore be regarded as: How to provide a further method for quantifying PD-1 expressing cells in T-lymphocytes from a tumor sample? The solution being claim 1.

3.4   The solution provided by the present application is not considered to be inventive. The reason is simply that the present application does not appear to solve the objective technical problem. Example 2 of the present application seemingly carries out such a method. However, the example basically does not provide enough details that explain how the method is exactly carried out. Also, the method does not appear to make any sense since it does not appear to be logical to use a reference obtained with PBMCs on cells derived from a solid tumor sample.

3.5   Claims 2-20 and 22 do not appear to contain any additional features which, in combination with the features of any claim to which they refer, meet the requirements with respect to inventive step, as the relevant subject matter is either disclosed in the cited prior art (see e.g. D1-D4) or also falls within the knowledge and ability of the skilled person.

Re Item VIII.

4   Clarity, support and disclosure

4.1   The claims are not clear and lack conciseness. The claims are drafted in such a manner that (based on the back references to other claims) it is very difficult to track down and understand the scope of each claim. It appears that the whole gist of the invention is based on claim 21, namely the manner in which a reference value 1 (REF1) is established. For the purpose of clarity and ease

of reading claims 21 and 22 should be promoted as claims 1 and 3 respectively. Claim 1 should be renumbered as claim 2. This would simplify extremely the understanding of the claims.

4.2 Claim 21 sets a limit of detection of 0,5-1,5% as the reference value for detecting M+/PD-1+ T-lymphocytes. However, this limit is based on example 1 of the application in which the T-lymphocyte marker is CD3. Thus, this limit cannot work for other markers such as CD4 or CD8. Indeed, it should be noted that in D3 the limit of detection for the reference value (i.e. healthy individuals) is set at 8.8% +/- 0.7% when using CD4 as the T-lymphocyte marker.

4.3 Most of the claims are not supported by the description, since the description does not sufficiently disclose in technical terms the subject-matter of the claims.

Furthermore, all of the product claims (i.e. independent claims 9 and 16-18) are so called reach through claims, i.e. the claims relate to an extremely large number of possible compounds. Support and disclosure is to be found however for none of the compounds claimed. The fact that any compound could be screened does not overcome this objection, as the skilled person would not have knowledge beforehand as to whether it would fall within the scope claimed. Undue experimentation would be required to screen compounds randomly.