BIOMARKERS FOR PREDICTING THE EFFICACY OF AN IMMUNOTHERAPY AGAINST CANCER

The present invention relates to methods for predicting the effect of an immunotherapy against cancer in a patient based on new biomarkers. The present invention furthermore relates to a prognosis regarding the outcome based on said biomarkers. The present invention furthermore relates to panels of biomarkers for use in the above methods.

Thus application claims the priorities of GB1021289.6, filed December 15, 2010; US 61/416,981, filed November 24, 2010; and US 61/423,652, filed December 16, 2010. This application includes a sequence listing including 50 sequences. For the purposes of the present invention, all references as cited herein are incorporated by reference in their entireties.

Background of the invention

Stimulation of an immune response is dependent upon the presence of antigens recognized as foreign by the host immune system. The discovery of the existence of tumor-associated and tumor-specific antigens has raised the possibility of using a host's immune system to intervene in tumor growth. Various mechanisms of harnessing both the humoral and cellular arms of the immune system are currently being explored for cancer immunotherapy.

Certain elements of the cellular immune response are capable of specifically recognizing and destroying tumor cells. The isolation of cytotoxic T-cells (CTLs) from tumor-infiltrating cell populations or from peripheral blood suggests that these cells play an important role in the natural immune defense against cancer (Cheever et al., Annals N.Y. Acad. Sci. 1993 690:101-112; Zeh HJ, Perry-Lalley D, Dudley ME, Rosenberg SA, Yang JC; J Immunol. 1999, 162(2):989-94). CD8-positive (CD8+) T-cells in particular, which recognize complexes of major histocompatibility complex (MHC) class I molecules and peptides of usually 8 to 10 amino acid residues derived from cytosolic proteins or defective ribosomal products (DRiPs) (Schubert U, Antón LC, Gibbs J, Norbury CC, Yewdell JW, Bennink JR. Nature 2000; 404(6779):770-774), play an important role in this response. Human MHC-molecules are also designated as human leukocyte antigens (HLA).

There are two classes of MHC-molecules: MHC class I molecules can be found on most cells having a nucleus and present peptides that result from proteolytic cleavage of endogenous proteins, DRiPs, and larger peptides. MHC class II molecules can be found predominantly on professional antigen presenting cells (APCs) and present peptides of exogenous proteins that are taken up by APCs, and are subsequently processed (Cresswell P. Annu. Rev. Immunol. 1994; 12:259-93). Complexes of peptide and MHC class I molecules are recognized by CD8+ CTLs bearing the appropriate T cell receptor (TCR), while complexes of peptide and MHC class II molecules are recognized by CD4+ helper-T-cells bearing the appropriate TCR. It is well known that the TCR, the peptide and the MHC are thereby present in a stoichiometric amount of 1:1:1.

For a peptide to elicit a cellular immune response, it must bind to an MHC-molecule. This process depends on the allele of the MHC-molecule and on the amino acid sequence of the peptide. MHC class I-binding peptides are usually 8, 9 or 10 amino acid residues in length and contain conserved residues ("anchors") in their sequences that interact with the corresponding binding groove of the MHC-molecule. Thus, each MHC allele has a "binding motif" determining which peptides can bind specifically to the binding groove (Rammensee H. G., Bachmann J. and Stevanovic, S; MHC Ligands and Peptide Motifs, Chapman & Hall 1998). To elicit an immune reaction, peptides not only have to be able to bind to certain MHC-molecules, they also have to be recognized by T-cells bearing specific TCRs. A further prerequisite for efficient immune reactions is the absence of immunological tolerance against the antigen.

Tumor-associated antigens (TAAs) from which epitopes recognized by CTLs are derived, can be molecules from all protein classes, such as enzymes, receptors, transcription factors, etc., which are upregulated in cells of the respective tumor. Furthermore, antigens can be tumor-specific, i.e. unique to tumor cells, for example as products of mutated genes or from alternative open reading frames (ORFs), or from protein splicing (Vigneron N, Stroobant V, Chapiro J, Ooms A, Degiovanni G, Morel S, van der Bruggen P, Boon T, Van den Eynde BJ. Science 2004 Apr 23; 304 (5670):587-90). Another important class of antigens are tissue-specific antigens, such as "cancer-testis-" (CT)-antigens that are expressed in different kinds of tumors and in healthy tissue of the testis.

Therefore, TAAs are a starting point for the development of a tumor vaccine. The methods for identifying and characterizing the TAAs are based e.g. on the use of CTLs that can be isolated from patients or healthy subjects, or on the generation of differential transcription profiles or differential peptide expression patterns between tumors and normal tissues (Lemmel C., Weik S., Eberle U., Dengjel J., Kratt T., Becker H. D., Rammensee H. G., Stevanovic S. Nat. Biotechnol. 2004 Apr.; 22(4):450-4, T. Weinschenk, C. Gouttefangeas, M. Schirle, F. Obermayr, S. Walter, O. Schoor, R. Kurek, W. Loeser, K. H. Bichler, D. Wernet, S. Stevanovic, and H. G. Rammensee. *Cancer Res.* 62 (20):5818-5827, 2002).

However, the identification of genes overexpressed or selectively expressed in tumor tissues or human tumor cell lines does not provide sufficient information if the corresponding antigen is a useful target for a T-cell based immunotherapy. This is because only an individual subpopulation of epitopes of these antigens are a) presented and b) recognized by T-cells with corresponding TCRs. In addition, immunological tolerance for this particular epitope needs to be absent or negligible. It is therefore important to select only those peptides from overexpressed or selectively expressed proteins that are presented in connection with MHC molecules and are targets of functional T-cells. A functional T-cell is defined as a T-cell that upon stimulation with a specific antigen can be clonally expanded and is able to execute effector functions ("effector T-cell").

T-helper cells play an important role in orchestrating the effector functions of CTLs in anti-tumor immunity. T-helper cell epitopes that trigger a T-helper cell response of the T_{H1} type support effector functions of CD8+ CTLs, which include cytotoxic functions directed against tumor cells displaying tumor-associated peptide/MHC complexes on their cell surfaces. In this way tumor-associated T-helper cell epitopes, alone or in combination with other tumor-associated peptides, can serve as active pharmaceutical ingredients of vaccine compositions which stimulate anti-tumor immune responses.

Since both types of response, CD8- and CD4-dependent, contribute jointly and synergistically to the anti-tumor effect, the identification and characterization of TAAs recognized by CD8+ CTLs (ligand: MHC class I molecule + peptide epitope) and of TAAs recognized by CD4+ T-helper cells (ligand: MHC class II molecule + peptide epitope) are both important in the development of effective tumor vaccines and an effective treatment based on these vaccines.

In Europe, renal cell carcinoma (RCC) ranks as the seventh most common malignancy in men, amongst whom there are 29,600 new cases each year (3.5% of all cancers). Among women, RCC ranks twelfth, with 16,700 cases a year (2.3% of all cancers). RCC is rare before the age of 40, and above this age it is twice as common in men as in women. Incidence by age rises rapidly from less than 2 per 100,000/year in patients under 40 years old to 38 per 100,000/year in the age group 65–69 years. Thereafter, it increases to 46 per 100,000/year in those older than 75 years.

A total of 25–30% of patients with RCC display overt metastases at initial presentation. About one third of patients with RCC will develop metastatic disease over time. Thus, nearly 50–60% of all patients with RCC will eventually present with metastatic disease. Among those with metastatic disease, approximately 75% have lung metastases, 36% lymph node and/or soft tissue involvement, 20% bone involvement, and 18% liver involvement.

RCC is the most lethal carcinoma of the genitourinary tumors with a 65% five-year survival rate compared to the 82% and 100% five-year survival rate for bladder or prostate cancer, respectively (US 1972-2001 data). Average survival rates at 5 years (up to 1999) after diagnosis (1990–1994) for kidney cancer were about 58% in Europe, and RCC was classified by several authors as a cancer with only moderate prognosis. Overall, RCC is fatal in nearly 80% of patients. This figure indicates a strong medical need for effective and early clinical follow-up and treatment for recurrences.

Survival strongly depends on the stage at which the tumor is diagnosed: 5-year survival is only 12% for patients bearing lesions with distant metastases, but 80% for those with localized malignancies.

Globally, colorectal carcinoma (CRC) is the third most common cancer. Colon and rectum cancer account for about 1 million new cases per year, and unlike for most other tumors, numbers are similar in men and women (ratio 1.2:1). In Europe, CRC is the second most common cancer and the second most common cancer-related cause of death in both men and women with approximately 380,000 new cases and about 200,000 disease-related deaths per year. The raw incidence rate in 2002 for men and women was 88.3 and 84.0/100,000, respectively; the raw mortality was 34.8 and 35.2/100,000, respectively. These data clearly reflect the significance of CRC as an enormous source of both individual and societal burden.

CRC is a cancer of the elderly population, as the mean age at the time of disease manifestation in men and women is 69 and 75 years, respectively. Besides dietary and lifestyle factors (e.g. obesity, lack of physical exercise, smoking, regular alcohol consumption), other risk factors are familial occurrence of CRC, hereditary types of CRC (familial adenomatous polyposis [FAP], attenuated FAP [attenuated adenomatous polyposis coli; AAPC], hereditary non-polyposis colorectal carcinoma [HNPCC], hamartomatous polyposis syndromes) and inflammatory bowel diseases such as ulcerative colitis or Crohn's disease.

CRC mostly occurs as adenocarcinoma of the mucous membranes in rectum, sigma, colon transversum/descendens, and colon ascendens/caecum. Early colorectal carcinoma may be cured by primary surgery. Distant metastases, however, spread to regional lymph nodes and to liver, lung, and other organs (such as CNS). Due to unspecific symptoms, CRC is often diagnosed at a relatively late stage and approximately 25% of patients with CRC have metastatic disease (mCRC) when first presented to their physicians. An additional 30% of newly diagnosed patients with localized resectable CRC subsequently develop metastatic recurrence.

EP2105740 describes that some proteins, including Apolipoprotein AI (APOA1), are regulated by c-myc overexpression in subjects suffering from or being susceptile to cancer. Consequently, EP2105740 describes the use of the biomarker APOA1 in the diagnosis, prognosis and/or treatment monitoring of cancer, in particular of lung cancer, but not a prediction of the effectiveness of a treatment, let alone an immunotherapy.

WO2010/076322 describes a method for predicting a response to and/or benefit from chemotherapy in a patient suffering from cancer involving (i) classifying a tumor into at least two classes, (ii) determining in a tumor sample the expression of at least one marker gene indicative of a response to chemotherapy for a tumor in each respective class, (iii) and depending on said gene expression, predicting said response and/or benefit; wherein one marker gene is CXCL13. WO2010/076322 also does not describe a prediction of the effectiveness of a treatment, let alone an immunotherapy.

Similarly, WO2010/003773 describes methods for predicting an outcome of cancer in a patient suffering from cancer, said patient having been previously diagnosed as node positive

and treated with cytotoxic chemotherapy; wherein one marker gene is CXCL13. WO2010/003773 does not describe a prediction of the effectiveness of an immunotherapy.

EP 1 777 523 A1 relates to the prognosis of the outcome of a cancer in a patient, which prognosis is based on the quantification of one or several biological markers that are indicative of the presence of, or alternatively the level of, the adaptive immune response of said patient against said cancer. Overall, an extremely large number of markers is disclosed. Furthermore, EP 1 777 523 A1 relates to a prognosis (and not prediction) for the outcome of a cancer in a patient, based on the detection and/or the quantification, of one or more biological markers indicative of the presence of, or alternatively of the level of, the adaptive immune response of said patient against said cancer at the tumor site.

Despite the recent progress in the diagnosis and management of many cancers described above, such as, for example, RCC and CRC, still biological markers are needed that can be used to achieve an improved diagnosis, and in particular a prediction of whether a beneficial effect of an immunotherapy can be expected, in order to further improve the survival and to better adjust the treatment of people in need. Furthermore, the markers should also allow for a prediction of the outcome of said treatment of cancer. It is therefore an object of the present invention to provide respective biological markers and diagnostic, predictive and prognostic methods.

In a first aspect of the present invention, said object is solved by providing a method for predicting an effect of an immunotherapy in a cancer patient, comprising a) determining the level of at least one marker selected from the group consisting of Apolipoprotein A1 (ApoA1), CCL17/TARC, eosinophils (in absolute numbers or %), monocytes (in absolute numbers or %), CD95/Fas, aspartate aminotransferase/serum glutamic oxaloaceticacid transaminase (ASAT/SGOT), cancer antigen 19-9 (CA19-9), lactate dehydrogenase (LDH), threonine, immunoglobulin E (IgE), and matrix metalloproteinase 3 (MMP-3) in a sample from said cancer patient, wherein a higher (or increased) level of the marker compared to the median of a given cancer patient population is indicative for a beneficial effect of an immunotherapy for said patient, or b) determining the level of at least one marker selected from the group consisting of CXCL13/BCA-1, neutrophils (in %), interleukin 6 (IL-6) and short-chain acylcarnitines in a sample from said cancer patient, wherein a lower (or

decreased) level of the marker compared to the median (+/-10%) of a given cancer patient population is indicative for a beneficial effect of an immunotherapy for said patient.

In this embodiment, the invention relates to univariate (also called "single" or "individual") markers for a prediction of an effect, particularly a beneficial effect of an immunotherapy in a cancer patient as described herein, such as longer overall survival, occurrence of single and/or multiple T-cell responses induced by the immunotherapy, retardation of tumor growth, tumor shrinkage or longer progression-free survival.

In the context of the present invention, the terms "marker", "biomarker", "analyte", or "parameter" are used interchangeably, and all relate to the marker(s) as analyzed in the context of the method(s) according to the present invention.

In the context of the present invention, a "prediction" or a "predictive marker" shall be based on markers according to the present invention that are informative for the effectiveness of an immunotherapy of cancer, such as, for example, using the vaccine(s) as described herein.

CXCL13/BCA-1, ApoA1, neutrophils, eosinophils, monocytes, CD95/Fas, ASAT/SGOT, CCL17/TARC, short-chain acylcarnitines and CA19-9 have been identified to be predictive for overall survival, and in some cases for T cell responses, by using univariate analyses.

Preferred is a method according to the present invention, wherein said marker is selected from ApoA1 and/or CCL17/TARC. CCL17/TARC levels above the median of the respective study population and ApoA1 levels above the median, as indicated by the respective assay provider, (e.g. Rules Based Medicine (RBM), 0.288 mg/ml) are positive.

Preferred is a method according to the present invention, wherein said marker is selected from CXCL13/BCA-1 or monocytes. Nevertheless, also combinations of the markers are included in the scope of the present invention, such as, for example, selected from ApoA1 and CCL17/TARC; ApoA1 and CXCL13/BCA-1; ApoA1, CXCL13/BCA-1, and monocytes; ApoA1, CCL17/TARC, CXCL13/BCA-1, and monocytes; CCL17/TARC and CXCL13/BCA-1; CCL17/TARC, CXCL13/BCA-1, and monocytes; CCL17/TARC, ApoA1 and monocytes; CCL17/TARC, ApoA1 and CXCL13/BCA-1; CCL17/TARC and monocytes;

CXCL13/BCA-1 and monocytes; ApoA1 and monocytes. All these marker combinations are particularly preferred.

Further preferred marker combinations are selected from CXCL13/BCA-1, neutrophils%, ApoA1, eosinophils%, eosinophils ABS (absolute), monocytes%, FAS, TARC; LDH, Thr, IL-6, albumin, IgE, MMP-3, CA19-9; monocytes ABS, ASAT, bilirubin, acylcarnitines (scAC); and IL-33. All these marker combinations are also particularly preferred.

ApoA1 is the main protein of high-density lipoprotein (HDL), and can be measured instead of HDL in clinical analyses. Under normal conditions, HDL shows anti-atherosclerotic, anti-oxidant, anti-thrombotic and anti-inflammatory properties. However, under chronic inflammatory/oxidative conditions (as e.g. in chronic infections, autoimmune diseases, metabolic syndrome and cancer), HDL loses these properties and gathers pro-inflammatory properties. In pro-inflammatory HDL (piHDL), ApoA1 – a negative acute phase protein – is reduced and other proteins like serum amyloid A (SAA) – a positive acute phase protein – are enriched. Thus, low ApoA1 levels are indicative of chronic inflammatory or oxidative conditions, and they might in turn promote such states, which are known to favor cancer development. Reduced levels of ApoA1 have been reported for many cancers and were also observed in RCC patients of the IMA901-202 (phase II) study cohort. Notably, the related protein ApoA2 has been described as positive prognostic in metastatic RCC, and a mouse model showed a functional involvement of ApoA1 in the suppression of cancer progression (Su et al., 2010; Vermaat et al., 2010). In addition to their cancer-promoting effects, chronic acute phase reactions and oxidative conditions are also known to disfavor adaptive immune responses (Haeryfar and Berczi, 2001; Muller et al., 2008; Vallejo et al., 2004).

CCL17/TARC is a chemokine that has originally been classified as chemokine attracting TH2 cells, but it also has other cellular targets, such as TH1- and TH2-type effector/memory T-cells, particularly those homing to the skin, a subset of CD8+ T-cells, TH17 memory T-cells, NK and NKT cells, dendritic cells, etc. Mouse studies showed that intra-tumoral CCL17/TARC expression is favorable for immunological rejection. Serum levels might indicate activity of myeloid dendritic cells, macrophages and monocytes, which are major sources of this chemokine. Steady-state production of CCL17 by dendritic cells has been shown to be a prerequisite for their unique function to trigger antigen-independent responses in T-cells. In contrast to conditions like atopic dermatitis and some other allergic or

autoimmune conditions, where CCL17/TARC is pathologically enhanced and considered to be indicative for increased TH2 activity, CCL17/TARC levels were within normal ranges in the IMA901-202 study population, and balanced with TH1 cytokines like IL-12 and IFN-gamma.

In the context of the present invention, levels of ApoA1 and levels of CCL17/TARC higher than the median of levels found in advanced RCC patients are considered favorable for treatment success. Patients with at least one factor above the indicated cutoffs (statistically around 75%) are predicted to show improved clinical outcome after vaccination with the cancer vaccine IMA901 (immatics Biotechnologies, Tübingen, Germany), compared to patients showing both factors below the cutoffs. Patients with both factors above the indicated cutoffs (statistically around 25%) are predicted to profit most from treatment. An LLN (lower limit of normal) for ApoA1 depends on the assay used for analysis. Using the Luminex bead-based multiplex technology (RBM), the LLN is 0.288 mg/ml. Using different assays, the LLN has to be adjusted either by using information indicated by the provider, by bridging experiments comparing the assay with the established one, or by measurement with a statistically relevant amount of healthy donor samples. The median of CCL17/TARC and/or ApoA1 levels in a given patient population depends on the population and on the assay used for quantification.

Preferred measurement methods for the markers, and specifically ApoA1 and CCL17/TARC, are selected from immunological assay such as ELISA, bead-, chip- or plate-based multiplex immunoassays, proteomics, or from mass spectrometry, biological assays, electrophoresis, immunonephelometry, immunoturbidimetry, enzymatic assays, colorimetric or fluorometric assays e.g. evaluable by photometry, and fluorescence-associated cell sorting (FACS)-based analyses. Neutrophils, eosinophils and monocytes can be determined by FACS or by other clinically established hematological assays. ApoA1 is highly correlated with HDL cholesterol and can therefore also be determined by all methods used to determine HDL cholesterol.

Furthermore, B-cell attracting chemokine 1 (CXCL13/BCA-1) has been identified as a marker. CXCL13/BCA-1 seems necessary for the organization of lymphocyte assembly in lymphoid organs. Elevated levels are associated with metastasis and poor prognosis in some tumor entities and probably also with impaired anti-tumor immune responses.

Eosinophils, whose levels in percent and absolute numbers were identified as a marker, may, given the contradicting reports on their effects, play several and opposing roles in cancer. Eosinophil recruitment to the tumor is apparently positive in several cases. Eosinophil recruitment to the site of GM-CSF injection was found in case of melanoma.

Monocytes, whose levels in percent and absolute numbers were identified as marker, are recruited to inflammatory sites or sites of GM-CSF administration. They are able to differentiate into macrophages or dendritic cells and can function as APCs themselves. They can replace emigrated tissue dendritic cells such as Langerhans cells. By contrast, high monocytes were described as a negative factor in cancer and IL-2 therapy of RCC, presumably due to reactive oxygen species (ROS) production and inhibition of NK and T cells.

Soluble CD95/Fas is generated by alternative splicing and competes with membrane-bound CD95 for binding of CD95L/FasL. Thus, it may prevent T cell apoptosis, e.g. at the tumor site (tumor counter-attack). It may also counteract the non-apoptotic functions of CD95 signaling, which promote motility and invasiveness of cancer cells. CD95 may also be related to eosinophils, preventing their apoptosis. By contrast, CD95 may also inhibit CD95L-mediated tumor killing by T cells; and in some entities it correlates with high tumor load and worse prognosis.

Neutrophils are a known negative prognostic marker in cancer. Particularly, a high ratio of neutrophils to lymphocytes indicates a negative prognosis in several cancer entities. Here an additional negative predictive influence on the immunotherapy of cancer is suggested. Neutrophils may counteract immune reactions by inhibition of T and NK cells.

Short-chain acylcarnitines are increased in case of a reduced glomerular filtration rate (GFR), indicating greater damage to the kidney which may result in decreased access of blood cells to the tumor (Wanner 1988). On the other hand, acid soluble (free and short-chain) acylcarnitines were reported to be lower in cancer patients than in controls (Sachean 1987).

CA19-9 is an epitope on the sialylated Lewis A structure, a carbohydrate antigen on mucins such as MUC1. The presence in serum might correlate with MUC1 expression in tumor and,

thus, with the presence of one of the vaccine antigens. By contrast, serum tumor markers are usually negative prognostic markers.

ASAT/SGOT is an enzyme involved in amino acid metabolism. Like in the case of LDH, increased occurrence in serum serves as indicator of enhanced cell turnover, as it occurs in the case of damage of the liver and other organs, as well as in cancer. Our results confirm that high ASAT/SGOT and LDH are negative prognostic factors in cancer, but unexpectedly also show that they appear to be positively predictive for the outcome of immunotherapies. The reason is yet unclear. It may be speculated that cell death inside the tumor, especially in the form of necrosis, favors immune reactions at the tumor site.

In another aspect thereof, the method according to the present invention further comprises c) determining at least one marker selected from the group consisting of albumin and direct bilirubin in said sample from said cancer patient, wherein a higher level compared to the median (+/-10%) of a given cancer patient population is indicative for a beneficial effect of an immunotherapy for said patient, or d) determining the level of the marker interleukin-33 (IL-33) in said sample from said cancer patient, wherein a lower level compared to the median (+/-10%) of a given cancer patient population is indicative for a beneficial effect of an immunotherapy for said patient.

In this aspect of the present invention, the markers as indicated are used in order to further support the univariate markers as mentioned above in the first aspect of the present invention. The markers thus are used in order to create a “multivariate” marker set or panel for the diagnosis.

Particularly preferred is a method according to the present invention, wherein said multivariate marker set or panel consists of the markers CXCL13/BCA-1, ApoA1, neutrophils, eosinophils (in percent and absolute numbers), monocytes (in percent and absolute numbers), CD95/FAS, ASAT/SGOT, CCL17/TARC, LDH, Thr, IL-6, short-chain acylcarnitines, albumin, bilirubin, IgE, IL-33, MMP-3, and CA19-9.

For the purpose of the present invention the marker reference value is a threshold value for each marker that defines if a patient will benefit from an immune therapy. Depending if the marker is a negative or positive marker, an higher or lower level in the patient compared to

the reference value is indicative for a beneficial immune therapy. The reference value is in one embodiment +/-10% of the median concentration observed in a given cancer patient population. More preferred is that the reference value is, depending if it is a negative or positive value, the upper or lower quartile, the upper or lower quintile or the upper or lower dezile of a given cancer patient population. Most preferred is that the reference value of a negative marker, up to which patients are considered to profit from treatment, is the 70th percentile, the 80th percentile, and most preferred the 90th percentile. For positive markers it is most preferred that the reference value, starting from which patients are considered to profit from treatment, is the 30th percentile, the 20th percentile, and most preferred the 10th percentile.

Another important aspect of the present invention relates to a method according to the present invention, wherein said immunotherapy comprises a vaccination with an anti-cancer vaccine, optionally together with an adjuvant, such as, for example, GM-CSF.

Immunotherapy and respective vaccines are described in the state of the art; immunotherapy in cancer patients aims at activating cells of the immune system specifically, especially the so-called cytotoxic T-cells (CTL, also known as “killer cells”, also known as CD8+ T-cells), against tumor cells but not against healthy tissue. Tumor cells differ from healthy cells by the expression of tumor-associated and tumor-specific proteins. HLA-molecules on the cell surface present parts of the cellular content to the outside, thus enabling a CTL to differentiate between a healthy and a tumor cell. This is realized by breaking down all proteins inside the cell into short peptides, which are then attached to HLA-molecules and presented on the cell surface. Peptides that are presented on tumor cells, but not or to a far lesser extent on healthy cells of the body, are called tumor-associated peptides (TUMAPs). The antigens from which the epitopes recognized by tumor-specific T-cells are derived can be molecules from all protein classes, such as enzymes, receptors, transcription factors, etc.

However, priming of one kind of CTL is usually insufficient to eliminate all tumor cells. Tumors are very mutagenic and, thus, able to respond rapidly to CTL attacks by changing their protein pattern to evade recognition by CTLs. To counter-attack the tumor evasion mechanisms, a variety of specific peptides is used for vaccination. In this way a broad simultaneous attack can be mounted against the tumor by several CTL clones simultaneously. This may decrease the chances of the tumor to evade the immune response. This hypothesis

has been recently confirmed in a clinical study treating late-stage melanoma patients. With only few exceptions, patients that had at least three distinct T-cell responses, showed objective clinical responses or stable disease as well as increased survival, while the vast majority of patients with less than three T-cell responses were diagnosed with progressive disease (Banchereau et al., 2001).

The preferred treatment/composition as used in the context of the methods of the present invention is a peptide-based tumor vaccine. Other preferred treatments include DNA- or RNA-based vaccines, for example as described by Weide et al. (Weide B, Garbe C, Rammensee HG, Pascolo S. Immunol. Lett. 2008 Jan 15;115(1):33-42. Epub 2007 Oct 26) dendritic cell-based vaccines, vaccines using lysates of primary tumor cells or cell lines, or selective components of tumor cells including whole proteins or heat shock proteins. The medicament may be administered directly into the patient, i.e. into the affected organ or systemically *i.d., i.m., s.c., i.p., p.o.* and *i.v.*, or applied *ex vivo* to cells derived from the patient or a human cell line which are subsequently administered to the patient, or used *in vitro* to select a subpopulation of immune cells derived from the patient, which are then re-administered to the patient. The vaccine antigens, e.g. peptides, may be substantially pure, or combined with an immune-stimulating adjuvant (see below) or used in combination with immune-stimulatory cytokines, or be administered with a suitable delivery system, for example liposomes. The peptides may also be conjugated to a suitable carrier such as keyhole limpet haemocyanin (KLH) or mannan (see WO 95/18145 and Longenecker et al (1993)). The peptides may also be tagged, may be fusion proteins, or may be hybrid molecules. The peptides whose sequences are given in the present invention are expected to stimulate CD4+ or CD8+ T cells. However, stimulation of CD+ 8 CTLs is more efficient in the presence of help provided by CD4+ T-helper cells. Thus, for MHC class I epitopes that stimulate CD8+ CTL, the fusion partner or sections of a hybrid molecule suitably provide epitopes which stimulate CD4+ T cells. CD4+-stimulating epitopes are well known in the art and include those identified in tetanus toxoid. In a further preferred embodiment the peptide is a fusion protein, in particular comprising N-terminal amino acids of the HLA-DR antigen-associated invariant chain (Ii). In one embodiment the peptide of the invention is a truncated human protein or a fusion protein of a protein fragment and another polypeptide portion, provided that the human portion includes one or more amino acid sequences of the present invention.

For use, the vaccine may also include one or more adjuvants. Preferred adjuvants are imiquimod, resiquimod, GM-CSF, cyclophosphamide, sunitinib, bevacizumab, interferon-alpha, CpG oligonucleotides and derivates, poly-(I:C) and derivates, RNA, sildenafil, and particulate formulations with PLG or virosomes. As mentioned, the medicament is used for parenteral administration, such as subcutaneous, intradermal, intramuscular or oral administration. For this, the peptides and optionally other molecules are dissolved or suspended in a pharmaceutically acceptable, preferably aqueous carrier. In addition, the composition can contain excipients, such as buffers, binding agents, blasting agents, diluents, flavors, lubricants, etc. The peptides can also be administered together with immune stimulating substances, such as cytokines. An extensive listing of excipients that can be used in such a composition, can be, for example, taken from A. Kibbe, Handbook of Pharmaceutical Excipients, 3rd Ed. 2000, American Pharmaceutical Association and pharmaceutical press. The composition can be used for a prevention, prophylaxis and/or therapy of cancerous diseases, such as, for example, RCC and CRC. Exemplary peptide combinations for vaccines to be used in the context of the present invention are listed in the following tables 1A to 1D, and are herein designated as IMA901, IMA910, IMA941, and IMA950, respectively.

Table 1A IMA901 (e.g. used in RCC)

SEQ ID No:	Abbrev.	Protein	Sequence
1	MMP-001	Matrix metalloproteinase 7	SQDDIKGIQKLYGKRS
2	ADF-002	Adipophilin	VMAGDIYSV
3	ADF-001	Adipophilin	SVASTITGV
4	APO-001	Apolipoprotein L1	ALADGVQKV
5	CCN-001	Cyclin D1	LLGATCMFV
6	GUC-001	GUCY1A3	SVFAGVVGV
7	K67-001	KIAA0367	ALFDGDPHL
8	MET-001	c met proto oncogene	YVDPVITSI
9	MUC-001	MUC1	STAPPVHNV
10	RGS-001	RGS 5	LAALPHSCL

Table 1B IMA910 (e.g. used in colon cancer)

SEQ ID No:	Abbrev.	Sequence
11	C20-001	ALSNLEVTL
12	NOX-001	ILAPVILYI
13	ODC-001	ILDQKINEV
14	PCN-001	KLMDLDVEQL
15	TGFBI-001	ALFVRLLALA

16	TOP-001	KIFDEILVNA
17	TGFBI-004	TPPIDAHTRNLLRNH
18	CEA-006	SPQYSWRINGIPQQHT
5	CCN-001	LLGATCMFV
9	MUC-001	STAPPVHNV
1	MMP-001	SQDDIKGIQKLYGKRS
19	CEA-004	YLSGANLNL
8	MET-001	YVDPVITSI

Table 1C IMA941 (e.g. used in gastric cancer)

SEQ ID NO	Peptide ID	Sequence
20	CDC2-001	LYQILQGIVF
21	ASPM-002	SYNPLWLRI
22	UCHL5-001	NYLPFIMEL
23	MET-006	SYIDVLPEF
24	PROM1-001	SYIIDPLNL
25	UQCRB-001	YYNAAGFNKL
26	MST1R-001	NYLLYVSNF
27	PPAP2C-001	AYLVYTDRL
28	SMC4-001	HYKPTPLYF
29	MMP11-001	VWSDVTPLTF

Table 1D IMA950 (e.g. used in glioblastoma)

SEQ ID NO	Peptide ID	Sequence
30	CSP-001	TMLARLASA
31	FABP7-001	LTFGDVVAV
32	NLGN4X-001	NLDTLMTYV
33	TNC-001	AMTQLLAGV
34	NRCAM-001	GLWHHQTEV
35	IGF2BP3-001	KIQEILTQV
36	BCA-002	ALWAWPSEL
37	MET-005	TFSYVDPVITSISPKYG

Table 1E IMA990a (e.g. used in prostate cancer)

SEQ ID NO	Peptide ID	Sequence
38	PSA-001	FLTPKKLQCV
39	PSA-002	KLQCVDLHV
40	PSA-003	VISNDVCAQV*
41	PSCA-001	ALQPGTALL*
42	PSCA-002	AILALLPAL
43	PSMA-001	LLHETDSAV*
44	PSMA-002	ALFDIESKV
45	Survivin-004	ELTLGEFLKL

46	Survivin-005	TLPPAWQPFL
47	TRP-P8-001	GLMKYIGEV
48	PROSTEIN-001	CLAAGITYV
49	PSMA-001	NYTLRVDCTPLMYSL
50	Survivin-001	TLGEFLKLDRERAKN

* Optionally left out

Therefore, in yet another preferred aspect of the method according to the present invention, said anti-cancer vaccine is selected from an anti-cancer vaccine comprising at least one immunogenic peptides selected from the group of SEQ ID No. 1 to 37, for example comprising SEQ ID No. 1 to 10; SEQ ID No. 11 to 19 and 1, 5, 8, and 9; SEQ ID No. 20 to 29, and SEQ ID No. 30 to 37.

In another aspect of the method according to the present invention, said patient is treated or has been pre-treated. Such pre-treatments may comprise for example curative surgery, radiotherapy and/or chemotherapy. A preferred pre-treatment is a therapy with an anti-cancer agent selected from cytokines, and tyrosine kinase inhibitors (TKI), such as sorafenib and sunitinib, and cyclophosphamide. In this aspect, said cancer treatment can be selected from therapies as described above, preferred is an immunotherapy, preferably comprising the use of an anti-cancer vaccine, optionally together with GM-CSF.

The cancers to be treated can be all cancers which are responsive to immunotherapy, and are preferably selected from RCC, CRC, gastric cancer (GC), melanoma, non-small-cell lung cancer (NSCLC), glioblastoma, and generally adenocarcinoma of any type.

The samples that are analyzed in the context of the present invention can be selected from whole blood, peripheral blood or fractions thereof, serum, buffy coat, tumor tissue, lymphatic fluid, urine, bone marrow, EDTA plasma, heparinized plasma, citrate plasma, heparinized whole blood, and frozen heparinized whole blood. The choice of the sample to be analyzed will also depend on the marker(s) that are to be analyzed, and the person of skill will be able to choose a suitable sample accordingly.

In a preferred embodiment thereof, the method according to the present invention can further comprise a prognosis of an effect of a cancer immunotherapy in a patient, wherein said patient preferably has been pre-treated with cyclophosphamide (as described above). In the context of

the present invention, a "prognosis" or a "prognostic marker" shall be based on markers as mentioned herein that are informative for an effect based on a regular or common chemotherapeutic cancer treatment, such as, for example, using taxoles, platinum compounds, and other common agents used in the chemotherapy of cancer(s). The prognosed effect can be selected from overall survival, occurrence of single and/or multiple T-cell responses against the immunotherapy, retardation of tumor growth or progression-free survival. Thus, the markers of the present invention can be used in "mixed" scenarios of predictive and prognostic uses.

In another aspect of the present invention, the method according to the present invention further comprises a monitoring of the effect of said cancer treatment in said patient, comprising repeating the determining step a) and/or b), and optionally c) and/or d), as disclosed herein at least once. Usually, a monitoring is performed in regular intervals during the treatment, such as weekly, twice weekly, or even monthly.

Preferred is a method according to the present invention, wherein said determining comprises at least one method selected from immunoassays, bead-based immunoassays, multiplex immunoassays, ELISA, microarray based assays, epigenetic assays, expression analysis, FACS analysis, mass spectrometry, methods of clinical hematology, and other routine clinical assays like electrophoresis, immunonephelometry, immunoturbidimetry, enzymatic assays, colorimetric or fluorometric assays. All these methods are well known to the person of skill in the art, and described in the literature.

Yet another preferred aspect of the present invention then relates to a diagnostic kit, comprising materials for performing a method according to the present invention as described herein, in one or separate containers, preferably comprising (i) at least one marker-specific antibody specific for ApoA1, CCL17/TARC, Fas, ASAT/SGOT, CA19-9, LDH, IgE, matrix metalloproteinase 3 (MMP-3), CXCL13/BCA-1, neutrophils, interleukin-6 (IL-6), interleukin-33 (IL-33), albumin, and bilirubin, optionally together with (ii) instructions for performing said method.

The kit may further comprise one or more of (iii) a buffer, (iv) a diluent, (v) a filter, (vi) a needle, or (v) a syringe. The container is preferably a bottle, a vial, a syringe or test tube; and it may be a multi-use container. The container may be formed from a variety of materials such

as glass or plastic. Preferably the kit and/or container contain/s instructions on or associated with the container that indicates directions for reconstitution and/or use. For example, the label may indicate that the lyophilized formulation is to be reconstituted to certain antibody concentrations as suitable for the above methods, such as ELISA.

Yet another preferred aspect of the present invention then relates to an improved method for treating cancer in a cancer patient in need thereof, comprising a) performing a method according to the present invention as above, and b) administering a suitable anti-cancer immunotherapy to said cancer patient based on the results as obtained in step a), and c) optionally, repeating steps a) and b).

In these therapeutic aspects of the present invention, the methods according to the present invention are used in order to provide improved treatment options in the therapy, in particular immunotherapy, of cancers. The methods according to the invention provide additional and early predictive or predictive and prognostic information regarding the need and the effect of an immunological treatment of cancer, and thus allow for more informed decisions regarding the further treatment of said cancer. Thus, preferred is a method according as above, which further comprises a monitoring of the effect of said cancer treatment in said patient, comprising repeating said determining step at least once..

As described above, preferred treatments or pre-treatments in addition to the immunotherapeutic vaccines as described above are selected from an anti-cancer agent selected from cytokines, sorafenib, sunitinib, cyclophosphamide, and tyrosine kinase inhibitors (TKI). The cancers to be treated can be all cancers which are responsive to immunotherapy, and are preferably selected from renal cell carcinoma (RCC), colorectal cancer (CRC), gastric cancer (GC), melanoma, non-small-cell lung cancer (NSCLC), glioblastoma, and adenocarcinoma.

It should be understood that the features of the invention as disclosed and described herein can be used not only in the respective combination as indicated but also in a singular fashion without departing from the intended scope of the present invention.

The invention will now be described in more detail in the examples with reference to the sequence listing. The following examples are provided for illustrative purposes only and are not intended to limit the invention.

In the Figures:

Figure 1 shows a Kaplan-Meier analysis for the effect of (A) ApoA1, (B) CXCL13/BCA-1, (C) monocytes and (D) CCL17/TARC on overall survival in all patients and subgroups. For each parameter, the upper figure shows the survival of all patients having high (red lines) vs. low (green lines) values of the parameter. The lower figure shows a subgroup analysis (+CY vs –CY) for patients with high values of the parameter (red vs blue lines), and for patients with low values of the parameter (green vs yellow lines).

Figure 2A shows the distribution of values of the univariate biomarkers ApoA1, CXCL13/BCA-1, monocytes and CCL17/TARC, as indicated in the figure, of the –CY (gray) and +CY (black) group, showing either no T-cell response (no), single peptide response (1) or multipeptide response (>1). Error bars represent the standard error of the mean. **Figure 2B** shows a different way of depicting the distribution of values of the multivariate biomarker in patients of the –CY (gray) and +CY (black) group, showing either no T-cell response (no), single peptide response (1) or multipeptide response (>1). Dots represent single values, lines represent the mean.

Figure 3 shows the Kaplan Meier analysis of the effect of a combination of ApoA1 and CCL17/TARC on overall survival in all patients and subgroups. In A), the biomarker-positive population was defined to consist of patients with at least one of the two parameters within the positive range (score = 1 or 2), while biomarker-negative patients showed no parameter within the positive range (score = 0). In B), the biomarker-positive population was defined to consist only of those patients showing both parameters within the positive range (score = 2), while patients with at least one parameter in the negative range (score = 0 or 1) were considered biomarker-negative. The upper figures show the survival of all biomarker-positive patients (green lines) vs all biomarker-negative patients (red lines). The lower figures show subgroup analyses (+CY vs –CY) for biomarker-positive patients (green lines vs yellow lines) and biomarker-negative patients (red lines vs blue lines).

Figure 4A shows the mean of the marker consisting of the combination of ApoA1 and CCL17/TARC in patients of the –CY (gray) and +CY (black) group, showing either no T-cell response (no), single peptide response (1) or multipeptide response (>1). Error bars represent the standard error of the mean. **Figure 4B** shows a different way of depicting the distribution of values of the binary biomarker in patients of the –CY (gray) and +CY (black) group, showing either no T-cell response (no), single peptide response (1) or multipeptide response (>1). Dots represent single values, lines represent the mean.

Figure 5 shows a Kaplan-Meier analysis for the effect of the multivariate biomarker on overall survival. The upper figure shows the survival of all patients having high (red lines) vs. low (green lines) values of the parameter. The lower figure shows a subgroup analysis (+CY vs –CY) for patients with high values of the parameter (red vs blue lines), and for patients with low values of the parameter (green vs yellow lines). The value of 0.019076043 points was used as cut-off separating biomarker high and low patients.

Figure 6A shows the mean values of the multivariate biomarker in patients of the –CY (gray) and +CY (black) group, showing either no T-cell response (no), single peptide response (1) or multipeptide response (>1). Error bars represent the standard error of the mean. **Figure 6B** shows a different way of depicting the distribution of values of the multivariate biomarker in patients of the –CY (gray) and +CY (black) group, showing either no T-cell response (no), single peptide response (1) or multipeptide response (>1). Dots represent single values, lines represent the mean.

SEQ ID No. 1 to 50 show the amino acid sequences of the peptides as listed above in table 1A to 1E.

Table 2

Univariate biomarkers	**HR and logrank p-values**			
	ApoA1	**CXCL13/BCA-1**	**Monocytes**	**CCL17/TARC**
All patients, Biomarker-positive vs -negative	0.42 (0.01)	0.39 (0.006)	0.44 (0.012)	0.41 (0.011)
+CY group, Biomarker-positive vs -negative	0.3 (0.017)	0.25 (0.004)	0.24 (0.001)	0.18 (0.003)

-CY group, Biomarker-positive vs -negative	0.6 (0.253)	0.7 (0.448)	0.93 (0.874)	0.67 (0.392)
Biomarker-positive group, +CY vs –CY arm	0.36 (0.061)	0.35 (0.051)	0.29 (0.014)	0.22 (0.017)
Biomarker-negative group, +CY vs –CY arm	0.84 (0.696)	1.07 (0.876)	1.31 (0.538)	0.85 (0.715)
Interaction	0.43 (0.24)	0.3271 (0.13)	0.22 (0.03)	0.26 (0.094)
Parameter is favourable if values are high/low	high	low	high	high
Cutoff	0.264 mg/ml (MEDIAN)	64.21 pg/ml (MEDIAN)	7.7% (MEDIAN)	161.99 pg/ml (MEDIAN)

Table 2 shows hazard ratios (HR) and logrank p-values (Cox proportional hazard analysis) for prediction of overall survival by the univariate biomarkers in all patients and +/-CY subgroups, HR and logrank p-values for the effect of cyclophosphamide pre-treatment within biomarker-positive and biomarker-negative patients, and for the interaction effect of the biomarkers with cyclophosphamide pre-treatment.

Table 3

p-values (Welch's t-test)	**ApoA1**	**CXCL13/BCA-1**	**Monocytes**	**CCL17/TARC**
All patients, responders	0.016	0.28	0.24	0.032
All patients, multipeptide responders	0.000074	0.031	0.34	0.0028
+CY group, responders	0.014	0.064	0.0021	0.013
+CY group, multipeptide responders	0.42	0.29	0.37	0.53
-CY group, responders	0.034	0.15	0.065	0.017
-CY group, multipeptide responders	0.00014	0.13	0.73	0.068
Parameter is favorable if values are high/low	high	low	high	high

Table 3 shows p-values calculated by univariate statistical analysis (Welch's t test) for the prediction of T cell responses and multipeptide T cell responses by the selected parameters for all patients and +/-CY subgroups.

Table 4

Multivariate biomarker (OSCY_CHEAP_UNIF_20)	HR (95% CI) and logrank p-values
All patients, Biomarker-positive vs -negative	0.47; P=0.041
+CY group, Biomarker-positive vs -negative	0.07; P=0.000008
-CY group, Biomarker-positive vs -negative	1.58; P=0.203
Biomarker-positive group, +CY vs –CY arm	0.08 (0.02-0.38); P=0.000135
Biomarker-negative group, +CY vs –CY arm	2.5 (0.95-6.58); P=0.055
Interaction	0.032 (0.0055-0.1863); P=0.000128
Parameter is favourable if values are high/low	High
Cutoff	0.019076043 points

Table 4 shows hazard ratios (HR) and logrank p-values for prediction of overall survival by the multivariate biomarker in all patients and +/-CY subgroups, HR and logrank p-values for the effect of cyclophosphamide pre-treatment within biomarker-positive and biomarker-negative patients, and for the interaction effect of the biomarker with cyclophosphamide pre-treatment.

Examples

1.Introduction

IMA901 is a peptide-based vaccine designed to induce specific T cell reactions against peptide-MHC complexes found on RCC. The IMA901-202 (phase II) study used univariate and multivariate analyses to identify parameters which alone or in combination serve as predictive biomarkers for the success in terms of immune response induction and prolongation of overall survival of IMA901 treatment of metastatic RCC patients.

To achieve this, around 450 parameters (patient- or tumor-related parameters as well as analytes measured in serum or urine and cellular parameters) were analyzed using pre-treatment samples of the IMA901-202 (phase II) study population. For multivariate analyses,

which selected a set of several parameters, those parameters that required preparation of peripheral blood mononuclear cells (PBMCs) were not included in order to limit measurement costs in future trials. Parameters were analyzed for their association with overall survival and T-cell responses.

Since IMA901 treatment showed much greater effects on overall survival after cyclophosphamide pre-treatment, and since only in the +CY arm the presence of immune responses was associated with improved overall survival, the +CY arm was considered the active arm of the study and compared to the –Cy arm as control. Thus, parameters were chosen that showed interaction with cyclophosphamide pre-treatment and/or that predicted overall survival better in the +CY arm than in the –CY arm. Thereby, the selection of purely prognostic markers, which by definition would show association with overall survival in both arms, was avoided.

2. Comparison of study patients with age-matched healthy donors

For comparison of pre-treatment levels with healthy donors, only intention-to-treat (ITT) patients below the age of 70 were selected to match the oldest healthy control donors. The resulting groups of patients (N=52) and healthy donors (N=22) were balanced with respect to age, gender and CMV (cytomegalovirus) seropositivity.

3. Materials and Methods

Sample collection

All samples were collected before any therapeutic study intervention, either three days before vaccination and immediately before administration of cyclophosphamide in the +CY arm, or immediately before vaccination. Serum samples were taken using a serum /gel-VACUTAINER (Becton Dickinson, 5 ml), inverted and incubated for a minimum of 30 minutes. Tubes were centrifuged for 15 minutes at at least 1200 x g, and serum (approximately 2 ml) was transferred into a NUNC cryotube (3.6 ml). Cryotubes were stored immediately at $\leq$ -20°C until measurement. EDTA blood for analysis of haematology parameters was collected in 3 ml EDTA-VACUTAINERs, which were not centrifuged, and stored at ambient temperature until analysis. Urine was collected shortly before vaccination. For dipstick examination, only fresh urine was used. Citrated plasma for analysis of coagulation was collected approximately two weeks before vaccination. Samples were taken

using a citrate-VACUTAINER and centrifuged within 30 minutes at at least 1200 x g at ambient temperature. Supernatant plasma was transferred into microtubes.

Measurements

Biomarker measurement methods included ELISA, multiplex immunoassays, epigenetic assays, mass spectrometry, FACS analyses, routine clinical methods of haematology, clinical chemistry and urine analysis, and other methods. The parameters that were finally selected were measured by multiplex assays provided by RBM (ApoA1, CD95/FAS, IL-6, IgE, MMP-3, CA19-9), Millipore (CXCL13/BCA-1, CCL17/TARC), IL-33), and by mass spectrometry provided by Biocrates (threonine, short-chain acylcarnitines). Further parameters were provided by the central laboratory and were measured by routine methods of haematology (neutrophils, eosinophils, monocytes) and clinical chemistry (ASAT/SGOT, LDH, albumin, bilirubin).

Assessment of T-cell responses.

As besides overall survival the occurrence of one or multiple T-cell responses had to be tested for association with biomarkers, specific T-cell responses were measured before and after several times after vaccination, using ELISPOT assays and tetramer stainings with the respective peptide-MHC complexes.

Univariate statistical Analysis:

The association of parameters with T-cell responses was assessed using Welch's t test. Parameters predictive for T cell responses in all patients and/or within the +CY group with $p < 0.05$ were considered significant.

The interaction of parameters with CY-pre-treatment was assessed using the Cox proportional hazards model. Candidates showing an interaction p value of < 0.05 at any cutoff value that was not at the extreme ends of the distribution (i.e. selecting or de-selecting > ~5% of patients) were considered as interesting.

Moreover, to exclude parameters with significant interaction due to inverse association of cyclophosphamide pre-treatment with overall survival in the unfavorable biomarker group, an additional criterion was significant association ($p < 0.05$) with overall survival in the positive biomarker group. A significant ($p < 0.05$) association with overall survival in the +CY group,

but not in the –CY group was considered to be an additional hint for the predictive instead of prognostic quality of a parameter.

Further criteria for parameter selection were based on their biology and on the persistence of their pattern of interaction with cyclophosphamide pre-treatment and association with overall survival in the +CY but not in the –CY arm in subgroups of patients, such as TKI-pre-treated and cytokine-pre-treated patients.

Correlations between the parameters were calculated. Parameters that were highly correlated with others in the remaining set (adjusted p-value < 0.005) were excluded.

After identifying single parameters using these steps, it was tested whether and which combinations of these parameters showed best performance with respect to prediction of overall survival and T-cell responses, and for stability in the subgroups of patients indicated above.

Multivariate statistical analyses:

Multivariate biomarkers were determined according to a Cox proportional hazard model, whose properties and extensions are described in the following.

As covariates in the generalized linear model, all parameters were considered, though linearly transformed to zero mean and unit variance ("standardized" parameters) in order to avoid undue influence for parameters that are represented in small units. Only parameters that did not require PBMC isolation were included, to limit measurement costs for the set. As the corresponding maximum partial likelihood problem was under-determined, i.e. the number of parameters exceeded the number of patients; the model was augmented with a Gaussian prior, which limits the relative influence of each parameter in the optimal biomarker. The corresponding maximum a-posteriori (MAP) optimization is also known to improve the predictive performance of the results ("regularization") over the maximum likelihood approach. The model was further augmented by a Laplacian prior, which causes the optimization to use only a subset of the considered parameters. By this means, the number of parameters included in the biomarker was limited to 20.

Further it was desired that the multivariate biomarker be not predictive in the -CY arm, or at least be less predictive for those patients than for the +CY arm. In order to achieve this, repeatedly predictive models were optimized on the -CY arm, and the resultant directions projected out from the parameter space, until only essentially unpredictive directions remained. The biomarker itself was then determined on the remaining subspace of the parameters on the +CY arm. In order to assess the predictive accuracy of the biomarker, the entire optimization process was applied in a leave-one-out fashion. The predictions for all patients were computed in the way, collected, and then compared to the known survival times.

Results: Univariate Analysis

Selection of parameters

Of the around 450 parameters tested, 59 significantly predicted the occurrence of T-cell responses either on all patients and/or in the +CY group of patients. 118 parameters showed significant interaction with cyclophosphamide pre-treatment at their optimal cutoff values. Using the above-mentioned criteria of T-cell response prediction, interaction with cyclophosphamide pre-treatment, better association with overall survival in the +CY than in the –CY group, non-redundancy with other parameters, and presence of a reasonable biological rationale, four parameters (ApoA1, CXCL13/BCA-1, monocytes and CCL17/TARC) could be identified as preferred univariate biomarker candidates.

Definition of cutoffs

For each of the four parameters identified as described above, a discrete cutoff was defined, separating patients into two groups (positive or negative for the biomarker) which showed a significantly different outcome of IMA901 treatment. The selection of cutoff values was required, as the parameters, although associated with overall survival and T-cell responses in a continuous rather than discrete fashion, should be used for yes/no treatment decisions. For that reason, appropriate cutoffs should a) include sufficient patients and should b) be objective.

In case of ApoA1, for which more than 50% of IMA901-202 patients showed pathologically reduced levels, the cutoff was chosen to be the LLN determined in healthy donors as indicated by the assay provider (RBM, 0.288 mg/ml), identifying the patients with ApoA1 levels within the normal range as biomarker-positive group. This cutoff value was close to the median of the IMA901-202 study patients (0.264 mg/ml). Thus, this cutoff is maximally objective and

associated with the biological rationale for ApoA1. Alternatively, for ApoA1 also the median was used. The other parameters were largely within normal ranges in IMA901-202 patients. For these parameters, the median of the study population was chosen as cutoff, as this is also objective and includes sufficient (50%) patients.

Prediction of overall survival and T-cell responses by single univariate biomarkers

Cox proportional hazard analyses showed that all of the four parameters selected by univariate analyses significantly predict overall survival in all patients and in the subgroup of patients pre-treated with cyclophosphamide, but not in the subgroup that did not receive cyclophosphamide pre-treatment (**Table 2, Figure 1**). In the biomarker-positive groups, as defined by the respective cutoff of the biomarker value, cyclophosphamide pre-treatment showed a significantly ($p < 0.05$) positive effect on overall survival (CCL17/TARC, monocytes) or a trend ($p < 0.1$) towards a positive effect on overall survival (ApoA1, CXCL13/BCA-1). Interaction analysis showed that the effect of cyclophosphamide-pretreatment was significantly different ($p < 0.05$) in patients classified as positive or negative for their monocyte levels. CCL17/TARC showed a trend for interaction with cyclophosphamide pre-treatment. ApoA1 and CXCL13/BCA-1 did not reach significance at the chosen cutoffs.

Notably, in the +CY group, the best hazard ratio (HR) could be achieved if patients were classified by their CCL17/TARC levels (**Table 2**, HR=0.18, p=0.003). Vice versa, CCL17/TARC-positive patients profited most from cyclophosphamide pre-treatment (**Table 2**, HR=0.22, p=0.017).

CCL17/TARC was also exceptional in consideration of the fact that 75% of CCL17/TARC-positive cyclophosphamide-pre-treated patients were still alive at the end of the study (Figure 1D).

The pattern observed with respect to overall survival prediction remained stable for the subpopulations of patients that had been pre-treated with TKI or cytokines (data not shown).

With respect to T cell response prediction, CCL17/TARC and ApoA1 performed best, both significantly predicting responders in all patients and both +CY and –CY subgroups, and

predicting multipeptide responders in all patients. By contrast, CXCL13/BCA-1 and monocytes were weak response predictors (**Table 3**).

5. Results: Multivariate Analysis

Selection of parameters

A 20-parameter biomarker set predicting overall survival in the +CY group of patients better than in the –CY group upon treatment with IMA901 was calculated for the total patient population. Parameters included in this set are listed in Table 4. They include cytokines, chemokines and other proteins measurable in serum, cellular parameters measurable by standard hematology assays, and metabolomic parameters measurable by mass spectrometry. Besides the optimization for all patients (train version), the biomarker set was calculated using leave-one-out cross-validation (test version). The latter analysis is more relevant, as it grants robustness of the results in follow-up studies. Data shown in these experiments represent the test results.

Table 5

Weight	Rank	Parameter name
-47.3867	1	CXCL13/BCA-1
38.38821	2	ApoA1
-38.1861	3	Neutrophils (%)
34.36199	4	Eosinophils (%)
34.10915	5	Monocytes (%)
33.89069	6	CD95/FAS
32.72272	7	ASAT/SGOT
32.01683	8	CCL17/TARC
31.7325	9	LDH
31.47377	10	Thr
-31.3839	11	IL-6
-31.2785	12	Short-chain acylcarnitines
30.83843	13	Albumin
30.78772	14	Eosinophils (abs.)
29.57142	15	Monocytes (abs.)
28.89882	16	Bilirubin (direct)
28.63935	17	IgE
-27.8701	18	IL-33
27.72058	19	MMP-3
27.204	20	CA19-9

Table 5 shows the parameters included in the 20-parameter multivariate biomarker, and the weights and ranks indicating their relative importance in the set.

The biomarker value for each patient is a linear combination of the values measured for the 20 parameters included. The constant factors in this equation were chosen in a way that the median of the biomarker distribution was close to zero. The biomarker values calculated in this way were approximately normally distributed in the population of IMA901-202 patients, and they were equally distributed between patients of the +CY and –CY arm.

Prediction of overall survival and T-cell responses by the multivariate parameter set

To demonstrate the predictive power of the biomarker, a survival analysis was performed by subdividing patients in two groups according to their biomarker value. The median of the density distribution of the biomarker over the whole patient population was chosen as cutoff, including 50% of patients with values above this cutoff in the biomarker-positive group. In the +CY group of patients, biomarker-positive patients profited highly significantly from vaccination, while the biomarker showed no effect in the –CY arm. Vice versa, in the biomarker-positive group, cyclophosphamide pre-treatment resulted in significantly higher overall survival, while the effect was not significant or even (tendentially) inverse in the biomarker-negative group (**Table 4; Figure 5**). Correspondingly, there was a highly significant interaction between the multivariate biomarker and cyclophosphamide pre-treatment.

Although the biomarker had not been optimized to predict immune responses, the inventors tested whether the distribution of biomarker values was associated with the presence or absence of T-cell responses as well. There was in fact a tendency towards higher biomarker values in responder- and multipeptide-responder groups, particularly in the +CY group of patients (**Figure 6**).

Prediction of overall survival and T-cell responses by a combination of parameters

Single parameters can be combined in a way that patients can be assigned a biomarker score, depending on the values of all the parameters included in the combination. In case of a two-parameter combination, each parameter could be either positive or negative according to its cutoff, and patients could have a biomarker score of two (both parameters positive), one (one parameter positive) or zero (no parameter positive) with respect to the combined marker.

Combining several parameters to calculate a biomarker score may be favorable for several reasons. First, it leads to greater robustness, as a) the problem of fixed cutoffs in spite of a

continuous association of parameters with outcome is overcome to some extent, b) the impact of measurement outliers is reduced, and c) a greater number of parameters enables insight into a larger number of biological processes that may be relevant for anti-cancer immunity. Second, a score model enables more flexibility with respect to patient selection: While a single parameter with the median as objective cutoff would necessarily select only 50% of patients for treatment, a two-parameter score (with cutoff of the single parameters at the median of the distribution) divides patients in three groups. If only patients without positive markers (i.e. with a biomarker score of zero) are excluded, about 75% of patients would be included in the treatment group. However, if no significance is reached in this group, the patients with scores of zero and one can be excluded, leaving the about 25% patients showing a biomarker score of two in the treatment group.

For these reasons, it was tested whether a combination of the single parameters identified above leads to better performance with respect to prediction of overall survival and T-cell responses. Moreover, the stability of the effect in the patient subpopulations indicated above was assessed. Several combinations of markers outperformed the use of single parameters in these respects, the best of which was a combination of ApoA1 and CCL17/TARC (**Figure 3, Figure 4**).

6. Marker Levels:

6.1 Serum Levels of ApoA1

Condition	**Source**	**ApoA1 [mg/ml]; Range or Average +/-STD**
Healthy controls	(Riesen, 2008)	1.0–1.5
	Manufacturer (RBM)	0.288–1.17
IMA901-202 study	Range (median)	0.0808–0.514 (0.264)
	Average +/- STD	0.27 +/- 0.09

6.2 Serum Levels of CCL17/TARC

Condition	**Source**	**TARC [pg/ml]; Range or Average +/-STD**
Healthy controls	(Shimada et al., 2004)	200 +/- 100
	(Fujii et al., 2004)	101 +/- 81
	(Sugawara et al., 2002)	31.9 +/- 14.8
	(Sekiya et al., 2002)	88.8 +/- 58.2
	(Echigo et al., 2006)	93.3 +/- 25.3
	(Saeki and Tamaki, 2006)	215.34 +/-26.79
Atopic dermatitis	(Leung et al., 2003)	1125-3070 (1469)

	(Shimada et al., 2004)	96400	+/- 38100
	(Sugawara et al., 2002)	325	+/-287
	(Saeki and Tamaki, 2006)	2338.7	+/-301.83
Asthma	(Sugawara et al., 2002)	271	+/- 264
	(Sekiya et al., 2002)	192.0	+/- 143.6
Allergic rhinitis	(Sugawara et al., 2002)	147	+/- 101
Systemic sclerosis	(Fujii et al., 2004)	313	+/- 294
Dermatomyositis		262	+/- 291
SLE		254	+/- 326
IMA901-202 study	Range (median)	25.5–623	(162)
	Average +/- STD	174	+/- 118

6.3 Serum levels of BCA-1

Condition	**Source**	**BCA-1 [pg/ml]; Range or Average +/-STD**
Healthy controls	(Panse et al., 2008)	~40
	(Sansonno et al., 2008)	48.2 +/-11.0
Disease	(Panse et al., 2008), breast cancer	~80
	(Sansonno et al., 2008), HCV-infected patients with and without mixed cryo-globulinemia	273.6 +/-98; 113.9 +/-40.2
IMA901-202 study	Range (median)	13.09–1429.6 (64.21)
	Average +/- STD	114.23 +/- 196.98

6.4 Levels of Neutrophils in percent and absolute numbers

Condition	**Source**	**Neutrophils; Range or Average +/-STD**
Healthy controls	LKF Kiel Laboratory, Germany	34–71 %; 1.5–6.2 x 10^9/L
Disease	(Rashid et al., 2010), oesophageal cancer	58–71 %
	(Donskov et al., 2006), RCC	2.6–15.9 x 10^9/L
IMA901-202 study	Range (median)	47–86.9 (67.3) %; 2.18–10.39 (4.44) x 10^9/L
	Average +/- STD	68.05 +/-8.2 %; 4.88 +/-1.86 x 10^9/L

6.5 Levels of Eosinophils in percent and absolute numbers

Condition	Source	Eosinophils; Range or Average +/-STD
Healthy controls	LKF Kiel Laboratory, Germany	0–7 %; 0.04–0.6 x 10^9/L
	(Simon and Simon, 2007)	0–0.4 x 10^9/L
Disease	(Moroni et al., 2000), RCC	0.15 +/-0.1 x 10^9/L
IMA901-202 study	Range (median)	0.2–6.5 (2) %; 0.02–0.55 (0.12) x 10^9/L
	Average +/-STD	2.35 +/-1.62 %; 0.16 +/-0.13 x 10^9/L

6.6 Levels of Monocytes in percent and absolute numbers

Condition	Source	Monocytes; Range or Average +/-STD
Healthy controls	LKF Kiel Laboratory, Germany	4–13 %; 0.2-0.9 x 10^9/L
Disease	(Sasaki et al., 2006), hepatocellular carcinoma	0.03–1.04 x 10^9/L
	(Donskov et al., 2006), RCC	0.69–0.74 x 10^9/L
IMA901-202 study	Range (median)	3.1–17.7 (7.6) %; 0.2–1.31 (0.48) x 10^9/L
	Average +/-STD	8.19 +/-3.15 %; 0.57 +/-0.25 x 10^9/L

6.7 Serum levels of soluble CD95/FAS

Condition	Source	CD95/FAS [ng/ml]; Range or Average +/-STD
IMA901-202 study	Range (median)	4.09–20.4 (9.89)
	Average +/-STD	9.76 +/-3.06

6.8 Serum levels of ASAT/SGOT

Condition	Source	ASAT/SGOT [U/L]; Range or Average +/-STD
Healthy controls	LKF Kiel	12–35.4
IMA901-202 study	Range (median)	14–109 (23.5)
	Average +/- STD	27.9 +/-17.3

6.9 Serum levels of LDH

Condition	Source	LDH [U/L]; Range or Average +/-STD
Healthy controls	LKF Kiel	129–230
IMA901-202 study	Range (median)	94–734 (161.5)
	Average +/- STD	190.63 +/-98.97

6.10 Serum levels of Threonine

Thr Min/Max: 21 – 157 µM

Thr AVG/STD: 84.5 +/- 24.1 µM
MEDIAN: 82 µM

6.11 Serum levels of albumin
Normal serum ranges and deviations under pathological conditions:
Laboratory: Albumin 35-50 g/l (3.5-5 g/dl)

parameter	AVG(l.val_float)	unit	STD(l.val_float)	MIN(l.val_float)	MAX(l.val_float)	MEDIAN(l.val_float)
albumin_blood	39.95	G/L	4.39	26	48	41

6.12 Levels of direct bilirubin
Laboratory: 0 – 5.1 µM

Serum range in IMA-901 study:
Min/Max: 0.5 – 4.4 µM (21 distinct values)
AVG/STD: 2.0 – 0.89 µM
MEDIAN: 1.7 µM

6.13 Levels of MMP-3
Range in IMA-901 study:
Min/Max: 1.85 – 41.2 ng/ml (only 3 were above 17; 58 discrete values)
AVG/STD: 8.5 +/- 6.0 ng/ml
MEDIAN: 6.7 ng/ml
Normal serum ranges: Range according to manufacturer: 0.2 – 2.17 ng/ml

6.14 Levels of CA19-9

High Serum Range (provider)	parameter	AVG	unit	STD	MIN	MAX	MED	Count
52	Cancer_Antigen_19-9	15.63	U/ml	29.73	0	167	5.94	45

6.15 Levels of IgE

parameter	AVG(l.val_float)	unit	STD(l.val_float)	MIN(l.val_float)	MAX(l.val_float)	MEDIAN(l.val_float)
IgE	104.09	ng/ml	261.00	0	1362	22.5

Provider (RBM): normal up to 606 ng/ml IgE

6.16 Levels of IL-6

parameter	AVG(l.val_float)	unit	STD(l.val_float)	MIN(l.val_float)	MAX(l.val_float)	MEDIAN(l.val_float)
IL-6	16.32	pg/ml	67.65	0	532	2.86

Provider (RBM): normal up to 42.6 pg/ml IL-6

6.17 Levels of IL-33

parameter	AVG(l.val_float)	unit	STD(l.val_float)	MIN(l.val_float)	MAX(l.val_float)	MEDIAN(l.val_float)
IL-33	166.95	pg/ml	293.28	55.0672	1867	75.73

n.a. normal ranges

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Claims

1. A method for predicting an effect of an immunotherapy in a cancer patient, comprising
a) determining the level of at least one marker selected from the group consisting of Apolipoprotein A1 (ApoA1), CCL17/TARC, eosinophils, monocytes, CD95/Fas, aspartate aminotransferase/serum glutamic oxaloaceticacid transaminase (ASAT/SGOT), cancer antigen 19-9 (CA19-9), lactate dehydrogenase (LDH), threonine, immunoglobulin E (IgE), and matrix metalloproteinase 3 (MMP-3) in a sample from said cancer patient, wherein an increase or higher level compared to the median of a given cancer patient population is indicative for a beneficial effect of an immunotherapy for said patient,
and/or
b) determining the level of at least one marker selected from the group consisting of B-cell attracting chemokine (CXCL13/BCA-1), neutrophils, interleukin-6 (IL-6), and short-chain acylcarnitines in a sample from said cancer patient, wherein a decrease or lower level compared to the median of a given cancer patient population is indicative for a beneficial effect of an immunotherapy for said patient.

2. The method according to claim 1, wherein said marker is selected from ApoA1 and/or CCL17/TARC; CXCL13/BCA-1, neutrophils%, ApoA1, eosinophils%, eosinophils ABS (absolute), monocytes%, FAS, TARC; LDH, Thr, IL-6, albumin, IgE, MMP-3, CA19-9; monocytes ABS, ASAT, bilirubin, acylcarnitines (scAC); and IL-33.

3. The method according to claim 1 or 2, further comprising
c) determining at least one marker selected from the group consisting of albumin, and direct bilirubin in said sample from said cancer patient, wherein an increase or higher level compared to the median of a given cancer patient population is indicative for a beneficial effect of an immunotherapy for said patient,
and/or
d) determining the level of the marker interleukin-33 (IL-33) in said sample from said cancer patient, wherein an decrease or lower level compared to the median of a given cancer patient population is indicative for a beneficial effect of an immunotherapy for said patient.

4. The method according to any of claims 1 to 3, wherein said immunotherapy comprises a vaccination with an anti-cancer vaccine, optionally together with an adjuvant, preferably GM-CSF.

5. The method according to any of claims 1 to claim 4, wherein said patient is treated or has been pre-treated with therapies including surgery, radiotherapy and/or chemotherapy, and preferably therapies wherein said patient is treated or has been pre-treated with an anti-cancer agent selected from cytokines, and tyrosine kinase inhibitors (TKI), such as sorafenib and sunitinib, and cyclophosphamide.

6. The method according to any of claims 1 to 5, wherein said cancer is selected from renal cell carcinoma (RCC), colorectal cancer (CRC), gastric cancer (GC), melanoma, non-small-cell lung cancer (NSCLC), glioblastoma, and adenocarcinomas of any type.

7. The method according to any of claims 1 to 6, wherein said sample is selected from whole blood, peripheral blood, or fractions thereof, serum, buffy coat, tumor tissue, lymphatic fluid, urine, bone marrow, EDTA plasma, heparinized plasma, citrate plasma, heparinized whole blood, and frozen samples thereof, such as frozen heparinized whole blood.

8. The method according to any of claims 1 to 7, further comprising prognosing an effect of said immunotherapy in said patient.

9. The method according to any of claims 1 to 8, wherein said effect is selected from overall survival, occurrence of single and/or multiple T-cell responses against the immunotherapy, retardation of tumor growth, tumor shrinkage or progression-free survival.

10. The method according to any of claims 1 to 9, further comprising a monitoring of the effect of said cancer treatment in said patient, comprising repeating said determining step at least once.

11. The method according to any of claims 1 to 10, wherein said determining comprises at least one method selected from immunoassays, bead-based immunoassays, multiplex immunoassays, ELISA, microarray based assays, epigenetic assays, expression analysis, FACS analysis, established methods of hematology, proteomics, and mass spectrometry.

12. The method according to any of claims 4 to 11, wherein said anti-cancer vaccine is selected from an anti-cancer vaccine comprising at least one immunogenic peptides selected from the group of SEQ ID No. 1 to 37, for example comprising SEQ ID No. 1 to 10; SEQ ID No. 11 to 19 and 1, 5, 8, and 9; SEQ ID No. 20 to 29, and SEQ ID No. 30 to 37.

13. A diagnostic kit comprising materials for performing a method according to any of claims 1 to 12 in one or separate containers, preferably comprising at least one marker-specific antibody, optionally together with instructions for performing said method.

14. An improved method for treating cancer in a cancer patient in need thereof, comprising
a) performing a method according to any of claims 1 to 3 and 5 to 11, and
b) using the results for treatment decisions, particularly administering a suitable anti-cancer immunotherapy to said cancer patient based on the results as obtained in step a), and
c) optionally, repeating steps a) and b).

15. The method according to claim 14, wherein said cancer is selected from renal cell carcinoma (RCC), colorectal cancer (CRC), gastric cancer (GC), melanoma, non-small-cell lung cancer (NSCLC), glioblastoma, and adenocarcinoma of any type.