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(54) **Title:** METHODS FOR PREDICTING THE SURVIVAL TIME OF SUBJECTS SUFFERING FROM MELANOMA METASTASES

(57) **Abstract:** The present invention relates to methods for predicting the survival time of subjects suffering from melanoma metastases. In particular, the present invention relates to a method for predicting the survival time of a subject suffering from melanoma metastases comprising i) determining the expression level of HVEM in a metastasis tissue sample ii) comparing the expression level determined at step i) with a predetermined reference value and iii) concluding that the subject will have a short survival time when the expression level determined at step i) is higher than the predetermined reference value or concluding that the subject will have a long survival time when the expression level determined at step i) is lower than the predetermined reference value.



METHODS FOR PREDICTING THE SURVIVAL TIME OF SUBJECTS SUFFERING FROM MELANOMA METASTASES

5 **FIELD OF THE INVENTION:**

The present invention relates to methods for predicting the survival time of subjects suffering from melanoma metastases.

BACKGROUND OF THE INVENTION:

10 T lymphocytes play a central role in anti-tumor responses. Their T cell antigen receptor (TCR) specifically recognizes antigen on tumor cells and elicits tumor destruction. T cell responses are tuned by signals that result from the engagement of several other surface receptors that convey positive (co-stimulatory) or negative (co-inhibitory) signals. Co-inhibitory molecules such as PD-1 and CTLA-4 dampen TCR activity and their blockade by
15 monoclonal antibodies reinvigorates the activity of the anti-tumor effectors T cells present in tumor infiltrates. Accordingly, metastatic melanoma was one of the poorest prognosis tumors at metastatic stage until the arrival of immunotherapies based on anti-CTLA-4 and anti-PD-1 antibodies that drastically improved progression free survival and overall survival of patients (1–3). However, T cells co-expressing PD-1 together with other co-inhibitory molecules may
20 be more profoundly hyporesponsive than those expressing PD-1 alone, accounting for those patients that do not respond to anti-PD-1 immunotherapies. Therefore, it is likely that combinatorial immunotherapy targeting several appropriate co-inhibitory pathways will be required for maximal therapeutic benefit. It is thus important to characterize novel co-inhibitory pathways and to validate them as target for novel therapy against human tumors.

25 Herpes virus entry mediator (HVEM; TNFRSF14) is a member of the tumor necrosis factor (TNF) receptor superfamily, which is expressed on several types of cells, including T cells, B cells, natural killer cells, dendritic cells, and myeloid cells, as well as on the parenchyma of tissues. HVEM functions as either a ligand or receptor in diverse physiological and pathological processes. HVEM is a ligand for the TNF superfamily members LIGHT and
30 Lymphotoxin α . Ligation of HVEM, expressed by antigen presenting cells, by LIGHT promotes T-cell proliferation and cytokine production via the transcription factor nuclear factor- κ B. In contrast, ligation of HVEM by the immunoglobulin superfamily members B-lymphocyte and T-lymphocyte attenuator (BTLA) and CD160 activates inhibitory signaling in T cells, resulting in decreased T-cell proliferation and cytokine production. Therefore,

HVEM has a dual functional activity for T-cell activation depending on the receptors engaged. Additionally, HVEM could function also as a receptor by it activating ligation to BTLA and CD160 (4,5) via the transcription factor nuclear factor-KB.

For instance, in *in vitro* assays, Derré *et al* have shown that HVEM on melanoma cells inhibited IFN γ production and the proliferation of tumor-specific CD8⁺ T cells via engagement of BTLA-expressing T cell, suggesting that inhibitory interactions of HVEM-BTLA play a role in evading host anti-tumor immunity. Along the same line, HVEM expression levels on human esophageal squamous cell carcinoma were inversely correlated with the presence of tumor-infiltrating CD4⁺ and CD8⁺ T cells and CD45RO memory T cells (6). Similar results have been obtained in colorectal cancer (7) and hepatocellular carcinoma (8).

SUMMARY OF THE INVENTION:

The present invention relates to methods for predicting the survival time of subjects suffering from melanoma metastases. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

Herpes Virus Entry Mediator (HVEM) has been recently suggested to play a role in several malignancies. A significant correlation exists between a high expression of HVEM and a reduced overall survival in human esophageal squamous cell carcinoma, colorectal cancer and hepatocellular carcinoma, with an independent prognosis value. The objective of the inventors was to correlate HVEM expression in melanoma metastases and clinical outcomes. HVEM expression was thus evaluated by immunohistochemistry in 126 formal-fixed paraffin embedded samples of melanoma metastases, collected during tumor excision for tumor mass sterilization or palliative care, between 2009 and 2012. The inventors confirmed the heterogeneous expression of HVEM in melanoma metastases, regardless of the metastatic site. They found that patients with high HVEM expression had a significantly poorer survival from the date of excision than those with a low expression. Moreover, in multivariate analysis, HVEM metastases status is an independent prognostic marker in melanoma. In conclusion, a high expression of HVEM by melanoma metastases is associated with a significantly poorer survival from the date of excision than a low HVEM expression. Therefore, high levels of HVEM expression on melanoma may dampen anti-tumor immune

responses, suggesting that together with its ligands, HVEM constitutes promising targets for antibody-mediated 'checkpoint blockade' therapy.

Accordingly a first object of the present invention relates to a method for predicting the survival time of a subject suffering from melanoma metastases comprising i) determining the expression level of HVEM in a metastasis tissue sample ii) comparing the expression level determined at step i) with a predetermined reference value and iii) concluding that the subject will have a short survival time when the expression level determined at step i) is higher than the predetermined reference value or concluding that the subject will have a long survival time when the expression level determined at step i) is lower than the predetermined reference value.

The method is particularly suitable for predicting the duration of the overall survival (OS), progression-free survival (PFS) and/or the disease-free survival (DFS) of the cancer subject. Those of skill in the art will recognize that OS survival time is generally based on and expressed as the percentage of people who survive a certain type of cancer for a specific amount of time. Cancer statistics often use an overall five-year survival rate. In general, OS rates do not specify whether cancer survivors are still undergoing treatment at five years or if they've become cancer-free (achieved remission). DSF gives more specific information and is the number of people with a particular cancer who achieve remission. Also, progression-free survival (PFS) rates (the number of people who still have cancer, but their disease does not progress) includes people who may have had some success with treatment, but the cancer has not disappeared completely. As used herein, the expression "short survival time" indicates that the subject will have a survival time that will be lower than the median (or mean) observed in the general population of subjects suffering from said cancer. When the subject will have a short survival time, it is meant that the subject will have a "poor prognosis". Inversely, the expression "long survival time" indicates that the subject will have a survival time that will be higher than the median (or mean) observed in the general population of subjects suffering from said cancer. When the subject will have a long survival time, it is meant that the subject will have a "good prognosis".

As used herein, the term "metastasis tissue sample" means any tissue metastasis tissue sample derived from a melanoma metastasis. Said tissue sample is obtained for the purpose of the in vitro evaluation. In some embodiments, the metastasis tissue sample may result from a

tumor excision. In some embodiments, the metastasis tissue sample may result from a biopsy performed in metastatic sample distant from the primary tumor of the patient. The metastasis tissue sample can, of course, be subjected to a variety of well-known post-collection preparative and storage techniques (e.g., fixation, storage, freezing, etc.). The sample can be
5 fresh, frozen, fixed (e.g., formalin fixed), or embedded (e.g., paraffin embedded).

As used herein, the term “HVEM” is intended to encompass all synonyms including, but not limited to, “Herpes Virus Entry Mediator”, “HVEA”, “Herpes Virus Entry Mediator A”, “TNFRSF14”, “Tumor Necrosis Factor Receptor Superfamily Member 14”, “TNR14”,
10 “LIGHTR”, “LIGHT receptor”, “TR2”, “TNF Receptor-like”, “ATAR”, “Another TRAF-Associated Receptor”. TNFRSF14 is the HUGO (Human Genome Organization) Gene Nomenclature Committee (HGNC) approved symbol. The UniProtKB/Swiss-Prot “Primary Accession Number” for HVEM is Q92956. The “Secondary Accession Numbers” are Q8WXR1, Q96J31 and Q9UM65.

In some embodiments, the level of HVEM is determined at the protein level by any well known method in the art. Typically, such methods comprise contacting the tumor tissue sample with at least one selective binding agent capable of selectively interacting with HVEM. The selective binding agent may be polyclonal antibody or monoclonal antibody, an
20 antibody fragment, synthetic antibodies, or other protein-specific agents such as nucleic acid or peptide aptamers. For the detection of the antibody that makes the presence of HVEM detectable by microscopy or an automated analysis system, the antibodies may be tagged directly with detectable labels such as enzymes, chromogens or fluorescent probes or indirectly detected with a secondary antibody conjugated with detectable labels.

In some embodiments, the expression level of HVEM is determined by immunohistochemistry (IHC). Accordingly, the metastasis tissue sample is firstly incubated the binding partners. After washing, the labeled antibodies that are bound to marker of interest are revealed by the appropriate technique, depending of the kind of label is borne by the
30 labeled antibody, e.g. radioactive, fluorescent or enzyme label. Multiple labelling can be performed simultaneously. Alternatively, the method of the present invention may use a secondary antibody coupled to an amplification system (to intensify staining signal) and enzymatic molecules. Such coupled secondary antibodies are commercially available, e.g. from Dako, EnVision system. Counterstaining may be used, e.g. H&E, DAPI, Hoechst. Other

staining methods may be accomplished using any suitable method or system as would be apparent to one of skill in the art, including automated, semi-automated or manual systems. For example, one or more labels can be attached to the antibody, thereby permitting detection of the target protein (i.e. HVEM). Exemplary labels include radioactive isotopes, fluorophores, ligands, chemiluminescent agents, enzymes, and combinations thereof. In some embodiments, the label is a quantum dot. Non-limiting examples of labels that can be conjugated to primary and/or secondary affinity ligands include fluorescent dyes or metals (e.g. fluorescein, rhodamine, phycoerythrin, fluorescamine), chromophoric dyes (e.g. rhodopsin), chemiluminescent compounds (e.g. luminal, imidazole) and bioluminescent proteins (e.g. luciferin, luciferase), haptens (e.g. biotin). A variety of other useful fluorescers and chromophores are described in Stryer L (1968) *Science* 162:526-533 and Brand L and Gohlke J R (1972) *Annu. Rev. Biochem.* 41:843-868. Affinity ligands can also be labeled with enzymes (e.g. horseradish peroxidase, alkaline phosphatase, beta-lactamase), radioisotopes (e.g. ^3H , ^{14}C , ^{32}P , ^{35}S or ^{125}I) and particles (e.g. gold). The different types of labels can be conjugated to an affinity ligand using various chemistries, e.g. the amine reaction or the thiol reaction. However, other reactive groups than amines and thiols can be used, e.g. aldehydes, carboxylic acids and glutamine. Various enzymatic staining methods are known in the art for detecting a protein of interest. For example, enzymatic interactions can be visualized using different enzymes such as peroxidase, alkaline phosphatase, or different chromogens such as DAB, AEC or Fast Red. In other examples, the antibody can be conjugated to peptides or proteins that can be detected via a labeled binding partner or antibody. In an indirect IHC assay, a secondary antibody or second binding partner is necessary to detect the binding of the first binding partner, as it is not labeled.

Immunohistochemistry typically includes the following steps i) fixing said metastasis tissue sample with formalin, ii) embedding said metastasis tissue sample in paraffin, iii) cutting said metastasis tissue sample into sections for staining, iv) incubating said sections with the binding partner specific for HVEM, v) rinsing said sections, vi) incubating said section with a biotinylated secondary antibody and vii) revealing the antigen-antibody complex with avidin-biotin-peroxidase complex.

The resulting stained specimens are each imaged using a system for viewing the detectable signal and acquiring an image, such as a digital image of the staining. Methods for image acquisition are well known to one of skill in the art. For example, once the sample has been stained, any optical or non-optical imaging device can be used to detect the stain or biomarker label, such as, for example, upright or inverted optical microscopes, scanning

confocal microscopes, cameras, scanning or tunneling electron microscopes, scanning probe microscopes and imaging infrared detectors. In some examples, the image can be captured digitally. The obtained images can then be used for quantitatively or semi-quantitatively determining the amount of HVEM in the sample. Various automated sample processing, scanning and analysis systems suitable for use with immunohistochemistry are available in the art. Such systems can include automated staining and microscopic scanning, computerized image analysis, serial section comparison (to control for variation in the orientation and size of a sample), digital report generation, and archiving and tracking of samples (such as slides on which tissue sections are placed). Cellular imaging systems are commercially available that combine conventional light microscopes with digital image processing systems to perform quantitative analysis on cells and tissues, including immunostained samples. See, e.g., the CAS-200 system (Becton, Dickinson & Co.). In particular, detection can be made manually or by image processing techniques involving computer processors and software. Using such software, for example, the images can be configured, calibrated, standardized and/or validated based on factors including, for example, stain quality or stain intensity, using procedures known to one of skill in the art (see e.g., published U.S. Patent Publication No. US20100136549). The image can be quantitatively or semi-quantitatively analyzed and scored based on staining intensity of the sample. Quantitative or semi-quantitative histochemistry refers to method of scanning and scoring samples that have undergone histochemistry, to identify and quantitate the presence of the specified biomarker (i.e. HVEM). Quantitative or semi-quantitative methods can employ imaging software to detect staining densities or amount of staining or methods of detecting staining by the human eye, where a trained operator ranks results numerically. For example, images can be quantitatively analyzed using a pixel count algorithms (e.g., Aperio Spectrum Software, Automated QUantitative Analysis platform (AQUA® platform), and other standard methods that measure or quantitate or semi-quantitate the degree of staining; see e.g., U.S. Pat. No. 8,023,714; U.S. Pat. No. 7,257,268; U.S. Pat. No. 7,219,016; U.S. Pat. No. 7,646,905; published U.S. Patent Publication No. US20100136549 and 20110111435; Camp et al. (2002) Nature Medicine, 8:1323-1327; Bacus et al. (1997) Analyt Quant Cytol Histol, 19:316-328). A ratio of strong positive stain (such as brown stain) to the sum of total stained area can be calculated and scored. The amount of the detected biomarker (i.e. HVEM) is quantified and given as a percentage of positive pixels and/or a score. For example, the amount can be quantified as a percentage of positive pixels. In some examples, the amount is quantified as the percentage of area stained, e.g., the percentage of positive pixels. For example, a sample

can have at least or about at least or about 0, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more positive pixels as compared to the total staining area. In

5 some embodiments, a score is given to the sample that is a numerical representation of the intensity or amount of the histochemical staining of the sample, and represents the amount of target biomarker (e.g., HVEM) present in the sample. Optical density or percentage area values can be given a scaled score, for example on an integer scale. Thus, in some

10 embodiments, the method of the present invention comprises the steps consisting in i) providing one or more immunostained slices of tissue section obtained by an automated slide-staining system by using a binding partner capable of selectively interacting with HVEM (e.g. an antibody as above described), ii) proceeding to digitalisation of the slides of step a. by high resolution scan capture, iii) detecting the slice of tissue section on the digital picture iv)

15 providing a size reference grid with uniformly distributed units having a same surface, said grid being adapted to the size of the tissue section to be analyzed, and v) detecting, quantifying and measuring intensity of stained cells in each unit whereby the number or the density of cells stained of each unit is assessed.

In some embodiments, the level of HVEM is determined at nucleic acid level.

20 Typically, the level of a gene may be determined by determining the quantity of mRNA. Methods for determining the quantity of mRNA are well known in the art. For example the nucleic acid contained in the samples (e.g., cell or tissue prepared from the subject) is first extracted according to standard methods, for example using lytic enzymes or chemical solutions or extracted by nucleic-acid-binding resins following the manufacturer's

25 instructions. The extracted mRNA is then detected by hybridization (e. g., Northern blot analysis, in situ hybridization) and/or amplification (e.g., RT-PCR). Other methods of Amplification include ligase chain reaction (LCR), transcription-mediated amplification (TMA), strand displacement amplification (SDA) and nucleic acid sequence based amplification (NASBA).

30 Nucleic acids having at least 10 nucleotides and exhibiting sequence complementarity or homology to the mRNA of interest herein find utility as hybridization probes or amplification primers. It is understood that such nucleic acids need not be identical, but are typically at least about 80% identical to the homologous region of comparable size, more preferably 85% identical and even more preferably 90-95% identical. In some embodiments,

it will be advantageous to use nucleic acids in combination with appropriate means, such as a detectable label, for detecting hybridization.

Typically, the nucleic acid probes include one or more labels, for example to permit detection of a target nucleic acid molecule using the disclosed probes. In various applications, such as in situ hybridization procedures, a nucleic acid probe includes a label (e.g., a detectable label). A “detectable label” is a molecule or material that can be used to produce a detectable signal that indicates the presence or concentration of the probe (particularly the bound or hybridized probe) in a sample. Thus, a labeled nucleic acid molecule provides an indicator of the presence or concentration of a target nucleic acid sequence (e.g., genomic target nucleic acid sequence) (to which the labeled uniquely specific nucleic acid molecule is bound or hybridized) in a sample. A label associated with one or more nucleic acid molecules (such as a probe generated by the disclosed methods) can be detected either directly or indirectly. A label can be detected by any known or yet to be discovered mechanism including absorption, emission and/ or scattering of a photon (including radio frequency, microwave frequency, infrared frequency, visible frequency and ultra-violet frequency photons). Detectable labels include colored, fluorescent, phosphorescent and luminescent molecules and materials, catalysts (such as enzymes) that convert one substance into another substance to provide a detectable difference (such as by converting a colorless substance into a colored substance or vice versa, or by producing a precipitate or increasing sample turbidity), haptens that can be detected by antibody binding interactions, and paramagnetic and magnetic molecules or materials.

Particular examples of detectable labels include fluorescent molecules (or fluorochromes). Numerous fluorochromes are known to those of skill in the art, and can be selected, for example from Life Technologies (formerly Invitrogen), e.g., see, The Handbook—A Guide to Fluorescent Probes and Labeling Technologies). Examples of particular fluorophores that can be attached (for example, chemically conjugated) to a nucleic acid molecule (such as a uniquely specific binding region) are provided in U.S. Pat. No. 5,866, 366 to Nazarenko et al., such as 4-acetamido-4'-isothiocyanatostilbene-2,2' disulfonic acid, acridine and derivatives such as acridine and acridine isothiocyanate, 5-(2'-aminoethyl) aminonaphthalene-1-sulfonic acid (EDANS), 4-amino -N-[3 vinylsulfonyl]phenyl]naphthalimide-3,5 disulfonate (Lucifer Yellow VS), N-(4-anilino-1-naphthyl)maleimide, antl1ranilamide, Brilliant Yellow, coumarin and derivatives such as coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifluoromethylcoumarin (Coumarin 151); cyanosine; 4',6-diarninidino-2-phenylindole

(DAPI); 5',5''dibromopyrogallol-sulfonephthalein (Bromopyrogallol Red); 7 -diethylamino -3 - (4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; 5-[dimethylamino] naphthalene-1-sulfonyl chloride (DNS, dansyl chloride);

5 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives such as eosin and eosin isothiocyanate; erythrosin and derivatives such as erythrosin B and erythrosin isothiocyanate; ethidium; fluorescein and derivatives such as 5-carboxyfluorescein (FAM), 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein (JOE),

10 fluorescein, fluorescein isothiocyanate (FITC), and QFITC Q(RITC); 2',7'-difluorofluorescein (OREGON GREEN®); fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferone; ortho cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthalaldehyde; pyrene and derivatives such as pyrene, pyrene butyrate and succinimidyl 1-pyrene butyrate; Reactive Red 4 (Cibacron Brilliant Red 3B-A);

15 rhodamine and derivatives such as 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride, rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, rhodamine green, sulforhodamine B, sulforhodamine 101 and sulfonyl chloride derivative of sulforhodamine 101 (Texas Red); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine

20 isothiocyanate (TRITC); riboflavin; rosolic acid and terbium chelate derivatives. Other suitable fluorophores include thiol-reactive europium chelates which emit at approximately 617 nm (Heyduk and Heyduk, *Analyt. Biochem.* 248:216-27, 1997; *J. Biol. Chem.* 274:3315-22, 1999), as well as GFP, LissamineTM, diethylaminocoumarin, fluorescein chlorotriazinyl, naphthofluorescein, 4,7-dichlororhodamine and xanthene (as described in U.S. Pat. No.

25 5,800,996 to Lee et al.) and derivatives thereof. Other fluorophores known to those skilled in the art can also be used, for example those available from Life Technologies (Invitrogen; Molecular Probes (Eugene, Oreg.)) and including the ALEXA FLUOR® series of dyes (for example, as described in U.S. Pat. Nos. 5,696,157, 6,130,101 and 6,716,979), the BODIPY series of dyes (dipyrrrometheneboron difluoride dyes, for example as described in U.S. Pat.

30 Nos. 4,774,339, 5,187,288, 5,248,782, 5,274,113, 5,338,854, 5,451,663 and 5,433,896), Cascade Blue (an amine reactive derivative of the sulfonated pyrene described in U.S. Pat. No. 5,132,432) and Marina Blue (U.S. Pat. No. 5,830,912).

In addition to the fluorochromes described above, a fluorescent label can be a fluorescent nanoparticle, such as a semiconductor nanocrystal, e.g., a QUANTUM DOTTM

(obtained, for example, from Life Technologies (QuantumDot Corp, Invitrogen Nanocrystal Technologies, Eugene, Oreg.); see also, U.S. Pat. Nos. 6,815,064; 6,682,596; and 6,649,138). Semiconductor nanocrystals are microscopic particles having size-dependent optical and/or electrical properties. When semiconductor nanocrystals are illuminated with a primary energy source, a secondary emission of energy occurs of a frequency that corresponds to the bandgap of the semiconductor material used in the semiconductor nanocrystal. This emission can be detected as colored light of a specific wavelength or fluorescence. Semiconductor nanocrystals with different spectral characteristics are described in e.g., U.S. Pat. No. 6,602,671. Semiconductor nanocrystals that can be coupled to a variety of biological molecules (including dNTPs and/or nucleic acids) or substrates by techniques described in, for example, Bruchez et al., *Science* 281 :20132016, 1998; Chan et al., *Science* 281:2016-2018, 1998; and U.S. Pat. No. 6,274,323. Formation of semiconductor nanocrystals of various compositions are disclosed in, e.g., U.S. Pat. Nos. 6,927,069; 6,914,256; 6,855,202; 6,709,929; 6,689,338; 6,500,622; 6,306,736; 6,225,198; 6,207,392; 6,114,038; 6,048,616; 5,990,479; 5,690,807; 5,571,018; 5,505,928; 5,262,357 and in U.S. Patent Publication No. 2003/0165951 as well as PCT Publication No. 99/26299 (published May 27, 1999). Separate populations of semiconductor nanocrystals can be produced that are identifiable based on their different spectral characteristics. For example, semiconductor nanocrystals can be produced that emit light of different colors based on their composition, size or size and composition. For example, quantum dots that emit light at different wavelengths based on size (565 nm, 655 nm, 705 nm, or 800 nm emission wavelengths), which are suitable as fluorescent labels in the probes disclosed herein are available from Life Technologies (Carlsbad, Calif.).

Additional labels include, for example, radioisotopes (such as ^3H), metal chelates such as DOTA and DPTA chelates of radioactive or paramagnetic metal ions like Gd^{3+} , and liposomes.

Detectable labels that can be used with nucleic acid molecules also include enzymes, for example horseradish peroxidase, alkaline phosphatase, acid phosphatase, glucose oxidase, beta-galactosidase, beta-glucuronidase, or beta-lactamase.

Alternatively, an enzyme can be used in a metallographic detection scheme. For example, silver in situ hybridization (SISH) procedures involve metallographic detection schemes for identification and localization of a hybridized genomic target nucleic acid sequence. Metallographic detection methods include using an enzyme, such as alkaline

phosphatase, in combination with a water-soluble metal ion and a redox-inactive substrate of the enzyme. The substrate is converted to a redox-active agent by the enzyme, and the redoxactive agent reduces the metal ion, causing it to form a detectable precipitate. (See, for example, U.S. Patent Application Publication No. 2005/0100976, PCT Publication No. 2005/003777 and U.S. Patent Application Publication No. 2004/ 0265922). Metallographic detection methods also include using an oxido-reductase enzyme (such as horseradish peroxidase) along with a water soluble metal ion, an oxidizing agent and a reducing agent, again to form a detectable precipitate. (See, for example, U.S. Pat. No. 6,670,113).

Probes made using the disclosed methods can be used for nucleic acid detection, such as ISH procedures (for example, fluorescence in situ hybridization (FISH), chromogenic in situ hybridization (CISH) and silver in situ hybridization (SISH)) or comparative genomic hybridization (CGH).

In situ hybridization (ISH) involves contacting a sample containing target nucleic acid sequence (e.g., genomic target nucleic acid sequence) in the context of a metaphase or interphase chromosome preparation (such as a cell or tissue sample mounted on a slide) with a labeled probe specifically hybridizable or specific for the target nucleic acid sequence (e.g., genomic target nucleic acid sequence). The slides are optionally pretreated, e.g., to remove paraffin or other materials that can interfere with uniform hybridization. The sample and the probe are both treated, for example by heating to denature the double stranded nucleic acids. The probe (formulated in a suitable hybridization buffer) and the sample are combined, under conditions and for sufficient time to permit hybridization to occur (typically to reach equilibrium). The chromosome preparation is washed to remove excess probe, and detection of specific labeling of the chromosome target is performed using standard techniques.

For example, a biotinylated probe can be detected using fluorescein-labeled avidin or avidin-alkaline phosphatase. For fluorochrome detection, the fluorochrome can be detected directly, or the samples can be incubated, for example, with fluorescein isothiocyanate (FITC)-conjugated avidin. Amplification of the FITC signal can be effected, if necessary, by incubation with biotin-conjugated goat antiavidin antibodies, washing and a second incubation with FITC-conjugated avidin. For detection by enzyme activity, samples can be incubated, for example, with streptavidin, washed, incubated with biotin-conjugated alkaline phosphatase, washed again and pre-equilibrated (e.g., in alkaline phosphatase (AP) buffer). For a general description of in situ hybridization procedures, see, e.g., U.S. Pat. No. 4,888,278.

Numerous procedures for FISH, CISH, and SISH are known in the art. For example, procedures for performing FISH are described in U.S. Pat. Nos. 5,447,841; 5,472,842; and 5,427,932; and for example, in Pirlkel et al., Proc. Natl. Acad. Sci. 83:2934-2938, 1986; Pinkel et al., Proc. Natl. Acad. Sci. 85:9138-9142, 1988; and Lichter et al., Proc. Natl. Acad. Sci. 85:9664-9668, 1988. CISH is described in, e.g., Tanner et al., Am. J. Pathol. 157:1467-1472, 2000 and U.S. Pat. No. 6,942,970. Additional detection methods are provided in U.S. Pat. No. 6,280,929.

Numerous reagents and detection schemes can be employed in conjunction with FISH, CISH, and SISH procedures to improve sensitivity, resolution, or other desirable properties. As discussed above probes labeled with fluorophores (including fluorescent dyes and QUANTUM DOTS®) can be directly optically detected when performing FISH. Alternatively, the probe can be labeled with a nonfluorescent molecule, such as a hapten (such as the following non-limiting examples: biotin, digoxigenin, DNP, and various oxazoles, pyrrazoles, thiazoles, nitroaryls, benzofurazans, triterpenes, ureas, thioureas, rotenones, coumarin, coumarin-based compounds, Podophyllotoxin, Podophyllotoxin-based compounds, and combinations thereof), ligand or other indirectly detectable moiety. Probes labeled with such non-fluorescent molecules (and the target nucleic acid sequences to which they bind) can then be detected by contacting the sample (e.g., the cell or tissue sample to which the probe is bound) with a labeled detection reagent, such as an antibody (or receptor, or other specific binding partner) specific for the chosen hapten or ligand. The detection reagent can be labeled with a fluorophore (e.g., QUANTUM DOT®) or with another indirectly detectable moiety, or can be contacted with one or more additional specific binding agents (e.g., secondary or specific antibodies), which can be labeled with a fluorophore.

In other examples, the probe, or specific binding agent (such as an antibody, e.g., a primary antibody, receptor or other binding agent) is labeled with an enzyme that is capable of converting a fluorogenic or chromogenic composition into a detectable fluorescent, colored or otherwise detectable signal (e.g., as in deposition of detectable metal particles in SISH). As indicated above, the enzyme can be attached directly or indirectly via a linker to the relevant probe or detection reagent. Examples of suitable reagents (e.g., binding reagents) and chemistries (e.g., linker and attachment chemistries) are described in U.S. Patent Application Publication Nos. 2006/0246524; 2006/0246523, and 2007/01 17153.

It will be appreciated by those of skill in the art that by appropriately selecting labelled probe-specific binding agent pairs, multiplex detection schemes can be produced to facilitate detection of multiple target nucleic acid sequences (e.g., genomic target nucleic acid

sequences) in a single assay (e.g., on a single cell or tissue sample or on more than one cell or tissue sample). For example, a first probe that corresponds to a first target sequence can be labelled with a first hapten, such as biotin, while a second probe that corresponds to a second target sequence can be labelled with a second hapten, such as DNP. Following exposure of the sample to the probes, the bound probes can be detected by contacting the sample with a first specific binding agent (in this case avidin labelled with a first fluorophore, for example, a first spectrally distinct QUANTUM DOT®, e.g., that emits at 585 nm) and a second specific binding agent (in this case an anti-DNP antibody, or antibody fragment, labelled with a second fluorophore (for example, a second spectrally distinct QUANTUM DOT®, e.g., that emits at 705 nm). Additional probes/binding agent pairs can be added to the multiplex detection scheme using other spectrally distinct fluorophores. Numerous variations of direct, and indirect (one step, two step or more) can be envisioned, all of which are suitable in the context of the disclosed probes and assays.

Probes typically comprise single-stranded nucleic acids of between 10 to 1000 nucleotides in length, for instance of between 10 and 800, more preferably of between 15 and 700, typically of between 20 and 500. Primers typically are shorter single-stranded nucleic acids, of between 10 to 25 nucleotides in length, designed to perfectly or almost perfectly match a nucleic acid of interest, to be amplified. The probes and primers are “specific” to the nucleic acids they hybridize to, i.e. they preferably hybridize under high stringency hybridization conditions (corresponding to the highest melting temperature T_m , e.g., 50 % formamide, 5x or 6x SCC. SCC is a 0.15 M NaCl, 0.015 M Na-citrate).

The nucleic acid primers or probes used in the above amplification and detection method may be assembled as a kit. Such a kit includes consensus primers and molecular probes. A preferred kit also includes the components necessary to determine if amplification has occurred. The kit may also include, for example, PCR buffers and enzymes; positive control sequences, reaction control primers; and instructions for amplifying and detecting the specific sequences.

In some embodiments, the methods of the invention comprise the steps of providing total RNAs extracted from cumulus cells and subjecting the RNAs to amplification and hybridization to specific probes, more particularly by means of a quantitative or semi-quantitative RT-PCR.

In some embodiments, the level is determined by DNA chip analysis. Such DNA chip or nucleic acid microarray consists of different nucleic acid probes that are chemically attached to a substrate, which can be a microchip, a glass slide or a microsphere-sized bead. A microchip may be constituted of polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, or nitrocellulose. Probes comprise nucleic acids such as cDNAs or oligonucleotides that may be about 10 to about 60 base pairs. To determine the level, a sample from a test subject, optionally first subjected to a reverse transcription, is labelled and contacted with the microarray in hybridization conditions, leading to the formation of complexes between target nucleic acids that are complementary to probe sequences attached to the microarray surface. The labelled hybridized complexes are then detected and can be quantified or semi-quantified. Labelling may be achieved by various methods, e.g. by using radioactive or fluorescent labelling. Many variants of the microarray hybridization technology are available to the man skilled in the art (see e.g. the review by Hoheisel, Nature Reviews, Genetics, 2006, 7:200-210).

In some embodiments, the nCounter® Analysis system is used to detect intrinsic gene expression. The basis of the nCounter® Analysis system is the unique code assigned to each nucleic acid target to be assayed (International Patent Application Publication No. WO 08/124847, U.S. Patent No. 8,415,102 and Geiss et al. Nature Biotechnology. 2008. 26(3): 317-325; the contents of which are each incorporated herein by reference in their entireties). The code is composed of an ordered series of colored fluorescent spots which create a unique barcode for each target to be assayed. A pair of probes is designed for each DNA or RNA target, a biotinylated capture probe and a reporter probe carrying the fluorescent barcode. This system is also referred to, herein, as the nanoreporter code system. Specific reporter and capture probes are synthesized for each target. The reporter probe can comprise at a least a first label attachment region to which are attached one or more label monomers that emit light constituting a first signal; at least a second label attachment region, which is non-over-lapping with the first label attachment region, to which are attached one or more label monomers that emit light constituting a second signal; and a first target- specific sequence. Preferably, each sequence specific reporter probe comprises a target specific sequence capable of hybridizing to no more than one gene and optionally comprises at least three, or at least four label attachment regions, said attachment regions comprising one or more label monomers that emit light, constituting at least a third signal, or at least a fourth signal, respectively. The

capture probe can comprise a second target-specific sequence; and a first affinity tag. In some embodiments, the capture probe can also comprise one or more label attachment regions. Preferably, the first target-specific sequence of the reporter probe and the second target-specific sequence of the capture probe hybridize to different regions of the same gene to be detected. Reporter and capture probes are all pooled into a single hybridization mixture, the "probe library". The relative abundance of each target is measured in a single multiplexed hybridization reaction. The method comprises contacting the tumor tissue sample with a probe library, such that the presence of the target in the sample creates a probe pair - target complex. The complex is then purified. More specifically, the sample is combined with the probe library, and hybridization occurs in solution. After hybridization, the tripartite hybridized complexes (probe pairs and target) are purified in a two-step procedure using magnetic beads linked to oligonucleotides complementary to universal sequences present on the capture and reporter probes. This dual purification process allows the hybridization reaction to be driven to completion with a large excess of target-specific probes, as they are ultimately removed, and, thus, do not interfere with binding and imaging of the sample. All post hybridization steps are handled robotically on a custom liquid-handling robot (Prep Station, NanoString Technologies). Purified reactions are typically deposited by the Prep Station into individual flow cells of a sample cartridge, bound to a streptavidin-coated surface via the capture probe, electrophoresed to elongate the reporter probes, and immobilized. After processing, the sample cartridge is transferred to a fully automated imaging and data collection device (Digital Analyzer, NanoString Technologies). The level of a target is measured by imaging each sample and counting the number of times the code for that target is detected. For each sample, typically 600 fields-of-view (FOV) are imaged (1376 X 1024 pixels) representing approximately 10 mm² of the binding surface. Typical imaging density is 100- 1200 counted reporters per field of view depending on the degree of multiplexing, the amount of sample input, and overall target abundance. Data is output in simple spreadsheet format listing the number of counts per target, per sample. This system can be used along with nanoreporters. Additional disclosure regarding nanoreporters can be found in International Publication No. WO 07/076129 and WO07/076132, and US Patent Publication No. 2010/0015607 and 2010/0261026, the contents of which are incorporated herein in their entireties. Further, the term nucleic acid probes and nanoreporters can include the rationally designed (e.g. synthetic sequences) described in International Publication No. WO 2010/019826 and US Patent Publication No. 2010/0047924, incorporated herein by reference in its entirety.

Expression level of a gene may be expressed as absolute level or normalized level. Typically, levels are normalized by correcting the absolute level of a gene by comparing its expression to the expression of a gene that is not a relevant for determining the cancer stage of the subject, e.g., a housekeeping gene that is constitutively expressed. Suitable genes for normalization include housekeeping genes such as the actin gene ACTB, ribosomal 18S gene, GUSB, PGK1 and TFRC. This normalization allows the comparison of the level in one sample, e.g., a subject sample, to another sample, or between samples from different sources.

In some embodiments, the predetermined reference value is a threshold value or a cut-off value. Typically, a "threshold value" or "cut-off value" can be determined experimentally, empirically, or theoretically. A threshold value can also be arbitrarily selected based upon the existing experimental and/or clinical conditions, as would be recognized by a person of ordinary skilled in the art. For example, retrospective measurement of level of HVEM in properly banked historical subject samples may be used in establishing the predetermined reference value. The threshold value has to be determined in order to obtain the optimal sensitivity and specificity according to the function of the test and the benefit/risk balance (clinical consequences of false positive and false negative). Typically, the optimal sensitivity and specificity (and so the threshold value) can be determined using a Receiver Operating Characteristic (ROC) curve based on experimental data. For example, after determining the expression level of HVEM in a group of reference, one can use algorithmic analysis for the statistic treatment of the measured levels of HVEM in samples to be tested, and thus obtain a classification standard having significance for sample classification. The full name of ROC curve is receiver operator characteristic curve, which is also known as receiver operation characteristic curve. It is mainly used for clinical biochemical diagnostic tests. ROC curve is a comprehensive indicator that reflects the continuous variables of true positive rate (sensitivity) and false positive rate (1-specificity). It reveals the relationship between sensitivity and specificity with the image composition method. A series of different cut-off values (thresholds or critical values, boundary values between normal and abnormal results of diagnostic test) are set as continuous variables to calculate a series of sensitivity and specificity values. Then sensitivity is used as the vertical coordinate and specificity is used as the horizontal coordinate to draw a curve. The higher the area under the curve (AUC), the higher the accuracy of diagnosis. On the ROC curve, the point closest to the far upper left of the coordinate diagram is a critical point having both high sensitivity and high specificity

values. The AUC value of the ROC curve is between 1.0 and 0.5. When $AUC > 0.5$, the diagnostic result gets better and better as AUC approaches 1. When AUC is between 0.5 and 0.7, the accuracy is low. When AUC is between 0.7 and 0.9, the accuracy is moderate. When AUC is higher than 0.9, the accuracy is quite high. This algorithmic method is preferably done with a computer. Existing software or systems in the art may be used for the drawing of the ROC curve, such as: MedCalc 9.2.0.1 medical statistical software, SPSS 9.0, ROCPOWER.SAS, DESIGNROC.FOR, MULTIREADER POWER.SAS, CREATE-ROC.SAS, GB STAT VI0.0 (Dynamic Microsystems, Inc. Silver Spring, Md., USA), etc.

In some embodiments, the predetermined reference value is determined by carrying out a method comprising the steps of a) providing a collection of samples; b) providing, for each sample provided at step a), information relating to the actual clinical outcome for the corresponding subject (i.e. the duration of the survival); c) providing a serial of arbitrary quantification values; d) determining the expression level of HVEM for each sample contained in the collection provided at step a); e) classifying said samples in two groups for one specific arbitrary quantification value provided at step c), respectively: (i) a first group comprising samples that exhibit a quantification value for level that is lower than the said arbitrary quantification value contained in the said serial of quantification values; (ii) a second group comprising samples that exhibit a quantification value for said level that is higher than the said arbitrary quantification value contained in the said serial of quantification values; whereby two groups of samples are obtained for the said specific quantification value, wherein the samples of each group are separately enumerated; f) calculating the statistical significance between (i) the quantification value obtained at step e) and (ii) the actual clinical outcome of the subjects from which samples contained in the first and second groups defined at step f) derive; g) reiterating steps f) and g) until every arbitrary quantification value provided at step d) is tested; h) setting the said predetermined reference value as consisting of the arbitrary quantification value for which the highest statistical significance (most significant) has been calculated at step g).

For example the expression level of HVEM has been assessed for 100 samples of 100 subjects. The 100 samples are ranked according to the expression level of HVEM. Sample 1 has the highest level and sample 100 has the lowest level. A first grouping provides two subsets: on one side sample Nr 1 and on the other side the 99 other samples. The next grouping provides on one side samples 1 and 2 and on the other side the 98 remaining

samples etc., until the last grouping: on one side samples 1 to 99 and on the other side sample Nr 100. According to the information relating to the actual clinical outcome for the corresponding subject, Kaplan Meier curves are prepared for each of the 99 groups of two subsets. Also for each of the 99 groups, the p value between both subsets was calculated. The predetermined reference value is then selected such as the discrimination based on the criterion of the minimum p value is the strongest. In other terms, the expression level of HVEM corresponding to the boundary between both subsets for which the p value is minimum is considered as the predetermined reference value.

It should be noted that the predetermined reference value is not necessarily the median value of levels of HVEM. Thus in some embodiments, the predetermined reference value thus allows discrimination between a poor and a good prognosis for a subject. Practically, high statistical significance values (e.g. low P values) are generally obtained for a range of successive arbitrary quantification values, and not only for a single arbitrary quantification value. Thus, in one alternative embodiment of the invention, instead of using a definite predetermined reference value, a range of values is provided. Therefore, a minimal statistical significance value (minimal threshold of significance, e.g. maximal threshold P value) is arbitrarily set and a range of a plurality of arbitrary quantification values for which the statistical significance value calculated at step g) is higher (more significant, e.g. lower P value) are retained, so that a range of quantification values is provided. This range of quantification values includes a "cut-off" value as described above. For example, according to this specific embodiment of a "cut-off" value, the outcome can be determined by comparing the expression level of HVEM with the range of values which are identified. In some embodiments, a cut-off value thus consists of a range of quantification values, e.g. centered on the quantification value for which the highest statistical significance value is found (e.g. generally the minimum p value which is found). For example, on a hypothetical scale of 1 to 10, if the ideal cut-off value (the value with the highest statistical significance) is 5, a suitable (exemplary) range may be from 4-6. For example, a subject may be assessed by comparing values obtained by measuring the expression level of HVEM, where values greater than 5 reveal a poor prognosis and values less than 5 reveal a good prognosis. In some embodiments, a subject may be assessed by comparing values obtained by measuring the expression level of HVEM and comparing the values on a scale, where values above the range of 4-6 indicate a poor prognosis and values below the range of 4-6 indicate a good prognosis, with values falling within the range of 4-6 indicating an intermediate occurrence (or prognosis).

The method of the present invention is also suitable for determining whether a patient is eligible to a treatment.

5 As used herein, the term "treatment" or "treat" refer to both prophylactic or preventive treatment as well as curative or disease modifying treatment, including treatment of subjects at risk of contracting the disease or suspected to have contracted the disease as well as subjects who are ill or have been diagnosed as suffering from a disease or medical condition, and includes suppression of clinical relapse. The treatment may be administered to a subject
10 having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay the onset of, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment. By "therapeutic regimen" is meant the pattern of treatment of an illness, e.g., the pattern of dosing used during therapy. A therapeutic regimen
15 may include an induction regimen and a maintenance regimen. The phrase "induction regimen" or "induction period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the initial treatment of a disease. The general goal of an induction regimen is to provide a high level of drug to a subject during the initial period of a treatment regimen. An induction regimen may employ (in part or in whole) a "loading regimen", which
20 may include administering a greater dose of the drug than a physician would employ during a maintenance regimen, administering a drug more frequently than a physician would administer the drug during a maintenance regimen, or both. The phrase "maintenance regimen" or "maintenance period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the maintenance of a subject during treatment of an
25 illness, e.g., to keep the subject in remission for long periods of time (months or years). A maintenance regimen may employ continuous therapy (e.g., administering a drug at a regular intervals, e.g., weekly, monthly, yearly, etc.) or intermittent therapy (e.g., interrupted treatment, intermittent treatment, treatment at relapse, or treatment upon achievement of a particular predetermined criteria [e.g., disease manifestation, etc.]).

30 In some embodiments, the treatment consists in administering to the subject having a short survival time with a therapeutically effective amount of an antagonist of the BTLA/HVEM interaction.

As used herein, the term “BTLA” has its general meaning in the art and refers to the B- and T-lymphocyte attenuator protein. The term includes any human isoform of BTLA that is capable of binding to HVEM.

5 In some embodiments, the antagonist is selected in the group consisting of HVEM ligand. Typically, said antagonist is a soluble HVEM ligand. The person skilled in the art may refer to the publication Pasero et al., "*The HVEM network : new directions in targeting novel costimulatory/co-inhibitory molecules for cancer therapy*", Current opinion in Pharmacology 2012; 12:478-485. Typically, said ligand is selected in the group consisting of BTLA,
10 LIGHT, LT α , glycoprotein D and CD160. Preferably, said soluble HVEM ligand is recombinant. In some embodiments, the antagonist is selected from the group consisting of a soluble recombinant BTLA, a soluble recombinant LIGHT, a soluble recombinant LT α , a soluble recombinant glycoprotein D, a soluble recombinant CD160 or a fragment thereof which blocks the interaction between BTLA and HVEM. More preferably, the antagonist is a
15 recombinant BTLA.

In some embodiments, the antagonist is chosen from antibodies directed against HVEM and fragments thereof which block the interaction between BTLA and HVEM. Typically, said antagonist is an antibody.

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The term “antibody” is further intended to encompass antibodies, digestion fragments, specified portions and variants thereof, including antibody mimetics or portions of antibodies that mimic the structure and/or function of an antibody or specified fragment or portion thereof, including single chain antibodies and fragments thereof. Functional fragments include
25 antigen-binding fragments that block the interaction between HVEM and BTLA.

In some embodiments, the antibody is chosen among polyclonal antibody, monoclonal antibody, chimeric antibody, humanized antibody, or an antibody fragments. As used herein, the term “human antibody” refers to an antibody in which a substantial portion of the
30 antibody molecule resembles, in amino acid sequence or structure, that of an antibody derived from human origin. The term “humanized antibody” refers to an antibody which has been modified by genetic engineering or by other means to be similar in structure or amino acid sequence to naturally occurring human antibodies. A “human antibody” or a “humanized antibody” may be considered more suitable in instances where it is desirable to reduce the

immunogenicity of the antibody for administration to humans for therapeutic, prophylactic or diagnostic purposes.

Antibodies specifically directed against HVEM may be derived from a number of species including, but not limited to, rodent (mouse, rat, rabbit, guinea pig, hamster, and the like), porcine, bovine, equine or primate and the like. Antibodies from primate (monkey, baboon, chimpanzee, etc.) origin have the highest degree of similarity to human sequences and are therefore expected to be less immunogenic. Antibodies derived from various species can be “humanized” by modifying the amino acid sequences of the antibodies while retaining their ability to bind the desired antigen. Antibodies may also be derived from transgenic animals, including mice, which have been genetically modified with the human immunoglobulin locus to express human antibodies. Procedures for raising “polyclonal antibodies” are well known in the art. For example, polyclonal antibodies can be obtained from serum of an animal immunized against HVEM, which may be produced by genetic engineering for example according to standard methods well-known by one skilled in the art. Typically, such antibodies can be raised by administering HVEM protein subcutaneously to New Zealand white rabbits which have first been bled to obtain pre-immune serum. The antigens can be injected at a total volume of 100 μ l per site at six different sites. Each injected material may contain adjuvants with or without pulverized acrylamide gel containing the protein or polypeptide after SDS-polyacrylamide gel electrophoresis. The rabbits are then bled two weeks after the first injection and periodically boosted with the same antigen three times at six weeks’ interval. A sample of serum is then collected 10 days after each boost. Polyclonal antibodies are then recovered from the serum by affinity chromatography using the corresponding antigen to capture the antibody. This and other procedures for raising polyclonal antibodies are disclosed by (Harlow et al., 1988), which is hereby incorporated in the references. Although historically monoclonal antibodies were produced by immortalization of a clonally pure immunoglobulin secreting cell line, a monoclonally pure population of antibody molecules can also be prepared by the methods of the present invention. Laboratory methods for preparing monoclonal antibodies are well known in the art.

A “monoclonal antibody” or “mAb” in its various names refers to a population of antibody molecules that contains only one species of antibody combining site capable of immunoreacting with a particular epitope. A monoclonal antibody thus typically displays a single binding affinity for any epitope with which it immunoreacts. Monoclonal antibody may

also define an antibody molecule which has a plurality of antibody combining sites, each immunospecific for a different epitope. For example, a bispecific antibody would have two antigen binding sites, each recognizing a different interacting molecule, or a different epitope. As used herein, the terms “antibody fragment”, “antibody portion”, “antibody variant” and the like include any protein or polypeptide containing molecule that comprises at least a portion of an immunoglobulin molecule such as to permit specific interaction between said molecule and an antigen (e.g. HVEM). The portion of an immunoglobulin molecule may include, but is not limited to, at least one complementarity determining region (CDR) of a heavy or light chain or a ligand binding portion thereof, a heavy chain or light chain variable region, a heavy chain or light chain constant region, a framework region, or any portion thereof, or at least one portion of a ligand or counter-receptor (e.g. LIGHT, BTLA or HSV-gD) which can be incorporated into an antibody of the present invention to permit interaction with the antigen (e.g. HVEM).

Monoclonal antibodies (mAbs) may be prepared by immunizing a mammal such as mouse, rat, primate and the like, with purified HVEM protein. The antibody-producing cells from the immunized mammal are isolated and fused with myeloma or heteromyeloma cells to produce hybrid cells (hybridoma). The hybridoma cells producing the monoclonal antibodies are utilized as a source of the desired monoclonal antibody. This standard method of hybridoma culture is described in (Kohler and Milstein, 1975). Alternatively, the immunoglobulin genes may be isolated and used to prepare a library for screening for reactive specifically reactive antibodies. Many such techniques including recombinant phage and other expression libraries are known to one skilled in the art. While mAbs can be produced by hybridoma culture the invention is not to be so limited. Also contemplated is the use of mAbs produced by cloning and transferring the nucleic acid cloned from a hybridoma of this invention. That is, the nucleic acid expressing the molecules secreted by a hybridoma of this invention can be transferred into another cell line to produce a transformant. The transformant is genotypically distinct from the original hybridoma but is also capable of producing antibody molecules of this invention, including immunologically active fragments of whole antibody molecules, corresponding to those secreted by the hybridoma. See, for example, U.S. Pat. No. 4,642,334 to Reading; PCT Publication No.; European Patent Publications No. 0239400 to Winter et al. and No. 0125023 to Cabilly et al. Antibody generation techniques not involving immunisation are also contemplated such as for example using phage display technology to examine naive libraries (from non-immunised animals); see (Barbas et al.,

1992, and Waterhouse et al. (1993). Antibodies of the invention are suitably separated from the culture medium by conventional immunoglobulin purification procedures such as, for example, affinity, ion exchange and/or size exclusion chromatography, and the like.

5 In some embodiments, the antibody of the invention may be a human chimeric antibody. Said human chimeric antibody of the present invention can be produced by obtaining nucleic sequences encoding VL and VH domains, constructing a human chimeric antibody expression vector by inserting them into an expression vector for animal cell having genes encoding human antibody CH and human antibody CL, and expressing the expression
10 vector by introducing it into an animal cell. The CH domain of a human chimeric antibody may be any region which belongs to human immunoglobulin, but those of IgG class are suitable and any one of subclasses belonging to IgG class, such as IgG1, IgG2, IgG3 and IgG4, can also be used. Also, the CL of a human chimeric antibody may be any region which belongs to Ig, and those of kappa class or lambda class can be used. Methods for producing
15 chimeric antibodies involve conventional recombinant DNA and gene transfection techniques are well known in the art (See Morrison SL. et al. (1984) and patent documents US5,202,238; and US5,204, 244).

 In some embodiments, said antibody may be a humanized antibody. Said humanized
20 antibody may be produced by obtaining nucleic acid sequences encoding for CDRs domain by inserting them into an expression vector for animal cell having genes encoding a heavy chain constant region identical to that of a human antibody; and a light chain constant region identical to that of a human antibody, and expressing the expression vector by introducing it into an animal cell. The humanized antibody expression vector may be either of a type in
25 which a gene encoding an antibody heavy chain and a gene encoding an antibody light chain exist on separate vectors or of a type in which both genes exist on the same vector (tandem type). In respect of easiness of construction of a humanized antibody expression vector, easiness of introduction into animal cells, and balance between the expression levels of antibody H and L chains in animal cells, a tandem type of the humanized antibody expression
30 vector is more preferable (Shitara K et al. 1994). Examples of the tandem type humanized antibody expression vector include pKANTEX93 (WO 97/10354), pEE18 and the like. Methods for producing humanized antibodies based on conventional recombinant DNA and gene transfection techniques are well known in the art (See, e.g. Riechmann L. et al. 1988; Neuberger MS. et al. 1985). Antibodies can be humanized using a variety of techniques

known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO91/09967; U.S. Pat. Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan EA (1991); Studnicka GM et al. (1994); Roguska MA. et al. (1994)), and chain shuffling (U.S. Pat. No. 5,565,332). The general recombinant DNA
 5 technology for preparation of such antibodies is also known (see European Patent Application EP 125023 and International Patent Application WO 96/02576).

In some embodiments, the fragment of the antibody which blocks the interaction between BTLA and HVEM interaction is chosen among Fab (e.g., by papain digestion), Fab'
 10 (e.g., by pepsin digestion and partial reduction) and F(ab')₂ (e.g., by pepsin digestion), facb (e.g., by plasmin digestion), pFc' (e.g., by pepsin or plasmin digestion), Fd (e.g., by pepsin digestion, partial reduction and reaggregation), Fv or scFv (e.g., by molecular biology techniques) fragments. Such fragments may be produced by enzymatic cleavage, synthetic or recombinant techniques, as known in the art and/or as described herein. Antibodies can also
 15 be produced in a variety of truncated forms using antibody genes in which one or more stop codons have been introduced upstream of the natural stop site. The various portions of antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques. Said Fab fragment of the present invention can be obtained by treating an antibody which specifically reacts with
 20 human HVEM with a protease, papaine. Also, the Fab may be produced by inserting DNA encoding Fab of the antibody into a vector for prokaryotic expression system or for eukaryotic expression system, and introducing the vector into a prokaryote or eucaryote to express the Fab. Said F(ab')₂ of the present invention may be obtained by treating an antibody which specifically reacts with HVEM with a protease, pepsin. Also, the F(ab')₂ can be produced by
 25 binding Fab' described below via a thioether bond or a disulfide bond. Said Fab' may be obtained by treating F(ab')₂ which specifically reacts with HVEM with a reducing agent, dithiothreitol. Also, the Fab' can be produced by inserting DNA encoding Fab' fragment of the antibody into an expression vector for prokaryote or an expression vector for eukaryote, and introducing the vector into a prokaryote or eukaryote to effect its expression. Said scFv
 30 fragment may be produced by obtaining cDNA encoding the VH and VL domains as previously described, constructing DNA encoding scFv, inserting the DNA into an expression vector for prokaryote or an expression vector for eukaryote, and then introducing the expression vector into a prokaryote or eukaryote to express the scFv. To generate a humanized scFv fragment, a well known technology called CDR grafting may be used, which

involves selecting the complementary determining regions (CDRs) from a donor scFv fragment, and grafting them onto a human scFv fragment framework of known three dimensional structure (see, e. g., W098/45322; WO 87/02671; US5,859,205; US5,585,089; US4,816,567; EP0173494).

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In some embodiments, monoclonal antibodies of the invention are monovalent, bivalent, multivalent, monospecific, bispecific, or multispecific.

In some embodiments, the anti-HVEM antibody is a monoclonal antibody obtainable
10 from the hybridoma deposited at the Collection Nationale de Cultures de Microorganismes (CNCM, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France), in accordance with the terms of Budapest Treaty, on May 16, 2013, under the number CNCM I-4752 such as described in WO2014/184360. As used herein, the expression "HVEM 18.10" refers to an isolated HVEM antibody which is obtainable from the hybridoma accessible
15 under CNCM deposit number I-4752. In some embodiments, the anti-HVEM antibody is a monoclonal antibody which comprises a variable light chain (VL) comprising all the CDRs of the VL chain of the antibody obtainable from hybridoma deposited as CNCM I-4752; and a variable heavy chain (VH) comprising all the CDRs of the VH chain of the antibody obtainable from hybridoma deposited as CNCM I-4752.

In some embodiments, the anti-HVEM antibody is a monoclonal antibody obtainable
20 from the hybridoma deposited at the Collection Nationale de Cultures de Microorganismes (CNCM, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France), in accordance with the terms of Budapest Treaty, on April 26, 2007, under the numbers CNCM I-3752, CNCM I-3753 and CNCM I-3754 such as described in WO2008/146101. In some
25 embodiments, the anti-HVEM antibody is a monoclonal antibody which comprises a variable light chain (VL) comprising all the CDRs of the VL chain of the antibody obtainable from hybridoma deposited as CNCM I-3752, CNCM I-3753 or CNCM I-3754; and a variable heavy chain (VH) comprising all the CDRs of the VH chain of the antibody obtainable from hybridoma deposited as CNCM I-3752, CNCM I-3753 or CNCM I-3754.

30

In some embodiments, said antagonist is chosen from antibodies directed against BTLA and fragments thereof which block the interaction between BTLA and HVEM.

The previously disclosed technical features apply to said antibodies directed against BTLA.

In some embodiments, the antagonist is an antibody obtainable from a hybridoma deposited at the Collection Nationale de Cultures de Microorganismes (CNCM, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France), in accordance with the terms of Budapest Treaty, on February 4, 2009, under the number CNCM I-4123 such as described in WO2010/106051 and WO2014/184360. As used herein, the expression "BTLA8.2" refers to an isolated BTLA antibody which is obtainable from the hybridoma accessible under CNCM deposit number I-4123. In some embodiments, the BTLA antibody which comprises a variable light chain (VL) comprising all the CDRs of the VL chain of the antibody obtainable from hybridoma deposited as CNCM I-4123; and a variable heavy chain (VH) comprising all the CDRs of the VH chain of the antibody obtainable from hybridoma deposited as CNCM I-4123.

In some embodiments, the treatment consists in administering to the subject having a short survival time with a therapeutically effective amount of an anti-HVEM antibody capable of antigen-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody.

It is indeed possible to modify an anti-HVEM antibody with respect to effector functions, e.g. so as to enhance antigen-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing inter-chain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and/or antibody-dependent cellular cytotoxicity (ADCC) (Caron PC. et al. 1992; and Shopes B. 1992).

In some embodiments, the treatment consists in administering to the subject having a short survival time with a therapeutically effective amount of an anti-HVEM antibody conjugates to cytotoxic moiety.

The cytotoxic moiety may, for example, be selected from the group consisting of taxol; cytochalasin B; gramicidin D; ethidium bromide; emetine; mitomycin; etoposide; tenoposide; vincristine; vinblastine; colchicin; doxorubicin; daunorubicin; dihydroxy anthracin dione; a tubulin- inhibitor such as maytansine or an analog or derivative thereof; an
5 antimitotic agent such as monomethyl auristatin E or F or an analog or derivative thereof; dolastatin 10 or 15 or an analogue thereof; irinotecan or an analogue thereof; mitoxantrone; mithramycin; actinomycin D; 1-dehydrotestosterone; a glucocorticoid; procaine; tetracaine; lidocaine; propranolol; puromycin; calicheamicin or an analog or derivative thereof; an antimetabolite such as methotrexate, 6 mercaptopurine, 6 thioguanine, cytarabine, fludarabin,
10 5 fluorouracil, decarbazine, hydroxyurea, asparaginase, gemcitabine, or cladribine; an alkylating agent such as mechlorethamine, thioepa, chlorambucil, melphalan, carmustine (BSNU), lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, dacarbazine (DTIC), procarbazine, mitomycin C; a platinum derivative such as cisplatin or carboplatin; duocarmycin A, duocarmycin SA, rachelmycin (CC-1065), or an analog or
15 derivative thereof; an antibiotic such as dactinomycin, bleomycin, daunorubicin, doxorubicin, idarubicin, mithramycin, mitomycin, mitoxantrone, plicamycin, anthramycin (AMC)); pyrrolo[2,1-c][1,4]-benzodiazepines (PDB); diphtheria toxin and related molecules such as diphtheria A chain and active fragments thereof and hybrid molecules, ricin toxin such as ricin A or a deglycosylated ricin A chain toxin, cholera toxin, a Shiga-like toxin such as SLT
20 I, SLT II, SLT IIV, LT toxin, C3 toxin, Shiga toxin, pertussis toxin, tetanus toxin, soybean Bowman-Birk protease inhibitor, Pseudomonas exotoxin, alorin, saporin, modeccin, gelanin, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolacca americana proteins such as PAPI, PAPII, and PAP-S, momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin,
25 phenomycin, and enomycin toxins; ribonuclease (RNase); DNase I, Staphylococcal enterotoxin A; pokeweed antiviral protein; diphtherin toxin; and Pseudomonas endotoxin.

In some embodiments, the anti-HVEM antibody is conjugated to an auristatin or a peptide analog, derivative or prodrug thereof. Auristatins have been shown to interfere with
30 microtubule dynamics, GTP hydrolysis and nuclear and cellular division (Woyke et al (2001) Antimicrob. Agents and Chemother. 45(12): 3580-3584) and have anti-cancer (US5663149) and antifungal activity (Pettit et al., (1998) Antimicrob. Agents and Chemother. 42: 2961-2965. For example, auristatin E can be reacted with para-acetyl benzoic acid or benzoylvaleric acid to produce AEB and AEVB, respectively. Other typical auristatin derivatives include

AFP, MMAF (monomethyl auristatin F), and MMAE (monomethyl auristatin E). Suitable auristatins and auristatin analogs, derivatives and prodrugs, as well as suitable linkers for conjugation of auristatins to Abs, are described in, e.g., U.S. Patent Nos. 5,635,483, 5,780,588 and 6,214,345 and in International patent application publications WO02088172, WO2004010957, WO2005081711, WO2005084390, WO2006132670, WO03026577, WO200700860, WO207011968 and WO205082023.

Techniques for conjugating molecule to antibodies, are well-known in the art (See, e.g., Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy," in *Monoclonal Antibodies And Cancer Therapy* (Reisfeld et al. eds., Alan R. Liss, Inc., 1985); Hellstrom et al., "Antibodies For Drug Delivery," in *Controlled Drug Delivery* (Robinson et al. eds., Marcel Dekker, Inc., 2nd ed. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review," in *Monoclonal Antibodies '84: Biological And Clinical Applications* (Pinchera et al. eds., 1985); "Analysis, Results, and Future Prospective of the Therapeutic Use of Radiolabeled Antibody In Cancer Therapy," in *Monoclonal Antibodies For Cancer Detection And Therapy* (Baldwin et al. eds., Academic Press, 1985); and Thorpe et al., 1982, *Immunol. Rev.* 62:119-58. See also, e.g., PCT publication WO 89/12624.) Typically, the nucleic acid molecule is covalently attached to lysines or cysteines on the antibody, through N-hydroxysuccinimide ester or maleimide functionality respectively. Methods of conjugation using engineered cysteines or incorporation of unnatural amino acids have been reported to improve the homogeneity of the conjugate (Axup, J.Y., Bajjuri, K.M., Ritland, M., Hutchins, B.M., Kim, C.H., Kazane, S.A., Halder, R., Forsyth, J.S., Santidrian, A.F., Stafin, K., et al. (2012). Synthesis of site-specific antibody-drug conjugates using unnatural amino acids. *Proc. Natl. Acad. Sci. USA* 109, 16101–16106.; Junutula, J.R., Flagella, K.M., Graham, R.A., Parsons, K.L., Ha, E., Raab, H., Bhakta, S., Nguyen, T., Dugger, D.L., Li, G., et al. (2010). Engineered thio-trastuzumab-DM1 conjugate with an improved therapeutic index to target human epidermal growth factor receptor 2-positive breast cancer. *Clin. Cancer Res.* 16, 4769–4778.). Junutula et al. (2008) developed cysteine-based site-specific conjugation called "THIOMABs" (TDCs) that are claimed to display an improved therapeutic index as compared to conventional conjugation methods. Conjugation to unnatural amino acids that have been incorporated into the antibody is also being explored for ADCs; however, the generality of this approach is yet to be established (Axup et al., 2012). In particular the one skilled in the art can also envisage Fc-containing polypeptide engineered with an acyl donor glutamine-containing tag (e.g., Gin-

containing peptide tags or Q- tags) or an endogenous glutamine that are made reactive by polypeptide engineering (e.g., via amino acid deletion, insertion, substitution, or mutation on the polypeptide). Then a transglutaminase, can covalently crosslink with an amine donor agent (e.g., a small molecule comprising or attached to a reactive amine) to form a stable and

5 homogenous population of an engineered Fc-containing polypeptide conjugate with the amine donor agent being site- specifically conjugated to the Fc-containing polypeptide through the acyl donor glutamine- containing tag or the accessible/exposed/reactive endogenous glutamine (WO 2012059882).

10 In some embodiments, the subject is administered with a further immune checkpoint inhibitor. As used herein, the term "immune checkpoint inhibitor" refers to molecules that totally or partially reduce, inhibit, interfere with or modulate one or more checkpoint proteins. Checkpoint proteins regulate T-cell activation or function. Numerous checkpoint proteins are known, such as CTLA-4 and its ligands CD 80 and CD86; and PD1 with its ligands PDL1 and

15 PDL2 (Pardoll, Nature Reviews Cancer 12: 252-264, 2012). These proteins are responsible for co-stimulatory or inhibitory interactions of T-cell responses. Immune checkpoint proteins regulate and maintain self-tolerance and the duration and amplitude of physiological immune responses. Immune checkpoint inhibitors include antibodies or are derived from antibodies. In some embodiments, the immune checkpoint inhibitor is an antibody selected from the group

20 consisting of anti-CTLA4 antibodies (e.g. Ipilimumab), anti-PD1 antibodies, anti-PDL1 antibodies, anti-TIMP3 antibodies, anti-LAG3 antibodies, anti-B7H3 antibodies, anti-B7H4 antibodies, and anti-B7H6 antibodies. Examples of anti-CTLA-4 antibodies are described in US Patent Nos: 5,811,097; 5,811,097; 5,855,887; 6,051,227; 6,207,157; 6,682,736; 6,984,720; and 7,605,238. One anti-CDLA-4 antibody is tremelimumab, (ticilimumab, CP-

25 675,206). In some embodiments, the anti-CTLA-4 antibody is ipilimumab (also known as 10D1, MDX-D010) a fully human monoclonal IgG antibody that binds to CTLA-4. Another immune checkpoint protein is programmed cell death 1 (PD-1). Examples of PD-1 and PD-11 blockers are described in US Patent Nos. 7,488,802; 7,943,743; 8,008,449; 8,168,757; 8,217,149, and PCT Published Patent Application Nos: WO03042402, WO2008156712,

30 WO2010089411, WO2010036959, WO2011066342, WO2011159877, WO2011082400, and WO2011161699. In some embodiments, the PD-1 blockers include anti-PD-L1 antibodies. In certain other embodiments the PD-1 blockers include anti-PD-1 antibodies and similar binding proteins such as nivolumab (MDX 1106, BMS 936558, ONO 4538), a fully human IgG4 antibody that binds to and blocks the activation of PD-1 by its ligands PD-L1 and PD-

L2; lambrolizumab (MK-3475 or SCH 900475), a humanized monoclonal IgG4 antibody against PD-1 ; CT-011 a humanized antibody that binds PD-1 ; AMP-224 is a fusion protein of B7-DC; an antibody Fc portion; BMS-936559 (MDX- 1105-01) for PD-L1 (B7-H1) blockade. Other immune-checkpoint inhibitors include lymphocyte activation gene-3 (LAG-3) inhibitors, such as IMP321, a soluble Ig fusion protein (Brignone et al., 2007, J. Immunol. 179:4202-4211). Other immune-checkpoint inhibitors include B7 inhibitors, such as B7-H3 and B7-H4 inhibitors. In particular, the anti-B7-H3 antibody MGA271 (Loo et al., 2012, Clin. Cancer Res. July 15 (18) 3834). Also included are TIM3 (T-cell immunoglobulin domain and mucin domain 3) inhibitors (Fourcade et al., 2010, J. Exp. Med. 207:2175-86 and Sakuishi et al., 2010, J. Exp. Med. 207:2187-94).

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1: HVEM expression is heterogeneous in melanoma metastasis. (A) Repartition of HVLE global score in the entire cohort: 61/126 were HVEM low (score 0-5) and 59/126 HVEM high score (6-12). (B) Distribution of high and low HVE expression among metastatic sites. (C) Representative case of low and high HVEM expression in melanoma metastasis.

Figure 2: Survival from the date of excision of 126 melanoma metastasis in relation to their tumor HVEM status. Patients with high HVEM expression had a significantly poorer prognosis than those with low expression.

Figure 3: Representation of the intake of HVEM expression to predict prognosis from biopsy: predictive comparison curves.

Figure 4: HVEM is widely expressed on melanoma cells whereas its ligands are not: flow cytometry. CEF : n=14 (HVEM), n=11 (CD160), n=8 (LIGHT), n=6 (BTLA).

Figure 5: HVEM and BTLA are largely expressed on melanoma TILS while LIGHT is expressed under specific circumstances and CD160 is not. n=17.

Figure 6: Anti-HVEM, anti-BTLA and anti-HVEM+BTLA antibodies induce an increase of TNF α production in CD8⁺ T cells isolated from Peripheral Blood Mononuclear Cells (PBMC). n=13 Wilcoxon matched pair signed rank tests NS.

EXAMPLES:**EXAMPLE 1:****Material & Methods*****Patients***

5 One hundred twenty-six formol-fixed paraffin embedded samples of melanoma metastases were collected consecutively to tumor excision, for tumor mass sterilization or palliative care, between 2009 and 2012, from the Departments of Pathology of La Timone Hospital, Marseille and from Guy de Chauliac Hospital, Montpellier. The 93 tissue samples from Marseille were analyzed by tissue microarrays (TMA), on four representative 0,6 millimeters
 10 cores taken from each tumor sample, after pathological selection, while the 33 samples from Montpellier were studied on whole slide cuts, to confirm the absence of massive tumor heterogeneity.

The clinical and pathological characteristics of the patients are summarized in **Table I**. The median follow-up for patients was 15.75 months with a range of 0 to 76.80 months. Written
 15 informed consent was obtained from all living patients and the present study approved by institutional board.

Immunohistochemistry

Immunohistochemistry (IHC) for HVEM was performed using anti-HVEM antibody (94804, R&D) used at a 1:150 dilution. Additional immunochemistries were performed using
 20 Melan-A (A103, Cell-marque) and PS100 (RP035, Cliniscience) to stain melanoma cells and CD68 (KP1, Dako), CD163 (10D6, Leica) to stain histiocytes/macrophages. Four micrometers thick sections were cut and mounted on SuperFrost Plus adhesive slides (Thermoshandon, Pittsburgh, PA). IHC was performed on an automated immunostainer Benchmark XT (Ventana Medical Systems Inc, Roche, Tucson, AZ, USA), using indirect
 25 biotin-free system based on polymer (Ultraview universal RED kit, Ventana medical Systems Inc.).

HVEM expression scoring

A pathologist without prior knowledge of the patient's clinical status reviewed each tumor's TMA spots for HVEM signal intensity. Normal lymphoid tissue, taken from a patient
 30 without history of malignancy, was used as a control and standard for HVEM IHC. HVEM signal intensity of each spot was scored from 0 to 3, relatively to normal lymphoid tissue, which was scored 2+. Thus, spot's intensity ranged from 0 (completely negative), 1 (weak: very faint cytoplasmic signal only visible at high magnification and inferior to the signal of the standard), 2 (intermediate signal, equals to the signals of standard lymphocytes) or 3 (the

most intense signal noted on the TMA matrix, superior to the signal of the standard) (**Fig 1.C**). To account for HVEM signal heterogeneity, the 4 spots were analyzed within a given tumor sample using a global score. This global score was calculated secondly to the initial evaluation and corresponded to the sum of the intensity of each of the 4 analyzed TMA spots for each sample and thus ranged from 0 to 12. When less than 4 TMA spots were analyzed, the global score was normalized. In a few cases, whole slides were analyzed and HVEM signal intensity was scored similarly, but using lymphocytes present on the slide as a 2+ standard. To account signal heterogeneity, HVEM was scored on 4 different but representative parts of the slide. For each parts of the slide reviewed, HVEM intensity was interpreted globally and only the highest score was retained when signal heterogeneity was present. A global score, equal to the sum of the intensity of the 4 part of the slide, was calculated. TMA spots or whole slide cases were excluded of the analysis when no tumor cells were present (confirmed by the absence of staining for Melan-A and/or PS100), when HVEM⁺ macrophages (identified using CD68 and/or CD163 expression) were numerous and interfered with interpretation and when melanin pigment would interfere with interpretation.

Statistical analysis

Survival curves were calculated using the Kaplan-Meier method to estimate the probability of survival and significance was assessed by the log-rank test. Multivariate analysis was performed by the Cox proportional hazards model. A p value < 0.05 was considered to be statistically significant.

Results

HVEM expression in melanoma metastasis

We first evaluated HVEM expression by immunohistochemistry in 126 melanoma metastases. Cancer cells positive staining for HVEM were observed at the cell membrane, the cytoplasm or both in 115 of 126 samples (91.3 %) and without intra-specimen heterogeneity in 90 % of cases.

To explore the relevance of HVEM expression in melanoma metastases, we divided the cohort in 2 groups (**Fig 1.A**), 53.2 % of the specimen had a low expression of HVEM (≤ 50 %) whereas 46.8 % of them had a high HVEM expression (>50 %). We also noted that HVEM showed an heterogeneous expression on melanoma metastasis regardless of their site of origin (**Fig 1.B**).

Therefore, the majority of the analyzed melanoma metastasis expressed HVEM and such expression straddles a large range of intensities that does not correlate with the metastatic site.

Correlating clinical characteristics and levels of HVEM expression

We correlated next the levels of HVEM expression with the different clinical and histological markers. Initials prognosis markers in melanoma (age, sex, Breslow index, presence of an ulceration, positive sentinel lymph node biopsy and AJCC stage) were found similarly distributed between the high and low HVEM expression group. Likewise AJCC stage at the date of tumor excision, the occurrence of treatment received at a pre- and post-excision stage and the B-Raf status were similarly distributed between the high and low HVEM expression groups (**Table 1**).

43 patients had received an adjuvant or curative treatment before the excision of the lesion subjected to our analysis. Although the number of analyzed patients is small, HVEM expression appeared unchanged regardless of the treatment received. On the other hand, analysis of the HVEM status of metastasis of patient that received targeted therapies intended to block the MAP kinase pathway indicated the presence of more responders (71 %) in the low expression HVEM group than in the high expression HVEM group (50 %), though these data need to be further confirmed using a larger cohort. In the case of other therapies used against melanoma such as immunotherapies (anti CTLA-4 and anti-PD-1) and conventional chemotherapies (Dacarbazine and Fotemustine) no difference of responses post-biopsy were found. Therefore, HVEM expression does not seem to be associated neither to classic histoprognostics indexes in melanoma, nor modified by prior treatment.

Impact on survival of the levels of HVEM expression on tumor cells

Next, we correlate the magnitude of HVEM expression by melanoma metastases with survival from the date of the metastatic biopsy. We found that patients with high HVEM expression had a significantly poorer survival from the date of excision than those with a low expression ($p = 0.0182$) with 12.5 months and 24.2 months median survival respectively (**Fig. 2**). For the 78 melanoma metastases treated for tumor mass sterilization, no difference in progression-free survival was shown.

Prognostic value of tumor HVEM expression in melanoma

Finally, we evaluated the prognostic value of the levels of HVEM expression by melanoma metastasis. In univariate analysis, excision goal (resection for palliative care or tumor mass excision), AJCC stage, presence of cerebral metastasis, number of metastatic sites, tumor volume, LDH (Lactate Deshydrogenase) level, occurrence of modern treatment (modern treatment = targeted therapies, anti-CTLA-4 or anti-PD1) and HVEM expression were all significantly associated with survival from the date of excision (**Table 2**). Multivariate analysis revealed that in term of survival from the date of excision, resection for palliative

care or tumor mass sterilization ($p=0.0376$), presence of cerebral metastasis ($p=0.0030$), tumor index level ($p=0.0019$), LDH level ($p=0.0302$), occurrence of modern treatment ($p=0.0043$) and HVEM expression ($p=0.0146$) were statistically significant.

Predictive comparison curves indicated that median survival without HVEM expression was evaluated at 18.5 months. The inclusion of HVEM expression splits the population in 2 groups: the first group expressed low level of HVEM and showed a 24.2 months median survival whereas the second group expressed high level of HVEM and showed a 13.7 months median survival (Fig.3). Altogether, our results confirmed the heterogeneous expression of HVEM in melanoma metastases, regardless of the metastatic site. We found that patients with a high HVEM expression in melanoma metastasis had a significantly poorer survival from the date of excision than those with a low expression of HVEM, with an independent prognosis value. Therefore, HVEM and its ligands likely constitute a candidate pathway of therapeutic interest.

Discussion:

Immunotherapies strategies based on the blockade of co-inhibitory molecules intend to boost host immune responses against tumors and circumvent tumor evasion strategies. In that context, blockade of the HVEM-BTLA pathways or enhancement of the HVEM/LIGHT pathway could be of therapeutic value in melanoma. Therefore, it was important to assess whether high levels of HVEM expression by melanoma metastases was associated with a poor clinical outcome or not.

For that purpose we confirmed that HVEM was expressed by melanoma metastases in that 91.3 % of the 126 specimens we analyzed, turned out to express HVEM. Our data are consistent with a previous study by Derré and colleagues (9) that showed the expression of HVEM on 87.5 % of the 16 analyzed metastases. Moreover, using a RED staining, allowing us to avoid biased interpretation due to the presence of melanin, our work genuinely demonstrates the presence of HVEM expression by melanoma cells, that HVEM expression shows heterogeneous levels permitting to define high and low expression groups, regardless of the metastatic localization. Importantly, analyzing 4 representatives tumor sample for each TMA spot and extending in a few cases our analysis to the whole slide revealed that only 10 % of the samples showed an intra-tumor heterogeneity.

To correlate the levels of HVEM expression to the clinical data, we limited the cohort to samples collected 3 years ago to allow a proper segregation between live and death events. Classical histoprognois factors at diagnosis, treatment received after the study and

B-Raf status were evenly distributed between the low and high HVEM expression group, despite our analysis of a limited number of patients. The presence or a highest rate of responders to B-Raf inhibitors in the low HVEM expression group (70.6%) than in the high expression group (50%) is likely due to the small amount of patients treated by targeted therapies in this study. But it need to be deepened as a recent publication demonstrates, in melanoma, that the absence of tumoral membrane PD-L1 staining were associated with a better responses to B-Raf inhibitor treatment (10). Thus, it could support the hypothesis that a more hostile tumor microenvironment, as overexpression of PD-L1 and HVEM, could be associated with a worse outcome in B-RAF V600 mutated melanoma patients receiving B-Raf inhibitors.

Finally, the most important finding of our studies was that patients with melanoma metastases expressing high levels of HVEM had a significant poorer survival from the date of excision than those with low HVEM expression in univariate and more important in multivariate analysis. These results matched prior results obtained in three other solid tumors: corresponding to esophageal squamous cell carcinoma (6), hepatocellular carcinoma (8) and colorectal cancer (7), suggesting a role of HVEM expression in tumor progression. The strength of the association in melanoma is lower than in hepatocellular carcinoma but similar to esophageal squamous cell carcinoma and colorectal cancer. This can be explained in part by the fact that we studied metastasis and not primitive tumors. The use of metastatic samples is due to their convenient access, in comparison to primitive tumors, that are often resected in private practices. However, the decision to perform a surgical procedure is not random but guided by a precise purpose, therefore causing a loss of representativeness of the cohort. To offset such possible bias, we added in the multivariate analysis the purpose of the surgery: tumor mass sterilization or palliative care. This last variable together with the confounding variables accounted for in the multivariate analysis was strongly positive in univariate analysis, indicating the absence of major sampling bias. In contrast to report on esophageal squamous cell carcinoma and colorectal cancer, progression free survival from the date of surgery was not significant for the 78 melanoma patients analyzed in this study, due either to the small size of our panel or to a lack of effect.

In conclusion, this preliminary study provides new information on the role of HVEM in melanoma, confirming the wide variation existing in HVEM expression among melanoma metastasis and establishing a significant correlation between high expression of HVEM in melanoma metastasis and poor clinical outcomes. Further studies will be needed

to decipher the mechanisms regulating the variable levels of HVEM regulation in solid tumors. Pre-clinical studies using mouse models will also permit to establish whether high levels of HVEM expression on melanoma dampen anti-tumor immune responses, making HVEM a promising target for antibody-mediated ‘checkpoint blockade’ therapy.

Variables	Cohort characteristics	HVEM Expression	
		Low	High
<u>At initial diagnosis</u>			
Sex			
men	72	34	38
women	54	33	21
Median age		56 (19-89)	54 (25-86)
Breslow index (B)			
B ≤ 1 mm	11	4	7
1,01 > B < 2,01 mm	32	16	16
2,01 > B < 4 mm	27	18	9
B > 4mm	35	18	17
Absence of primitive tumor	21	12	9
Tumor ulceration positive ^(a)	39 (83)	25 (46)	14 (37)
SLNB positives / nb of SLNB procedures	27 (49)	14 (28)	13 (21)
AJCC stage			
IA	9	4	5
IB	28	10	18
IIA	15	8	7
IIB	17	9	8
IIC	14	5	9
IIIA	13	7	6
IIIB	14	9	4
IIIC	12	6	6
IV	4	1	3
<u>At biopsy time</u>			
Highest TNM stage			
N1	8	5	3
N2	35	22	13
N3	25	13	12
M1a	4	1	3
M1b	7	5	2
M1c	47	21	26
Treatment received before the study^b			
Adjuvant interferon	20	9	11
Adjuvant mel 005	3	1	2
Targeted therapies	4	3	1
anti-CTLA 4	4	3	1
Dacarbazine /Fotemustine	16	9	7
Treatment received after the study^b			
Targeted therapies	20 (16)	17 (12)	8(4)
anti-CTLA 4	19 (11)	9(5)	10 (6)
anti-PD1	11 (11)	6 (6)	5 (5)
Dacarbazine /Fotemustine	33 (5)	20 (4)	13 (1)
Tumor B-Raf status ^(a)	30 (67)	19 (32)	11 (35)

Table 1: Clinicopathological characteristics according to HVEM expression in melanoma metastases.

Abbreviation: SLNB, Sentinel Lymph Node Biopsy.

a (Number of patients with data available)

b- One same patient could have received different treatments.

Variables at the time of excision	No.	Univariate analysis			Multivariate analysis		
		HR	95% IC	p ^a	HR	95% IC	p ^b
Palliative care / Tumor mass sterilization	48 / 78	3.27	2.94-8.00	<0.0001	2.15	1.05-4.44	0.0376
TNM-AJCC (M-IV / N-III)	58 / 68	1.98	1.36-3.23	<0.0001	1.70		0.1779
Cerebral metastasis (Yes / No)	32 / 93	2.15	1.55-4.26	0.0003	2.64	1.39-5.00	0.0030
Nb of metastatic sites (>3 / <3)	40/86	2.73	2.25-6.47	<0.0001	0.78	0.31-2.00	0.6118
Global tumor volume (High / Medium / Low) ^c	33/32/61			<0.0001	3.24	1.54-6.79	0.0019
LDH (High/ Low)	33/93	2.9	2.60-8.53	<0.0001	2.03	1.07-3.85	0.0302
Receiving Modern treatment (Yes/ No)	65/61	0.76	0.50-1.15	0.1952	2.02		0.0043
HVEM (High/ Low)	59/67	1.68	1.12-2.60	0.0136	1.70	1.11-2.61	0.0146

Table.2. Univariate and multivariate analysis of factors associated with survival from the time of excision.

a- data were obtained by the Logrank test

b- data were obtained from the Cox proportional hazards model.

c- Global tumor volume was assessed on CT scan : Low (<5cm²), Medium (5-10cm²) and High (>10cm²).

EXAMPLE 2:

The inventors show that HVEM is widely expressed on melanoma cells whereas its ligands are not (Figure 4). The inventors also show that HVEM and BTLA are largely expressed on melanoma TILS while LIGHT is expressed under specific circumstances and CD160 is not (Figure 5).

The inventors also demonstrated that anti-HVEM, anti-BTLA and anti-HVEM+BTLA antibodies induce an increase of TNF α production in CD8⁺ T cells isolated from Peripheral Blood Mononuclear Cells (PBMC) (Figure 6).

REFERENCES:

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

1. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 19 août 2010;363(8):711-23.

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Pembrolizumab versus Ipilimumab in Advanced Melanoma. *N Engl J Med.* 25 juin 2015;372(26):2521-2532.

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CLAIMS:

1. A method for predicting the survival time of a subject suffering from melanoma metastases comprising i) determining the expression level of HVEM in a metastasis tissue sample ii) comparing the expression level determined at step i) with a predetermined reference value and iii) concluding that the subject will have a short survival time when the expression level determined at step i) is higher than the predetermined reference value or concluding that the subject will have a long survival time when the expression level determined at step i) is lower than the predetermined reference value.
2. The method of claim 1 wherein the expression level of HVEM is determined by immunohistochemistry.
3. The method of claim 1 wherein the subject having a short survival time is administered with a therapeutically effective amount of an antagonist of the BTLA/HVEM interaction.
4. The method of claim 3 wherein the antagonist of the BTLA/HVEM interaction is an antibody directed against HVEM or BTLA.
5. The method of claim 1 wherein the subject having a short survival time is administered with a therapeutically effective amount of an anti-HVEM antibody conjugates to cytotoxic moiety.

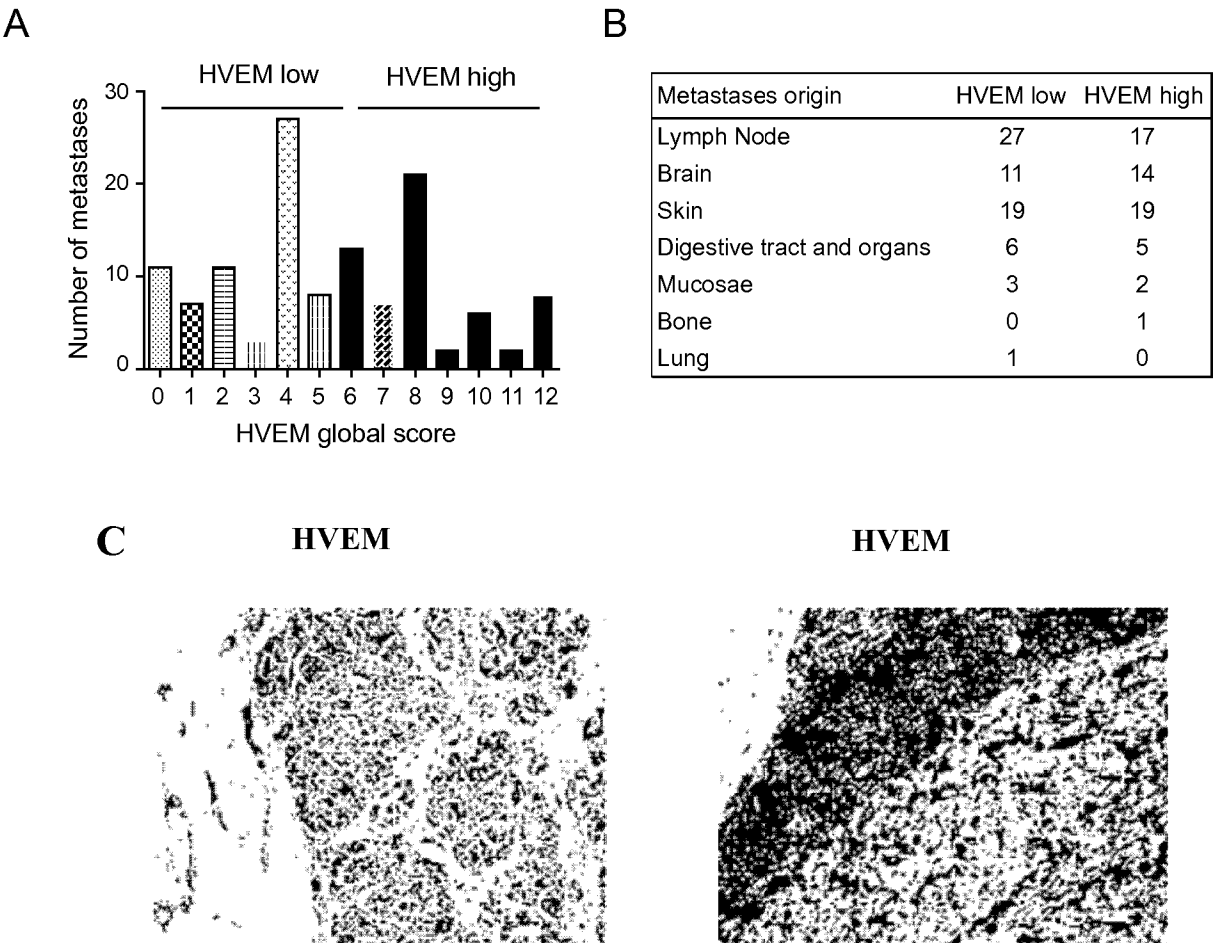


Figure 1

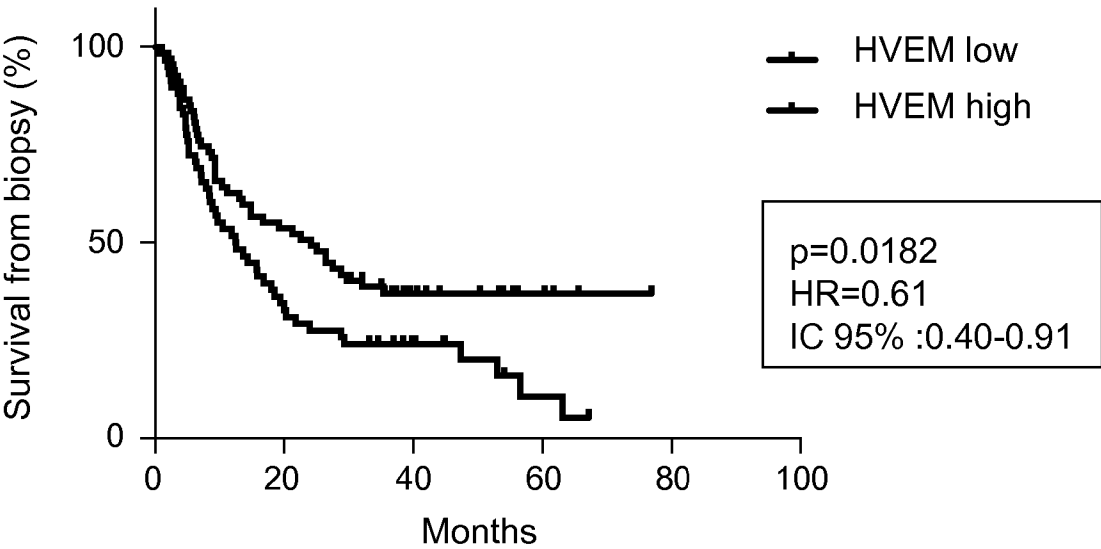


Figure 2

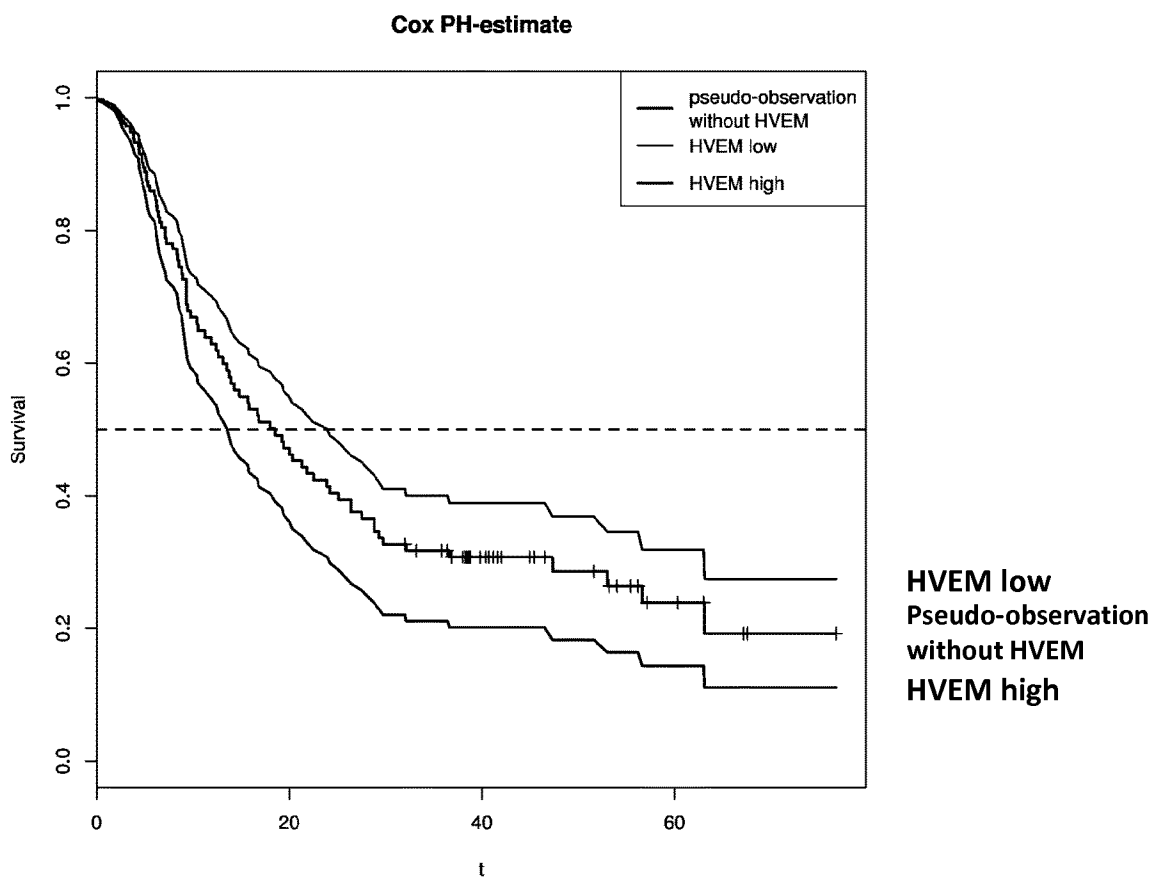


Figure 3

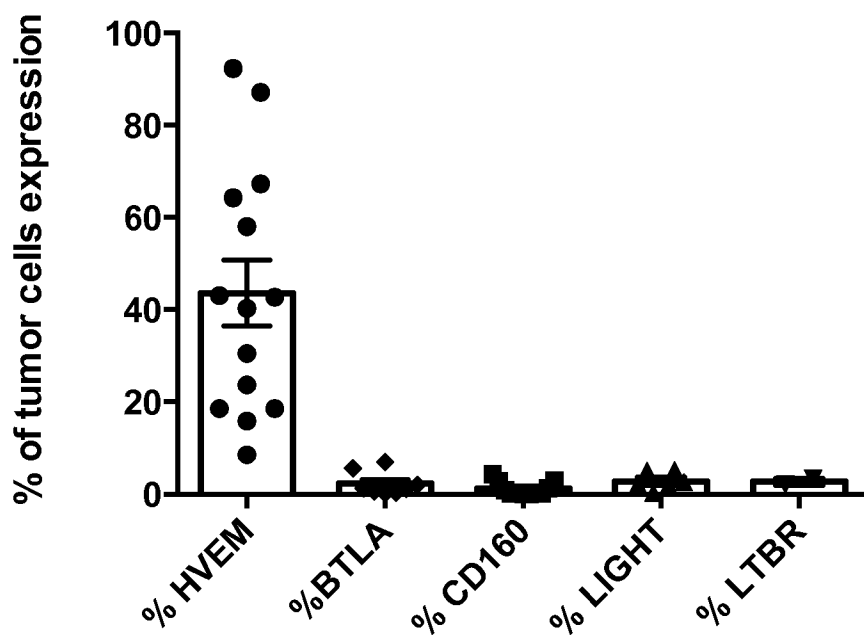


Figure 4

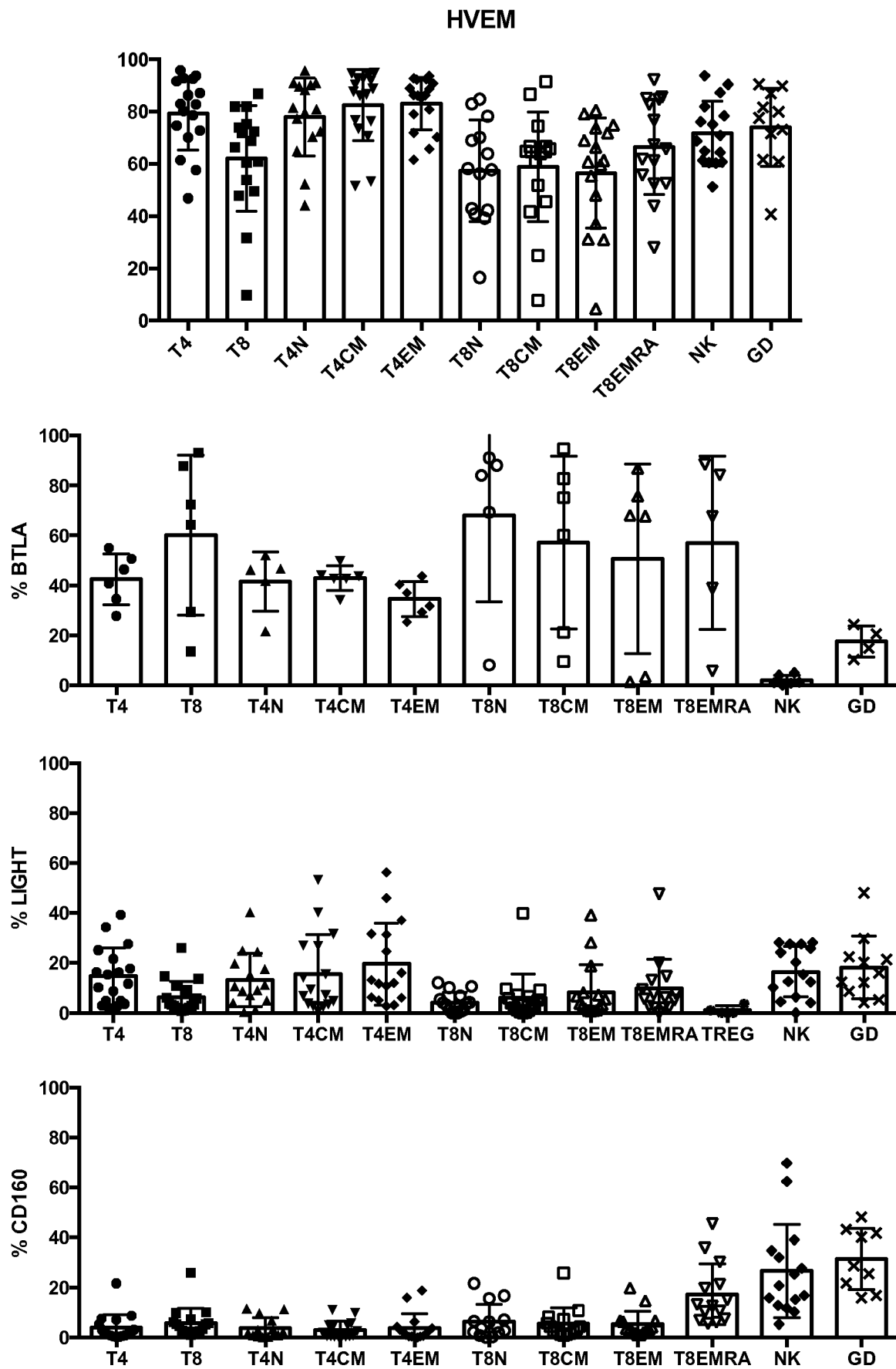


Figure 5

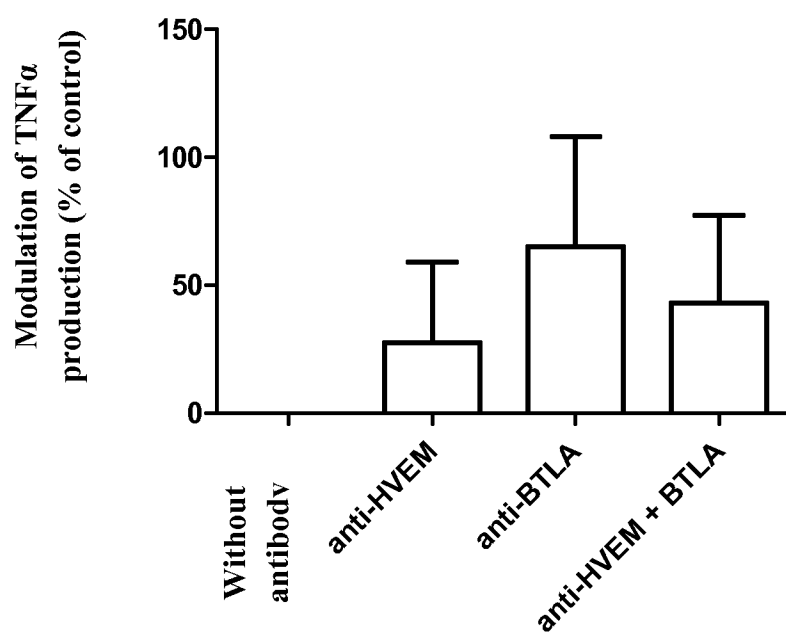


Figure 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/073951A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/574 A61K39/395
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

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

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

EPO-Internal, BIOSIS, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TAKASHI INOUE ET AL: "HVM expression contributes to tumor progression and prognosis in human colorectal cancer", ANTICANCER RESEARCH, vol. 35, no. 3, 1 March 2015 (2015-03-01), pages 1361-1368, XP055251707, Greece abstract ----- -/--	1-5



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 November 2016

Date of mailing of the international search report

02/12/2016

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Lunter, Pim

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/073951

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DERRÉ LAURENT ET AL: "BTLA mediates inhibition of human tumor-specific CD8+ T cells that can be partially reversed by vaccination", JOURNAL OF CLINICAL INVESTIGATION, AMERICAN SOCIETY FOR CLINICAL INVESTIGATION, US, vol. 120, no. 1, 28 December 2009 (2009-12-28), pages 157-167, XP009135446, ISSN: 0021-9738 abstract -----	1-5
A	CHRYSTAL M. PAULOS ET AL: "Putting the brakes on BTLA in T cell-mediated cancer immunotherapy", JOURNAL OF CLINICAL INVESTIGATION, vol. 120, no. 1, 4 January 2010 (2010-01-04), pages 76-80, XP055143199, ISSN: 0021-9738, DOI: 10.1172/JCI41811 abstract -----	1-5
A	CARA HAYMAKER: "BTLA: new biomarker for a highly proliferative CD8+ TIL subset associated with melanoma regression during adoptive cell therapy (P2189)", THE JOURNAL OF IMMUNOLOGY, vol. 190, 24 October 2013 (2013-10-24), page 170.51, XP055251709, abstract -----	1-5
A	CHRISTINE PASERO ET AL: "Interfering with coinhibitory molecules: BTLA/HVEM as new targets to enhance anti-tumor immunity", IMMUNOLOGY LETTERS., vol. 151, no. 1-2, 21 February 2013 (2013-02-21), pages 71-75, XP055251710, NL ISSN: 0165-2478, DOI: 10.1016/j.imlet.2013.01.008 abstract -----	3-5