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(54) Title: METHOD FOR PREDICTING THE OUTCOME OF A CANCER

(57) Abstract: The present invention relates to the prognostic of cancer and particularly renal cancer. Here, the inventors have investigated the presence and impact of C1q, produced by the macrophages, in the ccRCC TME and showed that it is associated with poor prognosis, particularly in patients with advanced and metastatic tumors. They propose that this is due to the activation of at least the early steps of the complement. Their data provide a novel mechanism of immune modulation of TME in ccRCC that may explain the particularly poor clinical impact of the TME in these tumors. Thus, the invention relates to a method for predicting the survival time of a patient suffering from a cancer by determining the expression level of C1q.

METHOD FOR PREDICTING THE OUTCOME OF A CANCER

FIELD OF THE INVENTION:

5 The present invention is in the field of cancer. More particularly, the invention relates to a method for predicting the survival time of a patient suffering from a cancer.

BACKGROUND OF THE INVENTION:

Renal cell cancer (RCC) is responsible for over 140 000 deaths per year. RCC 10 encompasses different histological subtypes, the far most frequent being the clear cell RCC (ccRCC), representing 75% of RCC. ccRCC still represents an unmet medical need, particularly at the metastatic stage when surgery is inappropriate or inefficient. Progress has, however, been made based on better knowledge of the histology of ccRCC. Mutations of the VHL gene, responsible for the Von Hippel-Lindau Syndrome are found in 80% of ccRCC. Such mutations 15 result in the activation of the transcription factor HIF (hypoxia inducible factor), which regulates genes involved in the angiogenesis, cell proliferation and survival. (1) This particular pathway has led to the successful development of anti-angiogenic treatment of ccRCC, which improves patients' clinical outcome. However, these treatments are efficient only in a subset of patients and acquired resistance limits their use. (2) More recently it has been reported that 20 some patients with ccRCC respond to checkpoint blockade therapy with antibody directed to PD-1 (programmed cell death protein 1), which allows revigoration of potential antitumoral T cells. A moderate but significant increase in overall survival (OS) has been reported in a subset of patients (3).

To advance in the conception of innovative therapies in ccRCC it is essential to better 25 understand the tumor and its microenvironment. (4) (+Nat rev Cancer) Indeed, ccRCC is particular in terms of its microenvironment and of how it impacts patients' clinical outcome. In addition to the high vascularization of the tumors (due in part to the VHL mutation) (1), many ccRCC tumors present with a strong and inflammatory cell infiltration. Precise analyses of ccRCC tumors revealed particular behaviors and clinical impact of the immune and 30 inflammatory tumor microenvironment (TME). In a subset of patients, tumors present with a structured immune landscape with mature dendritic cells (DC) within tertiary lymphoid structures (TLS) allowing T cell differentiation and regulation. (5) In these patients the density of CD8+ T cells in the TME correlates with longer progression free survival (PFS) and OS. (6) However, the vast majority of ccRCC tumors have either low density immune infiltrate or

disorganized TME with high density of CD8+ T cells correlating with shorter PFS and OS. The CD8+ T cells in these tumors show an exhausted phenotype, with expression of PD-1, LAG3 (CD223, Lymphocyte-activation gene 3) and TIM-3 (T-cell immunoglobulin and mucin-domain containing-3, HAVCR2), accompanied by expression of PD-1 ligands (PDL-1 and PDL-2) on tumor cells. These CD8+ T cells do not produce perforin and exhibit a very diverse inflammatory type T cell repertoire. A major characteristic of these tumors is a strong infiltration with inflammatory cells, particularly macrophages. One may hypothesize that this chronic inflammatory context, via factors locally produced by the inflammatory cells, may modulate the T cells content of the TME, making it deleterious for the patient. (7) Macrophages represent a major component of the TME and in most tumors, including ccRCC, are considered to be predominantly of M2 phenotype, favoring cancer growth, neovascularization and invasion (8, 9). Recent studies suggested a complex landscape of macrophages phenotypes in ccRCC, some of which (named M5) correlate with CD8 T cells exhaustion and decreased PFS. (10) Understanding the mechanisms by which the inflammatory cells and local inflammation modulate the clinical impact of the TME is essential to design novel targets for immunotherapy in order to restore an efficient immune reaction. Several factors, such as IDO, CTLA-4, LAG3, TIM3, TGF-b, VEGF, etc have been identified and immune modifying agents acting on these molecules have been subject to several studies for combination therapy with PD1/PDL1 inhibitors in order to improve clinical outcome (2, 11).

Complement system is one of the key factors for tissue inflammation (12) and the kidney is capable to produce the whole spectrum of complement components. (13, 14) Nevertheless, the role of complement activation for the progression of ccRCC remains poorly understood. In the present work we address the capacity of the components of the complement system to modulate the TME and to impact the clinical outcome. Complement is a major player in the anti-infections responses and also entertains chronic inflammation through the anaphylatoxins C3a and C5a, which are generated upon complement activation. (12) Complement activation can be induced by three different pathways – classical, lectin and alternative, depending on the context and the presence of distinct activator molecules. (15) Classical pathway is initiated by C1 complex activation, after recognition of the target structure (frequently, but not restricted to, IgG or IgM containing immune complexes) by C1q and subsequent activation of C1r and C1s. C1s is a serine protease, which cleaves C4 and C2, initiating thus the complement cascade. This may result in either beneficial effects, by killing the tumor cells by the lytic membrane attack complex or deleterious effects, through the production of pro-inflammatory and immunomodulatory complement fragments. (16) Mouse models, where complement genes

have been invalidated, showed that lack of C1q or later components may allow a better control of grafted tumors (17, 18). In the tested mouse melanoma model, though, the pro-tumoral role of C1q was considered complement-independent, related to direct effects of C1q on the tumor and endothelial cells (17).

5

SUMMARY OF THE INVENTION:

Here, the inventors have investigated the presence and impact of C1q, produced by the macrophages, in the ccRCC TME and showed that it is associated with poor prognosis, particularly in patients with advanced and metastatic tumors. They propose that this is due to
10 the activation of at least the early steps of the complement. Their data provide a novel mechanism of immune modulation of TME in ccRCC that may explain the particularly poor clinical impact of the TME in these tumors.

Thus, the present invention relates to a method for predicting the survival time of a patient suffering from a cancer comprising i) determining in a sample obtained from the patient
15 the expression level of C1q ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value. Particularly, the invention is defined by its claims.

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DETAILED DESCRIPTION OF THE INVENTION:

A first aspect of the invention relates to a method for predicting the survival time of a patient suffering from a cancer comprising i) determining in a sample obtained from the patient the expression level of C1q ii) comparing the expression level determined at step i) with its
25 predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

As used herein, the term "cancer" has its general meaning in the art and includes, but is
30 not limited to, solid tumors and blood borne tumors. The term cancer includes diseases of the skin, tissues, organs, bone, cartilage, blood and vessels. The term "cancer" further encompasses both primary and metastatic cancers. Examples of cancers that may be treated by methods and compositions of the present invention include, but are not limited to, cancer cells from the bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestine, gum, head,

kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, testis, tongue, or uterus. In addition, the cancer may specifically be of the following histological type, though it is not limited to these: neoplasm, malignant; carcinoma; carcinoma, undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoid tumor, malignant; branchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma; acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometroid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous; adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma; inflammatory carcinoma; paget's disease, mammary; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant; thecoma, malignant; granulosa cell tumor, malignant; and roblastoma, malignant; Sertoli cell carcinoma; leydig cell tumor, malignant; lipid cell tumor, malignant; paraganglioma, malignant; extra-mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; malig melanoma in giant pigmented nevus; epithelioid cell melanoma; blue nevus, malignant; sarcoma; fibrosarcoma; fibrous histiocytoma, malignant; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal sarcoma; mixed tumor, malignant; mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; mesenchymoma, malignant; brenner tumor, malignant; phyllodes tumor, malignant; synovial sarcoma; mesothelioma, malignant; dysgerminoma; embryonal carcinoma; teratoma, malignant; struma ovarii, malignant; choriocarcinoma; mesonephroma, malignant; hemangiosarcoma; hemangioendothelioma, malignant; kaposi's sarcoma; hemangiopericytoma, malignant; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; chondroblastoma, malignant;

mesenchymal chondrosarcoma; giant cell tumor of bone; ewing's sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic fibrosarcoma; pinealoma, malignant; chordoma; glioma, malignant; ependymoma; astrocytoma; protoplasmic astrocytoma; fibrillary astrocytoma; astroblastoma; glioblastoma; oligodendroglioma; oligodendroblastoma; primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemmoma, malignant; granular cell tumor, malignant; malignant lymphoma; Hodgkin's disease; Hodgkin's lymphoma; paragranuloma; malignant lymphoma, small lymphocytic; malignant lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides; other specified non-Hodgkin's lymphomas; malignant histiocytosis; multiple myeloma; mast cell sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia; basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; myeloid sarcoma; and hairy cell leukemia.

In some embodiments, the subject suffers from a cancer selected from the group consisting of pancreatic cancer, breast cancer, colon cancer, lung cancer, prostate cancer, testicular cancer, brain cancer, skin cancer, rectal cancer, gastric cancer, esophageal cancer, sarcomas, tracheal cancer, head and neck cancer, liver cancer, ovarian cancer, lymphoid cancer, cervical cancer, vulvar cancer, melanoma, mesothelioma, bladder cancer, thyroid cancer, bone cancers, carcinomas, sarcomas, and soft tissue cancers.

In one embodiment, the cancer is a renal cancer and more particularly a renal cell cancer (RCC) or clear cell renal cell cancer ccRCC).

In another embodiment, the renal cancer is a renal cancer of stages I, II, III or IV.

In another embodiment, the renal cancer is a metastatic renal cancer.

In another embodiment, the cancer is a cancer in which complement activation occur, especially in cancers in which infiltrating macrophages produce C1q and the tumor cells produce C1r and C1s, allowing formation of the C1 complex like renal cancer.

Another aspect of the invention relates to a method for predicting the overall survival (OS) of a patient suffering from a cancer comprising i) determining in a sample obtained from the patient the expression level of C1q ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad

prognosis when the expression level determined at step i) is higher than its predetermined reference value.

Another aspect of the invention relates to a method for predicting the progression free survival (PFS) of a patient suffering from a cancer comprising i) determining in a sample obtained from the patient the expression level of C1q ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

As used herein, the term “Overall survival (OS)” denotes the percentage of people in a study or treatment group who are still alive for a certain period of time after they were diagnosed with or started treatment for a disease, such as cancer and particularly a clear cell renal cell cancer (according to the invention).

As used herein, the term “progression free survival (PFS)” denotes the length of time after primary treatment for a cancer ends that the patient remains free of certain complications or events that the treatment was intended to prevent or delay. These events may include the return of the cancer or the onset of certain symptoms.

As used herein, the term “Good Prognosis” denotes a patient with more than 50% chance of survival for the next 5 years after the treatment.

Another aspect of the invention relates to a method for predicting the responsiveness of a patient affected with a cancer to an anti-cancer treatment comprising i) determining in a sample obtained from the patient the expression level of C1q ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

As used herein and according to all aspects of the invention, the term “C1q” has its general meaning in the art and refers to a protein involved in the complement system. C1q is part of the C1-complex. C1q can bind to the antibodies IgM and IgG (all but IgG4) when the antibody is bound to an antigen. When C1q binds to IgM or IgG complexed with antigen, the C1-complex is activated. Activation of the C1-complex initiates the classical complement pathway. C1q is a 400 kDa protein formed from 18 peptide chains in 3 subunits of 6. Each 6

peptide subunit consists of a Y-shaped pair of triple peptide helices joined at the stem and ending in a globular non-helical head. According to the invention, C1q also denotes the different chains C1qA, C1qB and C1qC (NCBI Reference Sequence: NP_001334395.1; NP_000482.3; AAH09016.1).

5 As used herein the term or “C1 complex” denotes a protein complex involved in the complement system, which is part of the innate immune system. The C1-complex is composed of 1 molecule of C1q, 2 molecules of C1r and 2 molecules of C1s, or C1qrs.

As used herein and according to all aspects of the invention, the term “sample” denotes surgical tumor specimen, tumor biopsy, bone marrow, peripheral blood mononuclear cell 10 (PBMC), macrophages, blood, serum or plasma.

Measuring the expression level of the biomarker of the invention (C1q and other biomarkers of the invention) may be obtained by determining the expression level of the genes or of the proteins corresponding of the biomarker of the invention (C1q and others biomarkers of the invention) in a sample. Measuring the expression level of the biomarkers may be also 15 obtained by using in situ labelling technique. In this case, the expression level of the biomarkers will be quantify thank to the staining coverage and/or the amount of present positive cells labelled with the antibody anti-C1q.

According to the invention, measuring the expression level of the biomarker C1q can be done by measuring the expression level of the different chains C1qA, C1qB and C1qC or of 20 the different genes coding for the chains C1qA, C1qB and C1qC.

In all case, a variety of techniques well known in the art can be thus performed.

Typically, the expression level of a gene may be determined by determining the quantity of mRNA (C1q gene or the genes coding for the chains C1qA, C1qB and C1qC). Methods for determining the quantity of mRNA are well known in the art. For example the nucleic acid 25 contained in the samples (e.g., cell or tissue prepared from the patient) 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 instructions. The extracted mRNA is then detected by hybridization (e. g., Northern blot analysis, in situ hybridization) and/or amplification (e.g., RT-PCR).

30 Other methods of Amplification include ligase chain reaction (LCR), transcription-mediated amplification (TMA), strand displacement amplification (SDA) and nucleic acid sequence based amplification (NASBA).

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 certain embodiments, it will be advantageous to use nucleic acids in combination with appropriate means, such as a detectable
5 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
10 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
15 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,
20 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.

25 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
30 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-trifluoromethylcouluarin (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'-disulfor1ic acid; 5-[dimethylamino] naphthalene-1-sulfonyl chloride (DNS, dansyl chloride); 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-(4,6dicl1lorotriazin-2-yDarninofluorescein (DTAF), 2'7'dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), 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-phthaldialdehyde; pyrene and derivatives such as pyrene, pyrene butyrate and succinimidyl 1-pyrene butyrate; Reactive Red 4 (Cibacron Brilliant Red 3B-A); 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 isothiocyanate (TRITC); riboflavin; rosolic acid and terbium chelate derivatives. Other suitable fluorophores include thiol-reactive europium chelates which emit at approximately 617 mn (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. 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 (dipyrrometheneboron difluoride dyes, for example as described in U.S. Pat. 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 handgap of the semiconductor material used in the semiconductor nanocrystal. This emission can he 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 he 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 Puhlication No. 2003/0165951 as well as PCT Puhlication No. 99/26299 (puhlished May 27, 1999). Separate populations of semiconductor nanocrystals can he produced that are identifiable based on their different spectral characteristics. For example, semiconductor nanocrystals can he produced that emit light of different colors hased on their composition, size or size and composition. For example, quantum dots that emit light at different wavelengths based on size (565 mn, 655 mn, 705 mn, or 800 mn emission wavelengths), which are suitable as fluorescent labels in the probes disclosed herein are available from Life Technologies (Carlshad, Calif.).

Additional labels include, for example, radioisotopes (such as 3 H), metal chelates such as DOTA and DPTA chelates of radioactive or paramagnetic metal ions like Gd3+, and liposomes.

Detectable labels that can he 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 hyhridization (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 Puhlication 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 Pir1kel 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. 5 85:9664-9668, 1988. CISH is described in, e.g., Tanner et al., Am. .1. 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 10 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, courmarin-based 15 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 20 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 25 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 30 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 he 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 he 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 he 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 mn) 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 mn). Additional probes/binding agent pairs can he added to the multiplex detection scheme using other spectrally distinct fluorophores. Numerous variations of direct, and indirect (one step, two step or more) can he 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 Tm, 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 a particular embodiment, 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 another preferred embodiment, the expression 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 expression 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).

Expression level of a gene may be expressed as absolute expression level or normalized expression level. Typically, expression levels are normalized by correcting the absolute expression 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 patient, 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. According to the invention the housekeeping genes used were GAPDH, GUSB, TBP and ABL1. This normalization allows the comparison of the expression level in one sample, e.g., a patient sample, to another sample, or between samples from different sources.

Measuring the expression level of a protein (C1q protein or the chains C1qA, C1qB and C1qC) can be performed by a variety of techniques well known in the art.

Typically protein concentration may be measured for example by capillary electrophoresis-mass spectroscopy technique (CE-MS) or ELISA performed on the sample.

Detection of protein concentration in the sample may also be performed by measuring the level of protein (C1q). In the present application, the “level of proteins” means the quantity or concentration of said proteins. In another embodiment, the “level of proteins” means the level of proteins fragments (C1q fragments for example).

Such methods comprise contacting a sample with a binding partner capable of selectively interacting with proteins present in the sample. The binding partner is generally an antibody that may be polyclonal or monoclonal, preferably monoclonal.

The presence of the protein can be detected using standard electrophoretic and immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, Western blots; agglutination tests; enzyme-labeled and mediated immunoassays, such as ELISAs; biotin/avidin type assays;

radioimmunoassays; immunoelectrophoresis; immunoprecipitation, capillary electrophoresis-mass spectroscopy technique (CE-MS).etc. The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, or other methods for detecting the formation of a complex between the antigen and the antibody or
5 antibodies reacted therewith.

The aforementioned assays generally involve separation of unbound protein in a liquid phase from a solid phase support to which antigen-antibody complexes are bound. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e. g., in membrane or microtiter well form); polyvinylchloride (e. g., sheets or
10 microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidine fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like.

More particularly, an ELISA method can be used, wherein the wells of a microtiter plate are coated with a set of antibodies against the proteins to be tested. A sample containing or
15 suspected of containing the marker protein is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate(s) can be washed to remove unbound moieties and a detectably labeled secondary binding molecule is added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate is washed and the presence of the secondary binding molecule is detected
20 using methods well known in the art.

Methods of the invention may comprise a step consisting of comparing the protein and fragments concentration in circulating cells with a control value. As used herein, "concentration of proteins" refers to an amount or a concentration of a transcription product, for instance the protein C1q. Typically, a level of a protein can be expressed as nanograms per microgram of
25 tissue or nanograms per milliliter of a culture medium, for example. Alternatively, relative units can be employed to describe a concentration. In a particular embodiment, "concentration of proteins" may refer to fragments of the proteins (C1q). Thus, in a particular embodiment, fragments of C1q may also be measured.

Measuring the expression level of the biomarker C1q may be also obtained by using in
30 situ labelling techniques. Method like Immunohistochemistry (IHC) and immunofluorescence (IF) are particularly useful to detect the presence of the biomarkers. In this case, the expression level of the biomarkers will be quantify thank to the staining coverage and/or the amount of present positive cells labelled with the antibody. More the biomarker will be present in a sample more the positive area will be high.

Immunohistochemistry typically includes the following steps i) fixing the tumor tissue sample with formalin, ii) embedding said tumor tissue sample in paraffin, iii) cutting said tumor tissue sample into sections for staining, iv) incubating said sections with the binding partner specific for the marker, v) rinsing said sections, vi) incubating said section with a secondary antibody typically biotinylated and vii) revealing the antigen-antibody complex typically with avidin-biotin-peroxidase complex. Accordingly, the tumor 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 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. Hematoxylin & Eosin, 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 the marker). 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, 14C, 32P, 35S or 125I) 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. 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, canning 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 the marker in the sample, or the absolute number of cells positive for the maker of interest, or the surface of cells positive for the maker of interest. Various automated sample processing, scanning and analysis systems suitable for use with IHC 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. the marker). 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 and tissue recognition pattern (e.g. Aperio Spectrum Software, Automated QUantitatative Analysis platform (AQUA® platform), or Tribvn with Ilastic and Calopix software), 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. the marker) 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. For example, the amount can be quantified as an absolute number of cells positive for the maker of interest. In 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., the marker) 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 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 the marker (e.g. an antibody as above described), ii) proceeding to digitalisation of the slides of step i).by high resolution scan capture, iii) detecting the slice of tissue section on the digital picture iv) 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 or the absolute number of stained cells in each unit whereby the number or the density of cells stained of each unit is assessed.

Immunofluorescence staining methods are widely used in research and diagnostics to demonstrate the presence of specific antigenic determinants on cells or tissues and to quantify the numbers of cells bearing particular determinants in a heterogeneous population.

Immunofluorescence staining methods can be divided into two categories, direct and indirect methods. In the direct staining method, a fluorophore is conjugated to an antibody (hereinafter called "the primary antibody") which is capable of binding directly to the cell surface antigen of interest. In the indirect staining method, the primary antibody is not fluorescently labeled; its binding is visualized instead by the binding of a fluorescently labeled

second-step antibody, which second-step antibody is capable of binding to the primary antibody. Typically, the second-step antibody is an anti-immunoglobulin antibody. ™Indirect immunofluorescence is advantageous in that it is more sensitive than direct immunofluorescence because for each molecule of the primary antibody which is bound, several molecules of the labeled second-step.antibody can bind. This leads to a geometric increase in the amount of cell-associated fluorescence. However, it is well known in the art that indirect immunofluorescence is more prone than direct immunofluorescence to nonspecific staining, that is, staining which is not due to the specific antigen-antibody interaction of interest. [Johnson et al , in Handbook of Experimental Immunology, D. M. Weir, ed., Oxford: Blackwell Publications (1979); Mishell et al., ed., Selected Methods in Cellular Immunology, San Francisco: W. H. Freeman (1980).]

Predetermined reference values used for comparison may comprise “cut-off” or “threshold” values that may be determined as described herein. Each reference (“cut-off”) value for each biomarkers of interest may be predetermined by carrying out a method comprising the steps of

a) providing a collection of samples from patients suffering of a cancer (particularly a renal cell cancer);

b) determining the expression level of the biomarkers for each sample contained in the collection provided at step a);

c) ranking the tumor tissue samples according to said expression level

d) classifying said samples in pairs of subsets of increasing, respectively decreasing, number of members ranked according to their expression level,

e) providing, for each sample provided at step a), information relating to the responsiveness of the patient or the actual clinical outcome for the corresponding cancer patient (i.e. the duration of the overall survival (OS), the progression-free survival (PFS) or both);

f) for each pair of subsets of samples, obtaining a Kaplan Meier percentage of survival curve;

g) for each pair of subsets of samples calculating the statistical significance (p value) between both subsets

h) selecting as reference value for the expression level, the value of expression level for which the p value is the smallest.

For example the expression level of a biomarker X has been assessed for 100 cancer samples of 100 patients. The 100 samples are ranked according to their expression level. Sample 1 has the best expression level and sample 100 has the worst expression 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
5 corresponding cancer patient, 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 reference value is selected such as the discrimination based on the criterion of the minimum p value is the strongest. In other terms, the expression level corresponding to the boundary between both subsets for which the p value is minimum is considered as the reference
10 value. It should be noted that the reference value is not necessarily the median value of expression levels.

In routine work, the reference value (cut-off value) may be used in the present method to discriminate cancer samples and therefore the corresponding patients.

Kaplan–Meier curves of percentage of survival as a function of time are commonly to
15 measure the fraction of patients living for a certain amount of time after treatment and are well known by the man skilled in the art.

The man skilled in the art also understands that the same technique of assessment of the expression level of a biomarker should of course be used for obtaining the reference value and thereafter for assessment of the expression level of a biomarker of a patient subjected to the
20 method of the invention.

Such predetermined reference values of expression level may be determined for any biomarker defined above.

In another embodiment, the method may comprise another biomarker like LAG3 and
25 PD1.

Thus, the invention may also relates to a method for predicting the survival time of a patient suffering from a cancer comprising i) determining in a sample obtained from the patient the expression level of at least 1 biomarker selected from the group consisting of C1q, LAG3 and PD1 ii) comparing the expression level determined at step i) with its predetermined
30 reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

As used herein, the term “LAG3” for “Lymphocyte-activation gene 3” also known as “CD223” has its general meaning in the art and denotes a cell surface molecule with diverse

biologic effects on T cell function. It is an immune checkpoint receptor and as such is the target of various drug development programs by pharmaceutical companies seeking to develop new treatments for cancer and autoimmune disorders. In soluble form it is also being developed as a cancer drug in its own right.

5 As used herein, the term “PD1” for “Programmed cell death 1” has its general meaning in the art and denotes a cell surface receptor that plays an important role in down-regulating the immune system and promoting self tolerance by suppressing T cell inflammatory activity. PD-1 is an immune checkpoint and guards against autoimmunity through a dual mechanism of promoting apoptosis (programmed cell death) in antigen specific T-cells in lymph nodes while 10 simultaneously reducing apoptosis in regulatory T cells (anti-inflammatory, suppressive T cells).

A further object of the invention relates to kits for performing the methods of the invention, wherein said kits comprise means for measuring the expression level or the staining pattern of the biomarkers of the invention in the sample obtained from the patient.

15 The kits may include probes, primers macroarrays or microarrays as above described. For example, the kit may comprise a set of probes as above defined, usually made of DNA, and that may be pre-labelled. Alternatively, probes may be unlabelled and the ingredients for labelling may be included in the kit in separate containers. The kit may further comprise hybridization reagents or other suitably packaged reagents and materials needed for the 20 particular hybridization protocol, including solid-phase matrices, if applicable, and standards. Alternatively the kit of the invention may comprise amplification primers that may be pre-labelled or may contain an affinity purification or attachment moiety. The kit may further comprise amplification reagents and also other suitably packaged reagents and materials needed for the particular amplification protocol.

25 In addition, the kit may comprise a selected set of antibodies, staining protocols and data interpretation algorithm and standards, allowing to perform IHC or IF on the patient’s surgical tumor specimen.

In another object, the invention also relates to an anti-cancer compound for use in the 30 treatment of a cancer in patient with a bad prognosis as described above.

In other words, the invention also relates to a method for treating a cancer in a patient with a bad prognosis as described above comprising administering to said patient an anti-cancer compound.

In one embodiment, the patient has a renal cell cancer with a bad prognosis.

According to the invention, patients with a higher expression level for C1q than their predetermined reference values as described above are eligible for a treatment using an anti-cancer compound according to the invention.

According to the invention, the methods of the invention may be used as a companion diagnostic under a treatment of patient affected with a cancer and particularly a renal cell cancer.

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 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 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 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 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.]).

For example, anti-cancer compounds denote all compounds use in chemotherapy and immunotherapy.

The term chemotherapy denotes all treatment with a chemotherapeutic agent. The term "chemotherapeutic agent" refers to chemical compounds that are effective in inhibiting tumor growth. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaorarnide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a carnptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CBI-TMI); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estrarnustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as the enediyne antibiotics (e.g. calicheamicin, especially calicheamicin (11 and calicheamicin 211, see, e.g., Agnew Chem Intl. Ed. Engl. 33:183-186 (1994); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromomophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, canninomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idanrbicin, marcellomycin, mitomycins, mycophenolic acid, nogalarnycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptomgrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophospharnide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an

epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; rhizoxin; sizofiran; spirogennanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylarnine; trichothecenes (especially T-2 toxin, verracurin A, roridinA and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobromtol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.].) and doxetaxel (TAXOTERE®, Rhone-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-1 1 ; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are antihormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and phannaceutically acceptable salts, acids or derivatives of any of the above.

The term radiotherapy denotes all treatment with a radiotherapeutic agent. The term "radiotherapeutic agent" as used herein, is intended to refer to any radiotherapeutic agent known to one of skill in the art to be effective to treat or ameliorate cancer, without limitation. For instance, the radiotherapeutic agent can be an agent such as those administered in brachytherapy or radionuclide therapy. Such methods can optionally further comprise the administration of one or more additional cancer therapies, such as, but not limited to, chemotherapies, and/or another radiotherapy.

The term immunotherapy denotes all treatment with an immunotherapeutic agent. The term "immunotherapeutic agent," as used herein, refers to a compound, composition or treatment that indirectly or directly enhances, stimulates or increases the body's immune response against cancer cells and/or that decreases the side effects of other anticancer therapies. Immunotherapy is thus a therapy that directly or indirectly stimulates or enhances the immune system's responses to cancer cells and/or lessens the side effects that may have been caused by other anti-cancer agents. Immunotherapy is also referred to in the art as immunologic therapy,

biological therapy biological response modifier therapy and biotherapy. Examples of common immunotherapeutic agents known in the art include, but are not limited to, cytokines, cancer vaccines, monoclonal antibodies and non-cytokine adjuvants. Alternatively the immunotherapeutic treatment may consist of administering the subject with an amount of immune cells (T cells, NK, cells, dendritic cells, B cells…). Immunotherapeutic agents can be non-specific, i.e. boost the immune system generally so that the human body becomes more effective in fighting the growth and/or spread of cancer cells, or they can be specific, i.e. targeted to the cancer cells themselves immunotherapy regimens may combine the use of non-specific and specific immunotherapeutic agents. Non-specific immunotherapeutic agents are substances that stimulate or indirectly improve the immune system. Non-specific immunotherapeutic agents have been used alone as a main therapy for the treatment of cancer, as well as in addition to a main therapy, in which case the non-specific immunotherapeutic agent functions as an adjuvant to enhance the effectiveness of other therapies (e.g. cancer vaccines). Non-specific immunotherapeutic agents can also function in this latter context to reduce the side effects of other therapies, for example, bone marrow suppression induced by certain chemotherapeutic agents. Non-specific immunotherapeutic agents can act on key immune system cells and cause secondary responses, such as increased production of cytokines and immunoglobulins. Alternatively, the agents can themselves comprise cytokines. Non-specific immunotherapeutic agents are generally classified as cytokines or non-cytokine adjuvants. A number of cytokines have found application in the treatment of cancer either as general non-specific immunotherapies designed to boost the immune system, or as adjuvants provided with other therapies. Suitable cytokines include, but are not limited to, interferons, interleukins and colony-stimulating factors. Interferons (IFNs) contemplated by the present invention include the common types of IFNs, IFN-alpha (IFN-α), IFN-beta (IFN-β) and IFN-gamma (IFN-γ). IFNs can act directly on cancer cells, for example, by slowing their growth, promoting their development into cells with more normal behaviour and/or increasing their production of antigens thus making the cancer cells easier for the immune system to recognise and destroy. IFNs can also act indirectly on cancer cells, for example, by slowing down angiogenesis, boosting the immune system and/or stimulating natural killer (NK) cells, T cells and macrophages. Recombinant IFN-alpha is available commercially as Roferon (Roche Pharmaceuticals) and Intron A (Schering Corporation). Interleukins contemplated by the present invention include IL-2, IL-4, IL-11 and IL-12. Examples of commercially available recombinant interleukins include Proleukin® (IL-2; Chiron Corporation) and Neumega® (IL-12; Wyeth Pharmaceuticals). Zymogenetics, Inc. (Seattle, Wash.) is currently testing a

recombinant form of IL-21, which is also contemplated for use in the combinations of the present invention. Colony-stimulating factors (CSFs) contemplated by the present invention include granulocyte colony stimulating factor (G-CSF or filgrastim), granulocyte-macrophage colony stimulating factor (GM-CSF or sargramostim) and erythropoietin (epoetin alfa, 5 darbepoietin). Treatment with one or more growth factors can help to stimulate the generation of new blood cells in subjects undergoing traditional chemotherapy. Accordingly, treatment with CSFs can be helpful in decreasing the side effects associated with chemotherapy and can allow for higher doses of chemotherapeutic agents to be used. Various-recombinant colony stimulating factors are available commercially, for example, Neupogen® (G-CSF; Amgen), 10 Neulasta (pelfilgrastim; Amgen), Leukine (GM-CSF; Berlex), Procrit (erythropoietin; Ortho Biotech), Epogen (erythropoietin; Amgen), Arnesp (erytropoietin). In addition to having specific or non-specific targets, immunotherapeutic agents can be active, i.e. stimulate the body's own immune response, or they can be passive, i.e. comprise immune system components that were generated external to the body. Passive specific immunotherapy typically involves 15 the use of one or more monoclonal antibodies that are specific for a particular antigen found on the surface of a cancer cell or that are specific for a particular cell growth factor. Monoclonal antibodies may be used in the treatment of cancer in a number of ways, for example, to enhance a subject's immune response to a specific type of cancer, to interfere with the growth of cancer cells by targeting specific cell growth factors, such as those involved in angiogenesis, or by 20 enhancing the delivery of other anticancer agents to cancer cells when linked or conjugated to agents such as chemotherapeutic agents, radioactive particles or toxins. Monoclonal antibodies currently used as cancer immunotherapeutic agents that are suitable for inclusion in the combinations of the present invention include, but are not limited to, rituximab (Rituxan®), trastuzumab (Herceptin®), ibritumomab tiuxetan (Zevalin®), tositumomab (Bexxar®), 25 cetuximab (C-225, Erbitux®), bevacizumab (Avastin®), gemtuzumab ozogamicin (Mylotarg®), alemtuzumab (Campath®), and BL22. Other examples include anti-CTLA4 antibodies (e.g. Ipilimumab), anti-PD1 antibodies (Nivolumab for example), anti-PDL1 antibodies, anti-TIMP3 antibodies, anti-LAG3 antibodies, anti-B7H3 antibodies, anti-B7H4 antibodies or anti-B7H6 antibodies. In some embodiments, antibodies include B cell depleting 30 antibodies. Typical B cell depleting antibodies include but are not limited to anti-CD20 monoclonal antibodies [e.g. Rituximab (Roche), Ibritumomab tiuxetan (Bayer Schering), Tositumomab (GlaxoSmithKline), AME-133v (Applied Molecular Evolution), Ocrelizumab (Roche), Ofatumumab (HuMax-CD20, Gemnab), TRU-015 (Trubion) and IMMU-106 (Immunomedics)], an anti-CD22 antibody [e.g. Epratuzumab, Leonard et al., Clinical Cancer

Research (Z004) 10: 53Z7-5334], anti-CD79a antibodies, anti-CD27 antibodies, or anti-CD19 antibodies (e.g. U.S. Pat. No. 7,109,304), anti-BAFF-R antibodies (e.g. Belimumab, GlaxoSmithKline), anti-APRIL antibodies (e.g. anti-human APRIL antibody, ProSci inc.), and anti-IL-6 antibodies [e.g. previously described by De Benedetti et al., J Immunol (2001) 166: 4334-4340 and by Suzuki et al., Europ J of Immunol (1992) 22 (8) 1989-1993, fully incorporated herein by reference]. The immunotherapeutic treatment may consist of allografting, in particular, allograft with hematopoietic stem cell HSC. The immunotherapeutic treatment may also consist in an adoptive immunotherapy as described by Nicholas P. Restifo, Mark E. Dudley and Steven A. Rosenberg "Adoptive immunotherapy for cancer: harnessing the T cell response, Nature Reviews Immunology, Volume 12, April 2012). In adoptive immunotherapy, the subject's circulating lymphocytes, NK cells, are isolated amplified in vitro and readministered to the subject. The activated lymphocytes or NK cells are most preferably be the subject's own cells that were earlier isolated from a blood or tumor sample and activated (or "expanded") in vitro.

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. C1q is associated with bad prognosis in human ccRCC. A) Prognostic value of C1q in cohort 1. The figures represent PFS and OS for the entire cohort and the distribution by stages. B) Prognostic value of C1q in cohort 2, composed of Stage IV patients only, on PFS (up) and OS (down).

EXAMPLES:

Example 1

Material & Methods

Patients

Two retrospective cohorts of ccRCC patients were available for this study. The first primary cohort includes 135 patients undergoing nephrectomy in the hospital Necker-Enfants Malades (Paris, France) between 1999 and 2003. The second retrospective cohort is composed by 51 primary ccRCC specimens with metastases operated in 3 French and 1 Belgian hospitals from 1994 to 2011. A third retrospective primary cohort includes 66 patients operated at the

Institut Mutualiste Montsouris (IMM) (Paris, France) between 2002 and 2010. Histopathologic features, such as the histologic subtype, tumor size, regional lymph node invasion, distant metastases, Furhman nuclear grade, TNM stage were available for almost all patients and the duration of follow-up was calculated from the date of the surgery to the date of cancer progression, last follow-up or death. Moreover, the TCGA-KIRC cohort composed by 537 primary ccRCC samples with clinical and expression data is also used in this study.

Immunohistochemistry (IHC) and immunofluorescence (IF) on human tumor specimens

The formalin-fixed-paraffin-embedded (FFPE) tumors specimens were cut at 3μm tick sections. The antigen retrieval were carried out on a PT-link (Dako) using the EnVision FLEX Target Retrieval Solutions (Dako) with low or high pH for the detection of C1q, CD163, LAG3 and PD-1 or with Proteinase K (Dako, S3020) for IgG staining. Endogenous peroxidase and non-specific staining were blocked with H2O2 3% (Gifrer, 10603051) and protein block (Dako, X0909) respectively. For immunohistochemistry studies, staining was revealed with 3-amino-9-ethylcarbazole substrate (Vector Laboratories, SK-4200). After mounting either by glycergel (Dako, C056330-2) for IHC or ProLongTM Gold antifade reagent with DAPI (Thermofisher, P36935) for IF the slides were scanned with Nanozommer (Hamamastu) for IHC or AxioScan (Zeiss) for IF. In this case, after the secondary HRP coupled antibody, an incubation with AF647 tyramide reagent (1:100 diluted) (B40958) was performed, followed by antibodies stripping at 97°C 10 minutes. This protocol was repeated for the second primary and secondary antibody and AF 546 tyramide reagent 1/100 diluted (B40954). Human FFPE tonsil sections were used as a positive control for C1q and liver sections – as positive controls for MBL. The specificity of the anti-C1q antibody was verified by a competition test with human C1q protein (Comptech, A099). C3 and C3d were used as negative controls for the competition studies respectively. For complement staining (C1q), the slides were classified into 2 groups according to the number of positive cells (> ou < to 30%) and the type of staining (cytoplasmic and deposits). This semi-quantification was performed by 3 independent observers to minimize analysis bias. For LAG3 and PD1, the quantification of the density of positive cells in the Invasive Margin (IM) and Tumor Center (TC) was realized by Calopix software (Tribvn).

Macrophage sorting and phenotype analysis

After tissue dissociation, fresh tumors were incubated for 1 hour at 4°C with Cell Recovery Solution (Fisher Scientific). To separate the immune population, a Ficoll-Paque PLUS (GE Healthcare Life Science) was performed. Cells were then stained with: CD14 APC, CD16 APC-H7, CD3 PE, CD66b PE, CD19 PE, CD56 PE, DAPI. Macrophages were sorted

(using FACSAria cytometer) and CD14+ cells were recovered with a purity over 95%. These macrophages were recovered in RLT β-mercaptoethanol solution and stored at -80°C.

The RNA was extracted from 8 frozen samples using RNAeasy microkit (Qiagen, 74004). The quality and quantity of RNA were determined with 2100 Bioanalyzer (Agilent) using Agilent RNA 6000 Pico assay kit (5067-1513). Reverse transcription and pre-amplification were conducted with the Ovation pico kit (Nugen, 3302). The quantitative gene expression was assessed by a custom low density array plate with 24 genes analyzed by an Applied Biosystem 7900HT Fast Real-Time PCR System. Expression levels were determined using threshold cycle (Ct) values normalized to GAPDH (ΔCt).

Cell lines

Human cell lines: Two human ccRCC cells lines - Caki-1 and A498 as well as two kidney-unrelated control cell lines from human colorectal cancer - HCT116 and SW620 were used in this study. Caki-1 and HCT116 were cultured in McCoy's medium (Gibco) + 10% FCS + 1x penicilin/streptomycin (Gibco), SW620 were cultured in Leibovitz medium (Gibco) + 10% FCS 1x penicilin/streptomycin (Gibco) and A498 - in EMEM medium (ATCC) + 10% FCS + 1x penicilin/streptomycin (Gibco).

Interaction of tumor cells with C1q

The interaction with immobilized C1q was studied using two human ccRCC cells lines - Caki-1 and A498 as well as for the mouse cancer cell line TC-1. 50 000 cells/well in Opti MEM medium were seeded in 96 wells flat bottom plates, coated either by bovine serum albumin, human C1q (Comptech, A099), fibronectin (Sigma, F1141) or polylysine (Sigma, P4862) at 20µg/mL. Ten minutes later, the wells were washed, the cells - fixed with paraformaldehyde (PFA) 0,5% and the nuclei - stained with Hoesch. The plates were scanned using BD pathway imager and the number of nucleus in each well was determined using ImageJ software. Alternatively, Superfrost plus slides were divided by Dakopen into 4 equivalent parts, coated either by bovine serum albumin, human C1q, fibronectin or polylysine 20µg/mL. 200 000 cells/quadrant, suspended in Opti MEM (Gibco, 31985-062) medium were placed in each part. After 10 minutes or an overnight incubation at 37°C, the cells on the slides were washed and fixed with PFA 4% for 30 minutes. After an antigen retrieval at low pH and a blocking with protein block (Dako, X0909), a goat anti mouse antibody Na/K ATP-ase followed by anti-mouse IgG-Cy3 was used. After nuclear staining with dapi and mounting with ProLongTM Gold antifade reagent (Thermofisher, P36934), the slides were scanned using AxioScan (Zeiss). The nuclei were counted using Visiopharm Software.

Complement genes expression

The expression of complement genes by the human and mouse cell lines was determined by real time PCR or custom low density array. After 48h of culture in a synthetic medium without serum Opti-MEM (ref), the cells were recovered in RLT- β-mercaptoethanol. The RNA was extracted with RNeasy mini kit (Qiagen, 74106). The quality and quantity of RNA were determined with 2100 Bioanalyzer (Agilent) using Agilent RNA 6000 Nano assay kit (5067-1511). The reverse transcription was made from 250ng RNA with the Applied Biosystem High capacity cDNA Reverse Transcription kit (Applied Biosystem, 4368814). The quantitative gene expression was assessed by a Taqman 96 wells plate read by an Applied Biosystem 7900HT Fast Real-Time PCR System. Expression levels were determined using threshold cycle (Ct) values normalized to GAPDH (ΔCt) and expressed with 2^(-ΔCt).

Detection of complement proteins by Western blot

After 48h of culture in a synthetic medium without serum Opti-MEM, the supernatants of the human and mouse cell lines were recovered and concentrated using amicon ultra, ultracel 3K (UFC 900324). The samples were prepared into NuPAGE® LDS sample buffer (4X) (Thermofisher) with or without reducing agent (DTT) and then denatured at 80°C for 10 minutes. Proteins were separated in NuPage 10% Bis-Tris gel (Thermofisher). The proteins were transferred on a nitrocellulose membrane using iBlot (Invitrogen). Further, the membranes were stained with the SNAP i.d. Protein Detection System (Millipore), using a primary goat anti-human C1s antiserum (Quidel, A302) 1/5000, a polyclonal rabbit anti-human C1r (Abcam, ab155060) 1/500, rabbit polyclonal anti-mouse C1r (Abcam ab205546, 1/500). Secondary antibodies were: rabbit anti-goat-HRP (Santa Cruz, H0712) or a goat anti-rabbit-HRP (Santa Cruz, J512) as a secondary antibody. After washes, the membranes were developed with an ECL reagent (Pierce #32106) and the chemiluminescence was detected with MyECL Imager (Thermo Scientific). The purified human proteins C1s (Comptech, A104) or C1r (Comptech, A102) were used as positive controls.

Assessment of the formation and activity of the C1 complex by ELISA

To test the formation of a C1 complex, a first ELISA assay was used, as described previously. (30) A polyclonal anti C1q (Dako, A0136), diluted 1/1000 in PBS, was coated overnight on 96 wells Nunc plates (Nunc maxisorb). A BSA 1% solution was then used for blocking for 1 hour at room temperature. The washing steps were performed with TBS Tween 0,05% CaCl2 1mM. The supernatants of cultured human or mouse cell lines supplemented with increasing doses of human C1q (Comptech) or mouse C1q (kind gift from Pierre Fabre Research Institute, France) respectively, from 0,125µg/mL to 4µg/mL diluted in washing buffer, were added to the plates and incubated for 1 hour at 37°C. Increasing doses of normal

human or mouse serum were added as a positive controls. A goat anti-C1s antiserum (Quidel, A302) 1/500 diluted in the washing buffer was used and incubated for 1 hour at 37°C and then a secondary rabbit anti goat HRP 1/2000 diluted (Santacruz, H0712) was added. The ELISA was revealed with SureBlue TMB Microwell Peroxidase Substrate (KPL) and the reaction was stopped with 2M sulfuric acid. The optical density at 450nm was measured by Multiskan Ex (Thermo Scientific).

To evaluate the functionality of this C1 complex, a second ELISA was set up as in (31). The 96 wells plates were coated with human IgG1, 50 µg/ml, for 1h at 37°C. BSA 1% solution was then used to block the plate during 1 hour at room temperature. The washing steps were performed with 10mM Hepes, 75mM NaCl, CaCl2 1mM, MgCl 1mM and 0.05% Tween 20. The supernatants of cultured cells lines and increasing doses of human C1q from 0,125µg/mL to 4µg/mL, diluted in this washing were added to the plates and incubated for 1 hour at 37°C. In the same plate, increasing dose of human serum diluted from 1/1280 to 1/40 were added as a positive control. A solution containing C2 protein 5µg/mL (Comptech, A112) were then added and incubated 2h30 at 37°C. The supernatant from the wells was recovered and the C2 cleavage was analyzed by western blot under reducing conditions using biotinilted anti-human C2 (R&D system, BAF1936) 1/400 diluted and then streptavidine HRP 1/3000 (Dako, P0397). The signal was revealed as above.

Statistical analysis

The survival analyses were realized with R software and the package survival. Impact on survial was assessed using Kaplan-Meier estimates and log-rank test, as well univariate and multivariate Cox regression analyses. The association between the distributions of different variable was assessed by a Fisher's exact test or a Mann-Whitney test. For quantitative variables, the cut-off was chosen according to the distribution density curves.

Mice tumor growth were analyzed using a two-way ANOVA test for the curve and independently to each days with a non-parametric Mann-Whitney test. Data from mice immunofluorescence quantifications, flow cytometry and RTqPCR were analysed using Mann-Whitney tests. These statistical analysis were performed using GraphPad Prism 6.

Results

C1q density is associated with poor prognosis in advanced ccRCC.

To search for the presence of C1q in ccRCC tumors, we used a rabbit polyclonal anti-human C1q antibody, which allowed us to detect specifically C1q-producing cells by immunohistochemistry (IHC) and immunofluorescence (IF) in paraffin-embedded tissue. It

recognized specifically C1q in tissues, since the staining in tonsils and ccRCC samples was inhibited after pre-incubation of the antibody with purified C1q but not with purified C3 (data not shown). Density of C1q-producing cells was semi-quantified as low (staining area, covering <50% of the surface of the tumor) and high (>50% of the surface of the tumor) (data not shown). We analyzed a first retrospective cohort of 135 patients with primary ccRCC "cohort 1" (previously described in (6)), for which we had C1q staining and survival data for 124 patients. High density of C1q producing cells correlated with a shorter PFS in this cohort, including for stages I+II and III when the patients were stratified by stage. A similar tendency was seen for stage IV patients, which did not reach significance due to the low number of patients (Figure 1B upper panel). In terms of OS, significance was reached in case of advanced stages III and IV (Figure 1A). Since we had access to small number of patients in stage IV in this cohort, we validated the significant association of high density of C1q producing cells with poor prognosis using an independent cohort of composed of 43 stage IV patients, all treated with Sunitinib (previously described in (2)) (Figure 1B).

C1q is mainly produced by macrophages

Transcriptomic analyses of C1qA gene expression of publically available databases of purified cells showed that it has expression restricted to myeloid cells, the highest expression being in (im)DC, macrophages and monocytes, as well as in peripheral blood mononuclear cells (PBMC) (data not shown). Similar results were obtained with C1qB and C1qC genes (data not shown). Using double labeling in IF we evidenced in ccRCC tumors that C1q was mainly produced by CD68+ cells, defined as macrophages (data not shown), the vast majority expressing the M2 marker CD163+ (data not shown). In addition, C1q positivity was detected in scarce neutrophils (double staining with CD66b, data not shown) and some endothelial cells (identified by double staining with CD31, data not shown). In an unsupervised gene clustering analysis for 8 patients, we found that C1qA gene expression was coordinated with the one of C1qC gene and other genes of macrophages activation, including PDL-2 (CD273, PDCD1LG2), HLA-DR, CD86, ADAP2, C3aR, and PDL-1 (CD274, PDCD1LG1) (data not shown). C1qA and C genes correlated to a less extend with classical M2 macrophages genes such as CD163, VSIG4 or LAIR-1. In these tumors C1qA and C genes were not correlated with genes encoding IL-10, CD206 (MRC1), C5aR1 (CD88), SIGLEC1 and VEGFA. Gene to gene correlation showed a strong coordination of C1qA with PDL-2, CD86, LAIR1 and HLA-DR but not with CD163 (data not shown), confirming that C1q may be produced by a population of activated macrophages that does not reflect only the presence of M2 macrophages.

High density of C1q producing cells is associated with an exhausted immune phenotype in ccRCC tumors

To search for a potential association between C1q and expression and immune exhaustion markers in ccRCC, we first analyzed publically available transcriptomic data in TCGA database, containing 537 ccRCC tumors samples. We found a strong correlation between C1qA gene expression and that of PDL-1 (CD274, p=0.00029), PDL-2 (CD273, PDCD1LG2, p=3.1x10-56), PD1 (PDCD1, p=1.5x10-70), LAG3 (p=2x10-70), TIM3 (HAVCR2, p=6.5x10-23) and CTLA-4 (p=1.3x10-39), (data not shown). Further, we analyzed the 89 tumors from cohort 1 by IHC and found a positive correlation between C1q+ cell density and that of PD1 (p=0.0077) as well as LAG3 (p= 2.6x10-5), (data not shown). Combining densities of the two markers showed a significantly shorter PFS in patients with high densities of both C1q+ and LAG3+ (p=0.019) (data not shown, data available for 117 patients), while the OS showed a tendency towards significance (0.094). The data for PD-1 showed the same tendency but did not reach significance (data not shown, data available for 115 patients). These results may suggest that C1q has a direct effect on T cells to induce or increase their exhaustion status, since T cells possess C1q receptors. (19) We, therefore, added C1q (surface immobilized or soluble) to in vitro cultures of human T-cells, purified from PBMC and activated with CD3/CD28 antibodies in presence or absence of supernatant of ccRCC cell line Caki-1. Albumin, added at the same molar concentration served as a negative control. We detected expression of exhaustion markers after incubation with ccRCC cells supernatants, but C1q did not show any significant effect (data not shown).

C1 activates the classical complement pathway

Since we could not evidence any direct effect of C1q on the T cells, we investigated whether C1q interacts directly with the tumor cells. Indeed, analysis of ccRCC tumors showed that, in addition to C1q producing cells, deposits of C1q could be found at the membrane of tumor cells in certain areas of a fraction of the tumors (data not shown). In vitro experiments showed that ccRCC cell lines Caki-1 and A498 grow better in C1q-coated wells, compared to albumin-coated wells, for A498 reaching density, similar to the positive control fibronectin at 12h (data not shown). Poly-L-lysine (PL) served as a non-specific adherence control. This higher density, though, was not due to increased proliferation rate (data not shown but due to a rapid direct interaction of the cells with C1q, resulting in better cell adherence at 10 min after inoculation (data not shown). In addition to direct interaction with the tumor cell surface, the presence of C1q deposits in ccRCC could be explained by presence of membrane-bound IgG (data not shown).

Therefore, we tested the hypothesis that the deleterious impact of C1q could be exerted through activation of the classical complement pathway. An interesting finding was that the two ccRCC cell lines Caki-1 and A498 expressed C1r and C1s mRNA (data not shown) and produced C1r and C1s proteins in the cultured supernatant in vitro (data not shown), contrary to two colon cancer cell lines (HCT116 and SW620) used as negative controls. The four tested cell lines did not produce detectable C1q mRNA and protein (data not shown). In vitro adding supernatant of Caki-1 and A498 ccRCC cell lines to purified human C1q allowed the formation of a C1 complex by the interaction of tumor cells-derived C1r and C1s with C1q (data not shown).

Example 2: new results

Tumors from C1q-/- mice are infiltrated by activated macrophages and have higher levels of CD8+ T-cells, NK cells and cDCs

Analysis of the TME content by flow cytometry revealed that TC-1 tumors from WT mice had a significantly lower infiltration of hematopoietic cells (CD45+) than C1q-/- mice even at day 10, when there was not yet a detectable difference in tumor volume between the two strains of mice (data not shown). This difference persisted until day 20, when the WT mice had a significantly larger tumor mass than C1q-/- mice. The composition of the TME was strikingly different between tumors growing in WT and C1q-/- mice, with significantly more macrophages in the WT mice (data not shown) and a lower proportion of IA/IE+ cells, IA/IE being an M1 phenotype marker (data not shown). Tumors from WT mice also exhibited lower percentages of antigen-presenting cDCs at both day 10 and day 20 (data not shown). In contrast, the tumors contained more potentially suppressive PMN-MDSCs at day 20 (data not shown), whereas no significant differences in M-MDSCs were found (data not shown). The WT mice showed fewer lymphocytes, particularly those with a cytotoxic potential, such as NK and CD8+ T-cells (data not shown), without reaching significance for CD4+ T-cells (data not shown).

The density of C1q-positive cells is associated with poor prognosis in advanced ccRCC

C1q staining and its correlation with clinical outcome was analyzed in primary tumors from a retrospective cohort of 106 patients with ccRCC. Compared to a low density, the high density of intratumoral C1q-producing cells had a significant negative impact on PFS (data not shown, p=0.008) and OS (data not shown, p=0.0016).

This finding was validated using another independent retrospective cohorts. In cohort 2 (154 patients), a negative clinical impact of a high density of C1q-positive cells on PFS was detected (data not shown, p=0.012).

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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.

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CLAIMS:

1. A method for predicting the survival time of a patient suffering from a cancer comprising i) determining in a sample obtained from the patient the expression level of C1q ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

2. A method according to claim 1 wherein the cancer is a renal cancer.

3. A method according to claim 2 wherein the renal cancer is a renal cell cancer (RCC) or a clear cell renal cell cancer ccRCC).

4. A method according to claim 2 wherein the renal cancer is a renal cancer of stages I, II, III or IV.

5. A method according to claim 2 wherein the renal cancer is a metastatic renal cancer.

6. A method for predicting the survival time of a patient suffering from a cancer comprising i) determining in a sample obtained from the patient the expression level of at least 1 biomarker selected from the group consisting of C1q, LAG3 and PD1 ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

7. An anti-cancer compound for use in the treatment of a cancer in patient with a bad prognosis according to claim 1.

8. A method for treating a cancer in a patient with a bad prognosis according to claim 1 comprising administering to said patient an anti-cancer compound.

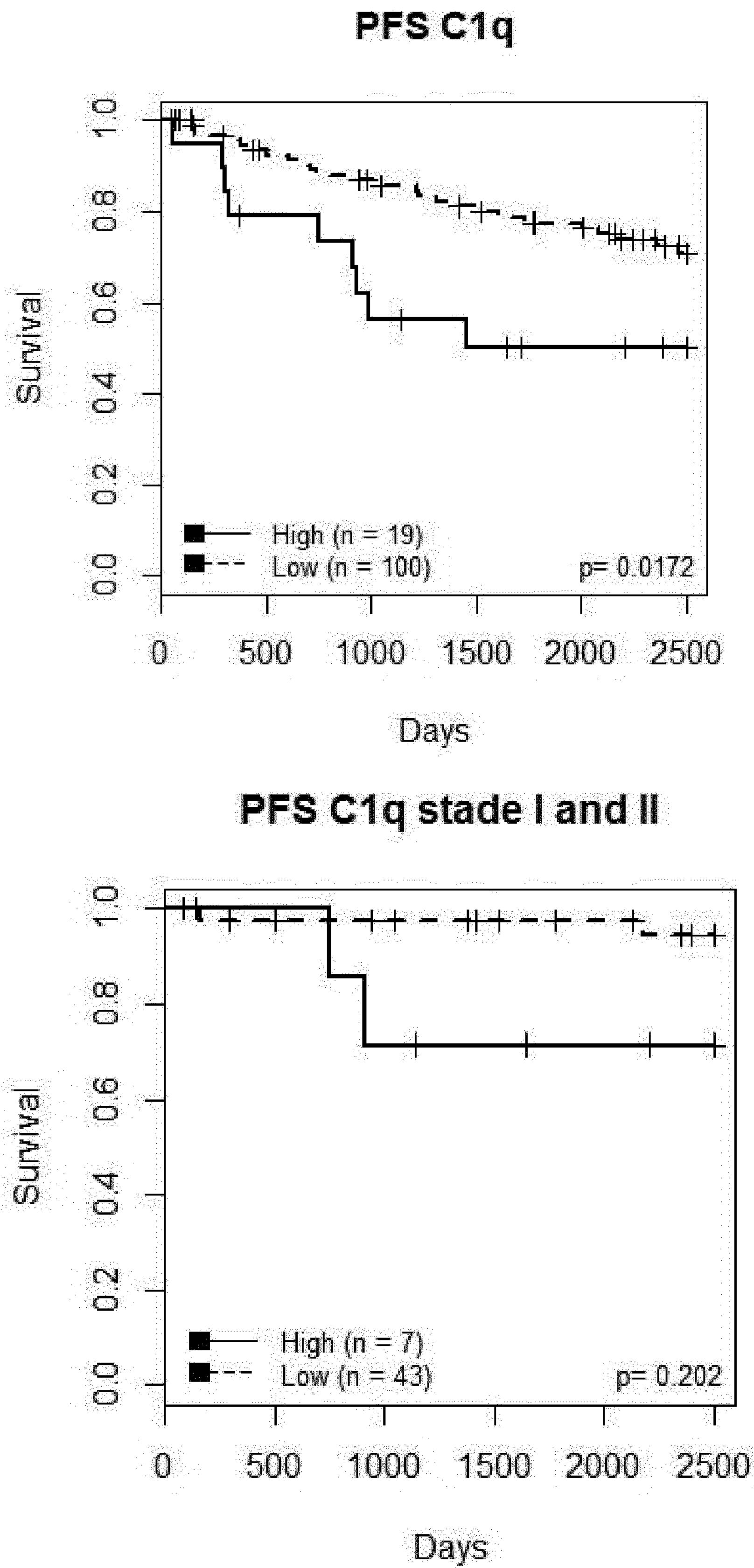
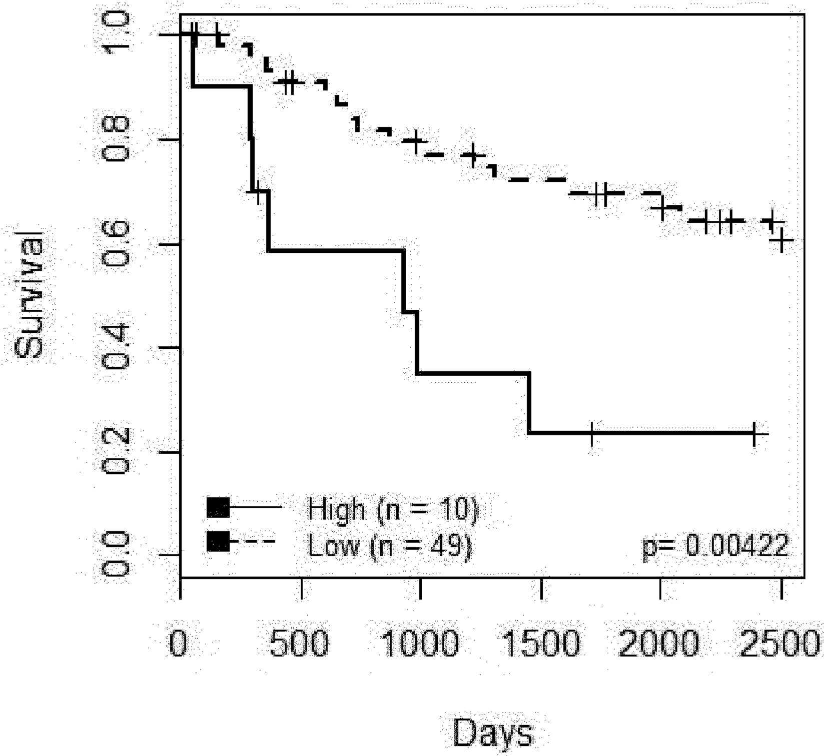


Figure 1A



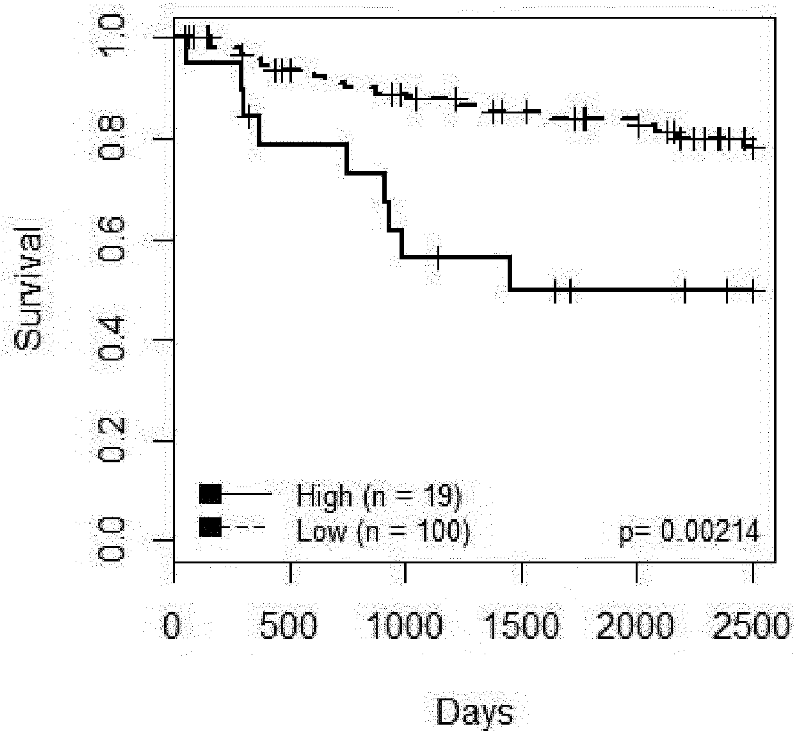
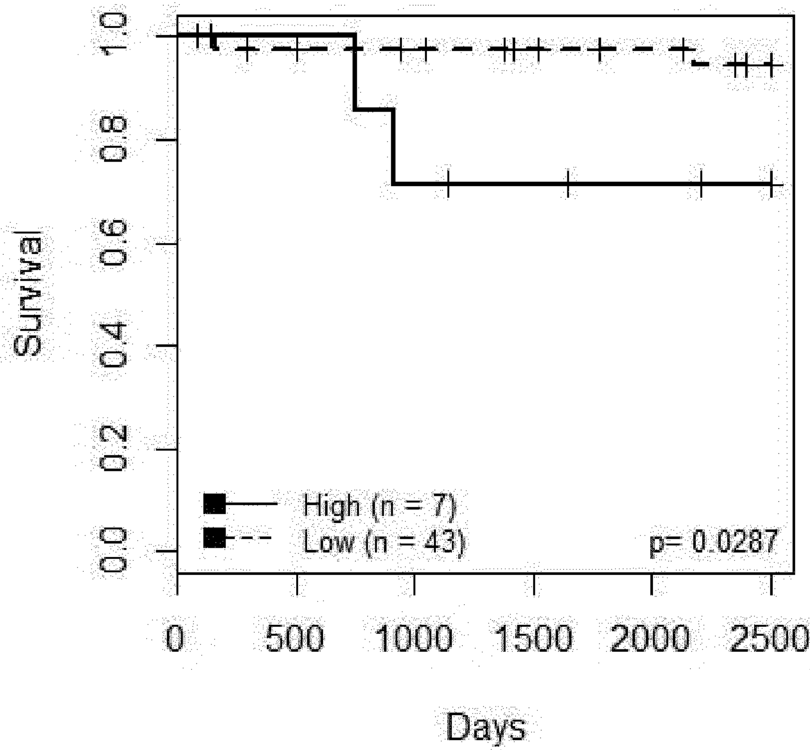


Figure 1A (following)



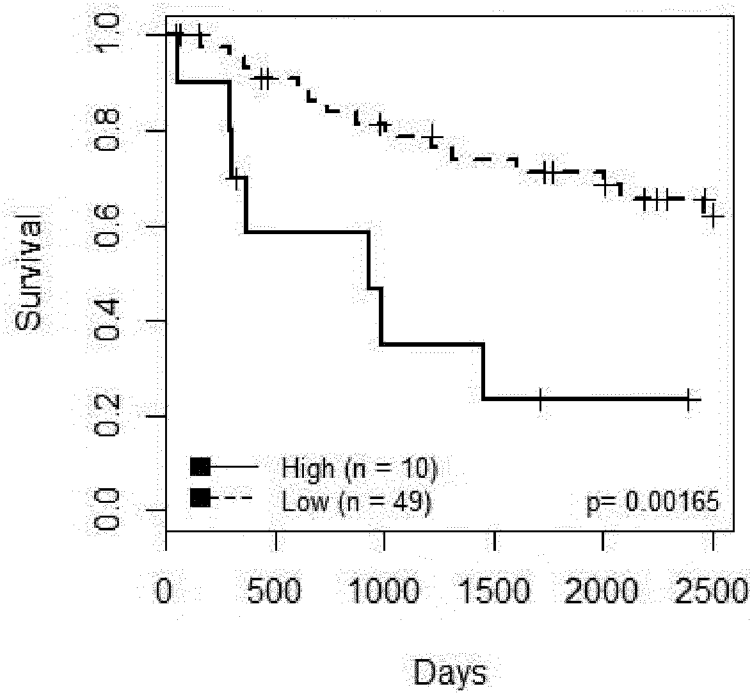
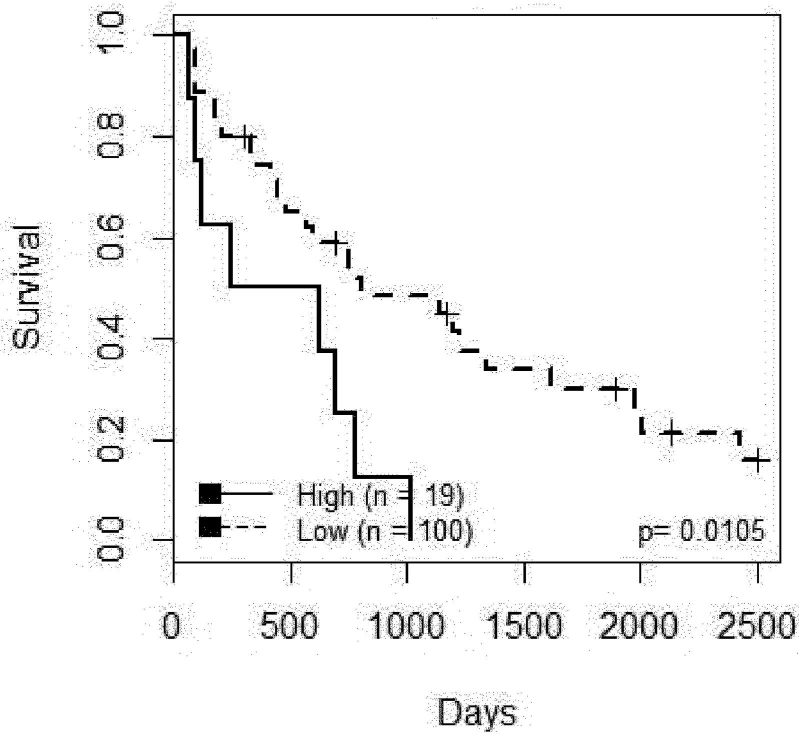


Figure 1A (following)



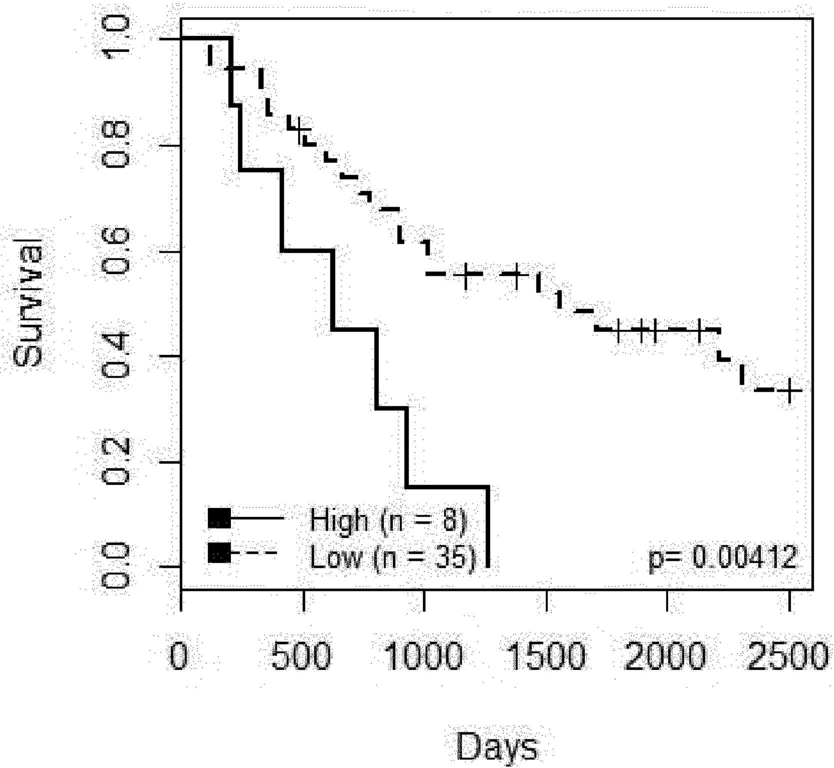


Figure 1B

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/073429

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/6886
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)
C12Q

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, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2017/004153 A1 (BROAD INST INC [US]; MASSACHUSETTS INST OF TECHNOLGY [US]; DANA-FARBER) 5 January 2017 (2017-01-05) paragraph [0495]; claims 1-6,78,95,120; figure 5	1-6
X	WO 2017/083640 A1 (DANA-FARBER CANCER INST INC [US]; HARVARD COLLEGE [US]) 18 May 2017 (2017-05-18) the whole document	1,6-8
A	WO 2010/145796 A2 (MERCK PATENT GMBH [DE]; STROH CHRISTOPHER [DE]; VON HEYDEBRECK ANJA [D) 23 December 2010 (2010-12-23) the whole document	1-8

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[X] Further documents are listed in the continuation of Box C.

[X] 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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 September 2018	02/10/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Cornelis, Karen

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROBERTA BULLA ET AL: "C1q acts in the tumour microenvironment as a cancer-promoting factor independently of complement activation", NATURE COMMUNICATIONS, vol. 7, 1 February 2016 (2016-02-01), page 10346, XP055449962, DOI: 10.1038/ncomms10346 the whole document -----	1,6
A	ANUVINDER KAUR ET AL: "Human C1q Induces Apoptosis in an Ovarian Cancer Cell Line via Tumor Necrosis Factor Pathway", FRONTIERS IN IMMUNOLOGY, vol. 7, 21 December 2016 (2016-12-21), XP055449982, DOI: 10.3389/fimmu.2016.00599 -----	1-8
A	JI-YEON KIM ET AL: "Immune signature of metastatic breast cancer: Identifying predictive markers of immunotherapy response", ONCOTARGET, vol. 8, no. 29, 18 July 2017 (2017-07-18), pages 47400-47411, XP055449988, DOI: 10.18632/oncotarget.17653 -----	1-8

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2018/073429

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 2017004153	A1	05-01-2017	EP	3314020	A1	02-05-2018
			US	2018100201	A1	12-04-2018
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