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(54) Title: METHOD FOR DIAGNOSING COLORECTAL CANCER

(57) Abstract: The present invention relates to the diagnosing of colorectal cancer. The inventors have thus designed an efficient, rapid and cost-effective method to purify and analyse mRNA from small volumes of blood plasma. They found that the RPS28, B2M, TIMP-1 and CLU levels were significantly higher in metastatic colorectal cancer patients. Thus, the present invention relates to a method for diagnosing a colorectal cancer in a subject in need thereof comprising i) determining in a sample obtained from the subject the expression levels of at least one biomarker selected from the group consisting of RPS28, B2M, TIMP-1 and CLU mRNAs.

METHOD FOR DIAGNOSING COLORECTAL CANCER

FIELD OF THE INVENTION:

The invention relates to a method for diagnosing a colorectal cancer in a subject in need thereof comprising i) determining in a sample obtained from the subject the expression levels of at least one biomarker selected from the group consisting of RPS28, B2M, TIMP-1 and CLU mRNAs.

BACKGROUND OF THE INVENTION:

In oncology field, cancer detection, cancer prognostic classification, monitoring of antineoplastic treatment response still need to be greatly improved. For instance, the large majority of cancers are diagnosed by histological examination of biopsy samples. However, their invasiveness can generate discomfort for patients. They are expensive and the results of the analyses are often long to obtain. In an attempt to overcome all these disadvantages, analyses of circulating nucleic acids as non-invasive biomarkers have generated great interest [1]. Indeed, fragments of cell-free DNA (cfDNA) as well as RNA (mRNA and microRNA), are released by the cells of the organism and carry valuable information that can be used as biomarker. Consequently, many studies have shown the clinical interest of circulating DNA analysis for cancer management [2]. A more emerging aspect of the study of circulating nucleic acids as a cancer biomarker deals with microRNAs since there are remarkably resistant to RNase activity. Indeed, the study and analysis of microRNAs are particularly interesting because of their role in the post-transcriptional regulation of gene expression. Many pathological conditions, including cancer, lead to changes in the expression of miRNAs hence the interest as a biomarker [3].

Nevertheless, it is necessary to multiply biomarkers in order to better characterize the disease and to move towards a perfectly personalized medicine. This is why in this study we looked at circulating messenger RNA present in plasma since they provide information about the expression level of their own genes, and when these genes are related to cancer, information about the disease as well. The first study showing the clinical value of the detection of tyrosinase mRNA in patients with metastatic melanoma date from 1999 [4]. However, the use of mRNA as a biomarker in liquid biopsy is facing difficulties due to its instability and low abundance, probably explaining why few studies have been published so far. On the contrary to whole-transcriptome sequencing that is an expensive method and only available in a few centers, we have designed an efficient, rapid and cost-effective RT-QPCR based-method to

purify and analyze mRNA from small volumes of blood plasma. This enabled us to show that the plasma concentration of some mRNAs is significantly higher in colorectal cancer (mCRC) patients than in the healthy population. Compared to the other method used in the art, the method of the present invention is faster, more efficient and less expensive.

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SUMMARY OF THE INVENTION:

The inventors have thus designed an efficient, rapid and cost-effective method to purify and analyse mRNA from small volumes of blood plasma. They found that the RPS28, B2M, TIMP-1 and CLU levels were significantly higher colorectal cancer patients. Thus, the present invention relates to a method for diagnosing a colorectal cancer in a subject in need thereof comprising i) determining in a sample obtained from the subject the expression levels of at least one biomarker selected from the group consisting of RPS28, B2M, TIMP-1 and CLU mRNAs. Particularly, the invention is defined by its claims.

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DETAILED DESCRIPTION OF THE INVENTION:

Method for diagnosing a colorectal cancer

A first aspect of the invention relates to a method for diagnosing a colorectal cancer in a subject in need thereof comprising i) determining in a sample obtained from the subject the expression levels of at least one biomarker selected from the group consisting of RPS28, B2M, TIMP-1 and CLU mRNAs ii) comparing the expression level determined at step i) with its predetermined reference value and iii) concluding that the subject in need thereof has a colorectal cancer when the expression level determined at step i) is higher than its predetermined reference value, or concluding that the subject in need thereof has not a colorectal cancer when the expression level determined at step i) is lower than its predetermined reference values.

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In a particular embodiment, the expression levels of 1, 2, 3 or 4 biomarkers selected from the group consisting of RPS28, B2M, TIMP-1 and CLU mRNAs can be determined according to the method of the invention.

Thus, in a particular embodiment, the expression levels of the 4 biomarkers can be determined.

Thus, in this particular embodiment, the invention relates to a method for diagnosing a colorectal cancer in a subject in need thereof comprising i) determining in a sample obtained from the subject the expression levels of the biomarkers selected from the group consisting of RPS28, B2M, TIMP-1 and CLU mRNAs ii) comparing the expression levels determined at step
5 i) with their predetermined reference values and iii) concluding that the subject in need thereof has a colorectal cancer when the expression levels determined at step i) are higher than their predetermined reference values, or concluding that the subject in need thereof has not a colorectal cancer when the expression levels determined at step i) are lower than their predetermined reference values.

10 In another particular embodiment, a step of addition of the expression levels of the 4 biomarkers of the invention can be realized to obtain the RBTC index.

Thus, the invention also relates to the invention relates to a method for diagnosing a colorectal cancer in a subject in need thereof comprising i) determining in a sample obtained
15 from the subject the expression levels of the biomarkers selected from the group consisting of RPS28, B2M, TIMP-1 and CLU mRNAs, ii) adding the expression levels of the 4 biomarkers to obtain the RBTC index iii) comparing RBTC index at step ii) with a predetermined reference values and iv) concluding that the subject in need thereof has a colorectal cancer when the RBTC index determined at step ii) is higher than its predetermined reference value, or
20 concluding that the subject in need thereof has not a colorectal cancer when the RBTC index determined at step ii) is lower than its predetermined reference value.

In a particular embodiment, the expression levels of B2M, TIMP-1 and CLU mRNAs can be determined according to the method of the invention.

25 The inventors show that if a high expression of the 3 biomarkers B2M, TIMP-1 and CLU mRNAs levels is measured, thus the subject is having all stage, in particular stage 4, of colorectal cancer.

In a particular embodiment, high expression levels of TIMP-1 and CLU mRNAs levels in a subject suffering from colorectal compared to the expression levels of TIMP-1 and CLU
30 mRNAs levels from healthy individuals (HI) indicates all stage, in particular stage 4, of colorectal cancer.

In another particular embodiment, the expression levels of TIMP-1 and CLU mRNAs can be determined according to the method of the invention.

The inventors show that if a high expression of the 2 biomarkers TIMP-1 and CLU mRNAs levels is measured, thus the subject is having all stage, in particular stage 4 of colorectal cancer.

In a particular embodiment, high expression levels of TIMP-1 and CLU mRNAs levels in a subject suffering from colorectal compared to the expression levels of TIMP-1 and CLU mRNAs levels from healthy individuals (HI) indicates all stage, in particular stage 4, of colorectal cancer.

Particularly, the methods of the invention are in-vitro methods.

Particularly, the colorectal cancer is a metastatic colorectal cancer.

As used herein and according to all aspects of the invention, the term “sample” denotes, blood, peripheral-blood, serum or plasma. Particularly, the sample is plasma.

According to the invention, the plasma can be collected in Streck Cell-free DNA BCT® tube or in tube with EDTA.

In a particular embodiment, the inventors show that in a tube streck, high expression of RPS28 B2M, TIMP-1 and CLU is equal to a stage 4 of colorectal cancer.

In a particular embodiment, the inventors show that in a EDTA tube, high expression of TIMP-1 and CLU is equal to all stage of colorectal cancer.

In a particular embodiment, the inventors show that in a EDTA tube, high expression of B2M, TIMP-1 and CLU is equal to a stage 4 of colorectal cancer.

Table A: biomarkers of the inventions:

Gene Name of the protein	Name	Protein ID (UniProt)	Gene ID (Entrez)
RPS28	40S ribosomal protein S28	P62857	6234
B2M	β2 microglobulin	P61769	567
TIMP-1	TIMP metalloproteinase inhibitor 1	P01033	7076
CLU	Clusterin	P10909	1191

Measuring the expression level of the 4 biomarkers mRNAs of the invention can be performed by a variety of techniques well known in the art. For example the nucleic acid contained in the samples (e.g., cell or tissue prepared from the subject) is first extracted according to standard methods, for example using lytic enzymes or chemical solutions or
5 extracted by nucleic-acid-binding resins following the manufacturer's instructions. The extracted mRNA is then detected by hybridization (e. g., Northern blot analysis and/or amplification (e.g., RT-PCR).

Other methods of Amplification include ligase chain reaction (LCR), transcription-mediated amplification (TMA), strand displacement amplification (SDA) and nucleic acid
10 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%
15 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 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,
20 such as in situ hybridization procedures, a nucleic acid probe includes a label (e.g., a detectable label). A "detectable label" is a molecule or material that can be used to produce a detectable signal that indicates the presence or concentration of the probe (particularly the bound or hybridized probe) in a sample. Thus, a labeled nucleic acid molecule provides an indicator of the presence or concentration of a target nucleic acid sequence (e.g., genomic target nucleic
25 acid sequence) (to which the labeled uniquely specific nucleic acid molecule is bound or hybridized) in a sample. A label associated with one or more nucleic acid molecules (such as a probe generated by the disclosed methods) can be detected either directly or indirectly. A label can be detected by any known or yet to be discovered mechanism including absorption, emission and/ or scattering of a photon (including radio frequency, microwave frequency,
30 infrared frequency, visible frequency and ultra-violet frequency photons). Detectable labels include colored, fluorescent, phosphorescent and luminescent molecules and materials, catalysts (such as enzymes) that convert one substance into another substance to provide a detectable difference (such as by converting a colorless substance into a colored substance or vice versa, or by producing a precipitate or increasing sample turbidity), haptens that can be

detected by antibody binding interactions, and paramagnetic and magnetic molecules or materials.

Particular examples of detectable labels include fluorescent molecules (or fluorochromes). Numerous fluorochromes are known to those of skill in the art, and can be selected, for example from Life Technologies (formerly Invitrogen), e.g., see, The Handbook—
 5 A Guide to Fluorescent Probes and Labeling Technologies). Examples of particular fluorophores that can be attached (for example, chemically conjugated) to a nucleic acid molecule (such as a uniquely specific binding region) are provided in U.S. Pat. No. 5,866, 366 to Nazarenko et al., such as 4-acetamido-4'-isothiocyanatostilbene-2,2' disulfonic acid, acridine
 10 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, ant1ranilamide, Brilliant Yellow, coumarin and derivatives such as coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-
 15 trifluoromethylcoumarin (Coumarin 151); cyanosine; 4',6-diaminidino-2-phenylindole (DAPI); 5',5"-dibromopyrogallol-sulfonephthalein (Bromopyrogallol Red); 7 -diethylamino -3 - (4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; 5-[dimethylamino] naphthalene-1-sulfonyl chloride (DNS, dansyl chloride);
 20 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives such as eosin and eosin isothiocyanate; erythrosin and derivatives such as erythrosin B and erythrosin isothiocyanate; ethidium; fluorescein and derivatives such as 5-carboxyfluorescein (FAM), 5-(4,6-dichloro-2-pyridinyl)amino-2,7-dimethoxy-4'-5'-dichloro-6-carboxyfluorescein (JOE),
 25 fluorescein, fluorescein isothiocyanate (FITC), and QFITC Q(RITC); 2',7'-difluorofluorescein (OREGON GREEN®); fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferone; ortho cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthalaldehyde; pyrene and derivatives such as pyrene, pyrene butyrate and succinimidyl 1-pyrene butyrate; Reactive Red 4 (Cibacron Brilliant Red 3B-A); rhodamine and
 30 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 nm (Heyduk and Heyduk, *Analyt. Biochem.* 248:216-27, 1997; *J. Biol. Chem.* 274:3315-22, 1999), as well as GFP, LissamineTM, diethylaminocoumarin, fluorescein chlorotriazinyl, naphthofluorescein, 4,7-dichlororhodamine and xanthene (as described in U.S. Pat. No. 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 bandgap of the semiconductor material used in the semiconductor nanocrystal. This emission can be detected as colored light of a specific wavelength or fluorescence. Semiconductor nanocrystals with different spectral characteristics are described in e.g., U.S. Pat. No. 6,602,671. Semiconductor nanocrystals that can be coupled to a variety of biological molecules (including dNTPs and/or nucleic acids) or substrates by techniques described in, for example, Bruchez et al., *Science* 281:2013-2016, 1998; Chan et al., *Science* 281:2016-2018, 1998; and U.S. Pat. No. 6,274,323. Formation of semiconductor nanocrystals of various compositions are disclosed in, e.g., U.S. Pat. Nos. 6,927,069; 6,914,256; 6,855,202; 6,709,929; 6,689,338; 6,500,622; 6,306,736; 6,225,198; 6,207,392; 6,114,038; 6,048,616; 5,990,479; 5,690,807; 5,571,018; 5,505,928; 5,262,357 and in U.S. Patent Publication No. 2003/0165951 as well as PCT Publication No. 99/26299 (published May 27, 1999). Separate populations of semiconductor nanocrystals can be produced that are identifiable based on their different spectral characteristics. For example, semiconductor nanocrystals can be produced that emit light of different colors based on their composition, size or size and composition. For example, quantum

dots that emit light at different wavelengths based on size (565 nm, 655 nm, 705 nm, or 800 nm emission wavelengths), which are suitable as fluorescent labels in the probes disclosed herein are available from Life Technologies (Carlsbad, Calif.).

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

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

Alternatively, an enzyme can be used in a metallographic detection scheme. For example, silver in situ hybridization (SISH) procedures involve metallographic detection schemes for identification and localization of a hybridized genomic target nucleic acid sequence. Metallographic detection methods include using an enzyme, such as alkaline phosphatase, in combination with a water-soluble metal ion and a redox-inactive substrate of the enzyme. The substrate is converted to a redox-active agent by the enzyme, and the redox-active agent reduces the metal ion, causing it to form a detectable precipitate. (See, for example, U.S. Patent Application Publication No. 2005/0100976, PCT Publication No. 2005/003777 and U.S. Patent Application Publication No. 2004/0265922). Metallographic detection methods also include using an oxido-reductase enzyme (such as horseradish peroxidase) along with a water soluble metal ion, an oxidizing agent and a reducing agent, again to form a detectable precipitate. (See, for example, U.S. Pat. No. 6,670,113).

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

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

equilibrium). The chromosome preparation is washed to remove excess probe, and detection of specific labeling of the chromosome target is performed using standard techniques.

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

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

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

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

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

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

The nucleic acid primers or probes used in the above amplification and detection method may be assembled as a kit. Such a kit includes consensus primers and molecular probes. A

preferred kit also includes the components necessary to determine if amplification has occurred. The kit may also include, for example, PCR buffers and enzymes; positive control sequences, reaction control primers; and instructions for amplifying and detecting the specific sequences.

5 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 (or q RT-PCR).

According to the invention, the expression level of the mRNA is expressed as absolute expression level (in copy genomic DNA equivalent/ml of plasma).

10 In a particular embodiment, when the RBTC index is obtained, a reference value (or cut-off or threshold) can be determined.

For example, and according to the invention, the cut-off value is 101516 for the RBTC index after a second centrifugation of the plasma at 160G and 546.5 for B2M after a second centrifugation of the plasma at 16000G, in absolute expression level.

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Methods of the invention may comprise a step consisting of comparing the mRNA of the invention with a control value. As used herein, "expression levels of an mRNA" refers to an amount or a concentration of the mRNA, for instance the biomarkers of the invention. Typically, a level of a mRNA can be expressed as nanograms per microgram of tissue or nanograms per milliliter of a culture medium, for example.

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

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a) providing a collection of samples from subjects suffering of a colorectal cancer;

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

c) ranking the tumor tissue samples according to said level or combine all the expression levels of the biomarkers of the invention to obtain a score;

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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 actual clinical outcome for the corresponding colorectal cancer subject;

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, sensitivity, specificity, AUC or Youden index) between both subsets

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

For the method of prediction according to the invention, the p-value can be calculated as well as others parameters (AIC, BIC, LLR, etc.).

For example the expression level of the biomarkers of the invention has been assessed
10 for 100 colorectal cancer samples of 100 subjects. 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
15 to 99 and on the other side sample Nr 100. According to the information relating to the actual clinical outcome for the corresponding colorectal cancer subject, Kaplan Meier curves are prepared for each of the 99 groups of two subsets. Also for each of the 99 groups, the p value between both subsets was calculated.

The reference value is selected such as the discrimination based on the criterion of the
20 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 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
25 to discriminate colorectal cancer samples and therefore the corresponding subjects.

Kaplan–Meier curves of percentage of survival as a function of time are commonly used to measure the fraction of subjects 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
30 expression level of a biomarker of the invention should of course be used for obtaining the reference value and thereafter for assessment of the expression level of a biomarker of the invention of a subject subjected to the method of the invention.

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 of the biomarkers of the invention.

The kits may include probes, primers macroarrays or microarrays as above described.

5 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 particular hybridization protocol, including solid-phase matrices, if applicable, and standards.

10 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.

15 The inventors have designed an efficient, rapid and cost-effective RT-QPCR based-method to purify and analyze mRNA from small volumes of blood plasma (see the results part).

Thus, in another aspect, the invention also relates to a method to analyse mRNA from a sample obtained from a subject comprising i) preparing a blood sample obtained from said subject to obtained plasma ii) doing a supplemental centrifugation of 160G or 16000G iii)

20 applying an optional freezing step of the plasma obtained in ii), iv) purifying the mRNA and v) doing a PCR assay like qPCR assay.

In a particular embodiment, a supplementation centrifugation of 160G or 16000G is done after the freezing.

According to the method of analyse of the mRNA, the supplemental centrifugation is of

25 160G.

Thus, in another aspect, the invention also relates to a method to analyse mRNA from a sample obtained from a subject comprising i) preparing a blood sample obtained from said subject to obtained plasma ii) doing a centrifugation of 160G or 16000G iii) applying a freezing step of the plasma obtained in ii), iv) doing a supplementation centrifugation of 160G or

30 16000G after the freezing, v) purifying the mRNA and vi) doing a PCR assay like qPCR assay.

According to the method of analyse of the mRNA, the preparation of blood sample in made by well-known techniques like decantation or centrifugation.

According to the method of analyse of the mRNA, the purification of the mRNA is made thank two different columns: the first to remove the residual gDNA and the second for purifying the total mRNA. Particularly, a Silicon-Carbide based technology column can be used.

According to the invention, the PCR assay can be a qPCR or RTPCR.

5

Method of treatment

The invention also relates to a method for treating a colorectal cancer in a subject diagnosed as having a colorectal cancer as described above comprising the administration to
10 said subject of an anti-colorectal cancerous agent.

Anti-colorectal cancer agents may be Melphalan, Vincristine (Oncovin), Cyclophosphamide (Cytosan), Etoposide (VP-16), Doxorubicin (Adriamycin), Liposomal doxorubicin (Doxil) and Bendamustine (Treanda).

Others anti-cancer agents may be for example cytarabine, anthracyclines, fludarabine,
15 gemcitabine, capecitabine, methotrexate, taxol, taxotere, mercaptopurine, thioguanine, hydroxyurea, cyclophosphamide, ifosfamide, nitrosoureas, platinum complexes such as cisplatin, carboplatin and oxaliplatin, mitomycin, dacarbazine, procarbazine, etoposide, teniposide, campathecins, bleomycin, doxorubicin, idarubicin, daunorubicin, dactinomycin, plicamycin, mitoxantrone, L-asparaginase, doxorubicin, epimbiom, 5-fluorouracil, taxanes
20 such as docetaxel and paclitaxel, leucovorin, levamisole, irinotecan, estramustine, etoposide, nitrogen mustards, BCNU, nitrosoureas such as carmustine and lomustine, vinca alkaloids such as vinblastine, vincristine and vinorelbine, imatinib mesylate, hexamethylmelamine, topotecan, kinase inhibitors, phosphatase inhibitors, ATPase inhibitors, tyrosine kinase inhibitors, protease inhibitors, inhibitors herbimycin A, genistein, erlotinib, and lapatinib. In one embodiment, additional
25 anticancer agents may be selected from, but are not limited to, one or a combination of the following class of agents: alkylating agents, plant alkaloids, DNA topoisomerase inhibitors, anti-folates, pyrimidine analogs, purine analogs, DNA antimetabolites, taxanes, podophyllotoxin, hormonal therapies, retinoids, photosensitizers or photodynamic therapies, angiogenesis inhibitors, antimitotic agents, isoprenylation inhibitors, cell cycle inhibitors,
30 actinomycins, bleomycins, MDR inhibitors and Ca²⁺ ATPase inhibitors.

Additional anti-cancer agents may be selected from, but are not limited to, cytokines, chemokines, growth factors, growth inhibitory factors, hormones, soluble receptors, decoy receptors, monoclonal or polyclonal antibodies, mono-specific, bi-specific or multi-specific antibodies, monobodies, polybodies.

Additional anti-cancer agent may be selected from, but are not limited to, growth or hematopoietic factors such as erythropoietin and thrombopoietin, and growth factor mimetics thereof.

In the present methods for treating cancer the further therapeutic active agent can be an
5 antiemetic agent. Suitable antiemetic agents include, but are not limited to, metoclopramide, domperidone, prochlorperazine, promethazine, chlorpromazine, trimethobenzamide, ondansetron, granisetron, hydroxyzine, acetylleucine monoemanolamine, alizapride, azasetron, benzquinamide, bietanautine, bromopride, buclizine, clebopride, cyclizine, dunenhydrinate, diphenidol, dolasetron, meclizme, methallatal, metopimazine, nabilone,
10 oxypemdy, pipamazine, scopolamine, sulpiride, tetrahydrocannabinols, thiephylperazine, thioproperazine and tropisetron. In a preferred embodiment, the antiemetic agent is granisetron or ondansetron.

In another embodiment, the further therapeutic active agent can be an hematopoietic colony stimulating factor. Suitable hematopoietic colony stimulating factors include, but are
15 not limited to, filgrastim, sargramostim, molgramostim and epoietin alpha.

In still another embodiment, the other therapeutic active agent can be an opioid or non-opioid analgesic agent. Suitable opioid analgesic agents include, but are not limited to, morphine, heroin, hydromorphone, hydrocodone, oxymorphone, oxycodone, metopon, apomorphine, nomioipine, etoipbine, buprenorphine, mepeddine, lopermide, anileddine,
20 ethoheptazine, piminidine, betaprodine, diphenoxylate, fentanyl, sufentanyl, alfentanyl, remifentanyl, levorphanol, dextromethorphan, phenazodone, pemazocine, cyclazocine, methadone, isomethadone and propoxyphene. Suitable non-opioid analgesic agents include, but are not limited to, aspirin, celecoxib, rofecoxib, diclofinac, diflusal, etodolac, fenoprofen, flurbiprofen, ibuprofen, ketoprofen, indomethacin, ketorolac, meclofenamate, mefanamic acid,
25 nabumetone, naproxen, piroxicam and sulindac.

In yet another embodiment, the further therapeutic active agent can be an anxiolytic agent. Suitable anxiolytic agents include, but are not limited to, buspirone, and benzodiazepines such as diazepam, lorazepam, oxazepam, chlorazepate, clonazepam, chlordiazepoxide and alprazolam.

30 In yet another embodiment, the further therapeutic active agent can be a checkpoint blockade cancer immunotherapy agent.

Typically, the checkpoint blockade cancer immunotherapy agent is an agent which blocks an immunosuppressive receptor expressed by activated T lymphocytes, such as cytotoxic T lymphocyte-associated protein 4 (CTLA4) and programmed cell death 1 (PDCD1,

best known as PD-1), or by NK cells, like various members of the killer cell immunoglobulin-like receptor (KIR) family, or an agent which blocks the principal ligands of these receptors, such as PD-1 ligand CD274 (best known as PD-L1 or B7-H1).

Typically, the checkpoint blockade cancer immunotherapy agent is an antibody.

5 In some embodiments, the checkpoint blockade cancer immunotherapy agent is an antibody selected from the group consisting of anti-CTLA4 antibodies, anti-PD1 antibodies, anti-PDL1 antibodies, anti-PDL2 antibodies, anti-TIM-3 antibodies, anti-LAG3 antibodies, anti-IDO1 antibodies, anti-TIGIT antibodies, anti-B7H3 antibodies, anti-B7H4 antibodies, anti-BTLA antibodies, and anti-B7H6 antibodies.

10 In another embodiment, the invention relates to a method for treating a colorectal cancer in a subject diagnosed as having a colorectal cancer as described above comprising the use to said subject of radiotherapy, heavy ion treatment, brachy-radiotherapy or radio-immunotherapy.

15 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:

20 **Figure 1: RPS28, B2M, TIMP-1 and CLU mRNA levels are increased in the plasma of metastatic colorectal cancer patients.**

Plasma mRNA levels of RPS28 (A), B2M (C) TIMP-1 (E) and CLU (G) in HI (n=53) compared with mCRC (n=103) after a second centrifugation of the plasma at 160G. Plasma mRNA levels of RPS28 (B), B2M (D) TIMP-1 (F) and CLU (H) in HI compared with mCRC
25 after a second centrifugation of the plasma at 16000G. Each dot represented as one case and mean $\pm$ SEM are indicated. Due to the logarithmic scale values that are at zero, 4 values in (A), 21 in (B), 2 in (C), 37 in (D), 24 in (F), 14 in (G) and 103 in (H) cannot be plotted. The plasma samples are collected in a Streck tube.

30 **Figure 2. Combination of RPS28, B2M, TIMP-1 and CLU mRNA as a biomarker of CRCm.**

The mRNA levels of RPS28, B2M, TIMP-1 and CLU were plotted for each patient and are connected by a line, (A) after a second centrifugation of plasma at 160G, (B) after a second centrifugation of plasma at 16000G, due to the logarithmic scale values that are at zero cannot be plotted. (C) Dot plot analysis showing the combination (RBTC index) of the level of RPS28,

B2M, TIMP-1 and CLU mRNA in HI (n=53) compared with mCRC (n=103) after a second centrifugation of the plasma at 160G. (D) Receiver operating characteristic curves showed that RBTS index discriminated between mCRC patients (n=103) and healthy controls (n=53) after a second centrifugation of the plasma at 160G. (E) Receiver operating characteristic curves showed the B2M mRNA level discriminated between mCRC patients (n=103) and healthy controls (n=53) after a second centrifugation of the plasma at 16000G. The plasma samples are collected in a Streck tube.

Figure 3: (A) B2M mRNA levels in plasma samples from HI (n=103) and patients with stage1 CRC (n=23), stage2 CRC (n=28), stage3 CRC (n=29) and stage4 CRC (n=38). (B) B2M mRNA levels in plasma samples from HI (n=103) and all stages CRC patients (n=116). Each dot represents one sample, the mean and standard deviation for each group is indicated. The absolute quantification was performed with human genomic DNA (gDNA) and the results are expressed in ng gDNA equivalent. Statistics: non-parametric Wilcoxon- Mann-Whitney test. The plasma samples are collected in EDTA tube.

Figure 4: (A) TIMP-1 mRNA levels in plasma samples from HI (n=103) and patients with stage1 CRC (n=23), stage2 CRC (n=28), stage3 CRC (n=29) and stage4 CRC (n=38). (B) TIMP-1 mRNA levels in plasma samples from HI (n=103) and all stages CRC patients (n=116). Each dot represents one sample, the mean and standard deviation for each group is indicated. The absolute quantification was performed with human genomic DNA (gDNA) and the results are expressed in ng gDNA equivalent. Statistics: non-parametric Wilcoxon- Mann-Whitney test. The plasma samples are collected in EDTA tube.

Figure 5: (A) CLU mRNA levels in plasma samples from HI (n=103) and patients with stage1 CRC (n=23), stage2 CRC (n=28), stage3 CRC (n=29) and stage4 CRC (n=38). (B) CLU mRNA levels in plasma samples from HI (n=103) and all stages CRC patients (n=116). Each dot represents one sample, the mean and standard deviation for each group is indicated. The absolute quantification was performed with human genomic DNA (gDNA) and the results are expressed in ng gDNA equivalent. Statistics: non-parametric Wilcoxon- Mann-Whitney test. The plasma samples are collected in EDTA tube.

Figure 6: (A) The TC index (i.e. the sum of the plasma levels of TIMP-1 and CLU mRNAs for each sample) in plasma samples from HI (n=103) and patients with stage1 CRC (n=23), stage2 CRC (n=28), stage3 CRC (n=29) and stage4 CRC (n=38). (B) The TC index in plasma samples from HI (n=103) and all stages CRC patients (n=116). Each dot represents one sample, the mean and standard deviation for each group is indicated. Statistics: non-parametric Wilcoxon- Mann-Whitney test. The plasma samples are collected in EDTA tube.

	Centrifugation: 160 G			Centrifugation: 16000 G		
	HI (n=53)	mCRC (n=103)	P value	HI (n=53)	mCRC (n=103)	P value
RPS28						
Median	488.7	1673	<0.0001 ****	239.7	335.7	0.152 ns
range	0-5463	0-29500		0-5607	0-2163	
Mean	851.1	3524		592.2	449.9	
Std. Error of Mean	142.5	439.4		154.7	43.46	
B2M						
Median	2023	77100	<0.0001 ****	133.7	859.7	<0.0001 ****
range	0-360000	131.3-3053333		0-20033	0-87933	
Mean	20817	380499		867.3	3260	
Std. Error of Mean	8543	63065		418.6	1019	
TIMP-1						
Median	7103	120784	<0.0001 ****	373.3	742.7	0.001 ***
range	400-186333	244-3453333		0-8863	0-69700	
Mean	17465	342903		964.3	4069	
Std. Error of Mean	4050	56555		222.9	1047	
CLU						
Median	515.7	17967	<0.0001 ****	0	0	0.1477 ns
range	0-77833	0-430333		0-1873	0-8857	
Mean	3620	45629		95.5	325.4	
Std. Error of Mean	1527	7314		39.7	112.9	
RBTC index						
Median	11101	259484	<0.0001 ****	698.2	2343	<0.0001 ****
range	750-628926	375.3-5636833		0-34842	0-168210	
Mean	42754	772555		2521	7909	
Std. Error of Mean	13919	107011		788.1	2094	

Table 1. Descriptive statistics of RPS28, B2M, TIMP-1 and CLU mRNA in HI and CRCm groups. All values are in genomic DNA copies equivalent per ml. P values are calculated using the non-parametric Wilcoxon- Mann-Whitney tests.

5

	Centrifugation : 160G	Centrifugation : 16000G
RPS28		
AUC	0.768	0.547
Std. Error	0.038	0.048
95% confidence interval	0.6932 to 0.8421	0.4530 to 0.6417
B2M		
AUC	0.873	0.736
Std. Error	0.02972	0.04154
95% confidence interval	0.8147 to 0.9312	0.6548 to 0.8176
TIMP-1		
AUC	0.897	0.650
Std. Error	0.02516	0.0448
95% confidence interval	0.8475 to 0.9461	0.5618 to 0.7374
CLU		
AUC	0.857	0.559
Std. Error	0.03027	0.04710
95% confidence interval	0.7977 to 0.9164	0.4671 to 0.6518

RBTC index		
AUC	0.9000	0.682
Std. Error	0.02576	0.04423
95% confidence interval	0.8495 to 0.9505	0.5955 to 0.7689

Table 2. ROC curve analysis

B2M	HI	Stage1	Stage2	Stage3	Stage4	All Stages
Number of values	103	23	28	29	38	116
Minimum	4.178	608.6	39.80	143.8	4.661	4.661
Median	5839	4803	9726	8026	14270	10515
Maximum	78180	142544	33125	100877	56579	142544
Mean	11323	14669	11688	16960	19710	16325
Std. Deviation	15393	29005	10826	23266	16202	20309
Std. Error of Mean	1517	6048	2046	4320	2628	1886

B2M	HI/Stage1	HI/Stage2	HI/Stage3	HI/Stage4	HI/All Stages
Mann Whitney test					
P value	0.6018	0.3934	0.1525	0.002	0.0136
P value summary	ns	ns	Ns	**	*
Significantly different (P < 0.05)?	No	No	No	Yes	Yes
Area under the ROC curve	0.5352	0.5531	0.5874	0.6686	0.5967

Table 3: Statistics regarding the analysis of plasma B2M mRNA in different stages of colorectal

5 cancer.

TIMP-1	HI	Stage1	Stage2	Stage3	Stage4	All Stages
Number of values	103	23	28	29	38	116
Minimum	3.641	31.74	2.789	26.49	10.19	2.789
Median	148.9	375.0	525.2	415.6	916.1	646.4
Maximum	5000	9134	3266	4068	6678	9134
Mean	304.2	903.1	778.4	857.7	1778	1160
Std. Deviation	550.5	1872	832.6	1017	1830	1531
Std. Error of Mean	54.24	390.3	157.3	188.9	296.9	142.2

TIMP-1	HI/Stage1	HI/Stage2	HI/Stage3	HI/Stage4	HI/All Stages
Mann Whitney test					
P value	0.0129	0.0022	0.0004	<0.0001	<0.0001
P value summary	*	**	***	****	****
Significantly different (P < 0.05)?	Yes	Yes	Yes	Yes	Yes
Area under the ROC curve	0.6650	0.6865	0.7121	0.8472	0.7449

Table 4: Statistics regarding the analysis of plasma TIMP-1 mRNA in different stages of colorectal cancer.

CLU	HI	Stage1	Stage2	Stage3	Stage4	All Stages
Number of values	103	23	28	29	38	116
Minimum	0.000	0.9023	5.691	0.6530	0.000	0.000
Median	66.78	163.5	648.6	432.0	1371	648.0
Maximum	22599	11316	11327	6107	10832	11327
Mean	590.6	930.4	1575	1068	2489	1646
Std. Deviation	2323	2335	2569	1658	2841	2497
Std. Error of Mean	228.9	486.9	485.6	308.0	460.8	231.9

TIMP-1	HI/Stage1	HI/Stage2	HI/Stage3	HI/Stage4	HI/All Stages
Mann Whitney test					
P value	0.0483	0.0002	0.0007	<0.0001	<0.0001
P value summary	*	***	***	****	***
Significantly different (P < 0.05)?	Yes	Yes	Yes	Yes	Yes
Area under the ROC curve	0.6319	0.7261	0.7029	0.7500	0.7150

Table 5: Statistics regarding the analysis of plasma CLU mRNA in different stages of colorectal cancer.

TC index	HI	Stage1	Stage2	Stage3	Stage4	All Stages
Number of values	106	21	28	29	38	116
Minimum	0.000	85.03	8.480	39.99	10.19	8.480
Median	216.2	530.3	1315	882.7	2485	1519
Maximum	27599	20450	13860	10088	16424	20450
Mean	869.5	1983	2353	1926	4267	2806
Std. Deviation	2801	4339	3236	2567	4447	3844
Std. Error of Mean	272.1	946.9	611.6	476.7	721.3	356.9

TC index	HI/Stage1	HI/Stage2	HI/Stage3	HI/Stage4	HI/All Stages
Mann Whitney test					
P value	0.0031	0.0005	<0.0001	<0.0001	<0.0001
P value summary	**	***	****	****	****
Significantly different (P < 0.05)?	Yes	Yes	Yes	Yes	Yes
Area under the ROC curve	0.7017	0.7119	0.7339	0.8337	0.7554

Table 6: Statistics regarding the analysis of plasma TC index in different stages of colorectal cancer.

5 EXAMPLE 1:

Material & Methods

Patients and sample collection

Blood samples from patients with metastatic colorectal cancer we analyzed were obtained from a part of samples from PANIRINOX clinical trial that is still the in course. PANIRINOX is a Phase II randomized study comparing efficacy of FOLFIRINOX + Panitumumab versus mFOLFOX6 + Panitumumab in metastatic colorectal cancer patients selected by RAS and B-RAF status from circulating DNA analysis (Protocol n° UC-0110/1608. EudraCT n°: 2016-001490-33). PANIRINOX study that involves 31 french hospitals and cancer centers in France was reviewed and approved by the human investigations committee Sud Méditerranée IV. All patients provided written informed consent before the screening procedure. This cohort study, along with other trial-related documents, received approval from Unicancer, the sponsor of the PANIRINOX study, which received authorization from the

Agence Nationale de Sécurité du Médicament et des Produits de Santé and the Comités de Protection des Personnes, according to French national regulatory requirements. Blood samples from healthy volunteers were provided by the “Etablissement Français du Sang (E.F.S),” the blood transfusion center of Montpellier, France (Convention EFS-PM N° 21PLER2015-0013).

5 Whole blood was collected in Streck Cell-free DNA BCT® tubes then shipped and stored at room temperature before plasma preparation. Blood was centrifuged at $1200 \times g$ for 10 min at 4 °C to separate plasma. To be sure not to contaminate the plasma with blood cells, 1 ml of plasma above the buffy coat was not collected. From the total plasma, 1 ml was reserved for our analysis, 500 µl was centrifuged at 160G for 10min at 4 °C and 500 µl at 16000G 160G for
10 10min at 4 °C then 250 µl of double-spun plasma aliquots were stored by at -20°C until RNA purification.

RNA isolation, reverse transcription and quantitative real-time PCR (qRT-PCR)

Plasma aliquots are thawed on ice and RNA is prepared from 200µl using Norgen Plasma/Serum RNA/DNA Purification Mini Kit columns (reference 55200). The first column
15 run is used to remove contaminating DNA, the second column is used to purify the total RNA which is eluted in 12 µl. QuantiTect Reverse Transcription Kit from Qiagen (reference 20531) was used for reverse transcription reaction. Briefly, purified RNA is incubated in gDNA wipeout buffer at 42°C for 5 minutes to thoroughly eliminate contaminating genomic DNA. Then, the reverse transcription reaction was conducted in a final volume of 20µl during 30 min.

20 Quantitation of mRNA was carried out by qRT-PCR using iQ™ SYBR® Green Supermix (BIO-RAD) according to the manufacturer’s instructions with a LightCycler® 480 (Roche). Absolute quantification was performed with human genomic DNA from Promega (reference G1471). The results are therefore expressed in genomic DNA copy equivalent considering that the weight of one copy of human male DNA is 3.3 pg [5]. All values below
25 100 copies per ml which corresponds to one copy in the QPCR assay are considered to be equal to zero copies detected. To certify that the prepared cDNA is free of contaminating genomic DNA, q-PCR quantification with primers that hybridize only to genomic DNA and not to RNA-derived cDNA sequences is routinely performed as a control. This allowed us to determine that
30 passage through a DNA purification column followed by a gDNA wipeout treatment are necessary for our method to be effective. Consequently, we have never detected any contaminating gDNA in our analyses.

Primer sequences were used to amplify genomic DNA and the biomarkers of the invention.

Statistical analysis

Differences between groups (HI and mCRC patients) in terms of transcript levels in plasma were assessed by using non-parametric Wilcoxon-Mann-Whitney tests. To evaluate the biomarker accuracy to identify mCRC patients, the receiver operating characteristic (ROC) curve and the area under the curve (AUC) were determined for each biomarker. AUC are presented with their 95% confidence intervals (95% CI). An optimal threshold value for biomarker combination (or for single biomarker) was determined by using the Youden's index (defined as the sum of the sensitivity and specificity minus 1). All p-values reported are two-sided and the significance level was set at 0.05. Statistical analyses were conducted with the Prism8 software.

Results

Development of the plasma RNA preparation method

Blood samples from healthy individuals (HI) and from mCRC patients were collected in identical conditions (Streck cell-free DNA BCT® tubes). These types of sampling tubes are now widely used to analyze circulating DNA from blood samples that have been in shipment for several days [6,7]. After preparation of the plasma as recommended by the tube supplier, and despite the fact that about 1 ml of plasma above the buffy coat is removed, the presence of residual cells in the collected plasma cannot be completely excluded if the buffy coat has been accidentally disturbed during the pipetting process.

To eliminate these eventual contaminating cells before preparing plasma RNA, we performed a supplementary centrifugation and compared two different centrifugation speeds (data not shown):

- a 160G centrifugation that is classically used in cell culture to pellet cells without breaking them in order to avoid they release their RNA.

- a 16000G centrifugation that allows the removal of solid particles such as cell debris, residual cells, residual platelets and large vesicles.

The plasmas that have undergone a second centrifugation are immediately frozen at -20°C. Thus, the analyses by QPCR can be temporally deferred.

To prepare RNA, we have avoided the use of phenol, which is extremely harmful. The Silicon-Carbide based technology column kit (Norgen) we used allows to eliminate efficiently the genomic DNA which could interfere with our QPCR assay thanks to a first column, a second column allows afterwards to purify the RNA. The absence of genomic DNA was systematically tested by QPCR using specific primers. To attest the presence of mRNA in our preparation we chose to assay mRNA encoding the Ribosomal Protein S28 (RPS28) and beta-glucuronidase

(GUS) that are both housekeeping genes classically used as references. RPS28 was consistently detected in the plasma of healthy individuals (data not shown) while GUS levels were lower and sometimes not detectable (not shown). The copy number of RPS28 mRNA was frequently higher when the plasmas underwent a second centrifugation at 160G than when the centrifugation was at 16000G, suggesting that some of the RPS28 mRNAs are associated with solid particles. These results show that the methodology we have developed is able to easily and rapidly determine the copy number in genomic DNA equivalent of a mRNA present in plasma.

We observed that the time between blood collection and plasma preparation (data not shown), the age of the donor (data not shown) and the sex of the donor (data not shown) had no impact on the efficiency of our analyses.

Selection of cancer related mRNA

We then investigated whether any mRNAs encoding proteins of interest in colorectal cancer were detectable with our method in healthy individual. Among candidate CRC-related biomarker proteins identified from literature searches we analyzed, we found that Beta2-microglobulin (B2M), tissue inhibitor of metalloproteinase 1 (TIMP-1) and Clusterin (CLU) mRNAs have copies levels per ml of plasma consistent with proper QPCR analysis regardless of the type of second centrifugation (data not shown).

RPS28, B2M, TIMP-1 and CLU mRNA levels in patients with metastatic colorectal cancer

We then sought to determine if the expression levels of RPS28, B2M, TIMP-1 and CLU mRNAs were significantly different in patients with metastatic colorectal cancer compared to healthy individuals and for this purpose 53 healthy individuals were compared to 103 patients. Surprisingly, plasma level of RPS28 mRNA in the cancer group was significantly increased compared to healthy controls (**Table 1, Figure 1A**) when the second centrifugation occurred at 160G. For the centrifugation of plasma at 16000G the difference was not significantly different (**Table 1, Figure 1B**). Regarding B2M mRNA levels, the difference is statistically significant between the plasmas of healthy individuals and patients regardless the speed of the second centrifugation applied to the plasmas (**Table 1, Figure 1C and 1D**). There is also a highly significant difference in TIMP-1 mRNA level when plasmas are centrifuged at 160 G (**Figure 1E**) and for centrifugation at 16000G (**Table 1, Figure 1F**). The difference of CLU mRNA level is significant between the plasmas of healthy individuals and patients when the second

centrifugation occurred at 160G (**Table 1, Figure 1G**), however when the second centrifugation occurred at 16000G the difference is not significant (**Table 1, Figure 1H**). The plasma level of the four mRNAs in each patient was represented (**Figure 2A and 2B**) and the dots representing each mRNA for each patient are connected by a line. We observed that the lines of the graph often intersect. This revealed that even if B2M and TIMP-1 are the higher, the profiles of each patient are different and that it is therefore clearly useful to analyze all markers for a given patient, because in the future they could give valuable indications about the disease. In addition, the cumulative values of the four markers can also be an additional data to take into account.

The combination (RPS28, B2M, TIMP-1, and CLU) of plasma mRNAs as a marker of mCRC

We tried to find a way to provide a simple result from this test. It appeared to us that the sum of the level of each mRNA (RBTC index) could give a more straightforward indicative result than all the separate results. We first analyzed the results obtained after the 160G second centrifugation and it emerged, as shown in **Figure 2C and Table 1**, that the difference of RBTC index was highly significant ($p < 0.0001$) which is a very simple way to interpret the results of this test. The test's diagnostic power was evaluated through the area under the curve (AUC) of the receiver operator characteristic curve (ROC). The RBTC index showed a diagnostic biomarker potential in distinguishing mCRC patients from healthy controls, with an AUC of 0.900 (**Figure 2D and Table 2**). Thus, even if it is close, the AUC of RBTC index is higher than the AUCs of the four mRNAs (RPS28, B2M, TIMP-1, and CLU) calculated individually (**Table 2**). A RBTC index level cutoff of 101516 was determined using the Youden's index; it has 81.6% sensitivity and 90.6% specificity to discriminate CRCm from healthy controls. In contrast, the ROC analysis of the four RNAs and RBTC index when the second centrifugation is performed at 16000G shows that the best AUC (0.736) is for B2M (**Figure 2E and Table 2**). This indicates that if the plasma has already undergone a second centrifugation at 16000 G, like in the case of plasmas prepared for circulating DNA analysis, single B2M measurement can give informative results. However, with a cutoff of 546.5 for the B2M level the sensitivity is only 60.2% and the specificity 83%. The cutoff value for the RBTC index when the second centrifugation is performed at 16000G was determined at 1154; sensitivity and specificity associated with this cutoff were 68.9% and 64.2%, respectively.

EXAMPLE 2:

Method

Whole blood samples were collected in EDTA tubes. Blood was centrifuged at $1200 \times g$ at 4°C for 10 min to separate plasma. The time interval between blood collection and plasma preparation is at most 4 hours. To avoid plasma contamination by blood cells, the first 1 ml of plasma above the buffy coat was not collected. Then the plasma is promptly frozen at -80°C . Plasma aliquots (250 μl) were thawed on ice and were then centrifuged at $160 \times g$ at 4°C for 10 min to remove cryo-precipitates. RNA were purified from 200 μl of plasma using the Plasma/Serum RNA/DNA Purification Mini Kit according to the manufacturer's protocol.

Results

The inventors show that the B2M mRNA levels (**Figure 3A and table 3**), the TIMP-1 mRNA levels (**Figure 4A and table 4**), the CLU mRNA levels (**Figure 5A and table 5**) and the sum of the plasma levels of TIMP-1 and CLU mRNAs levels (**Figure 6A and table 6**) in plasma samples from patients with stage 4 CRC were higher than respectively B2M mRNA levels, TIMP-1 mRNA levels, CLU mRNA levels and sum of the plasma levels of TIMP-1 and CLU mRNAs levels in plasma samples from healthy individuals (HI).

The inventors also show that the B2M mRNA levels (**Figure 3B**), the TIMP-1 mRNA levels (**Figure 4B**), the CLU mRNA levels (**Figure 5B**) and the sum of the plasma levels of TIMP-1 and CLU mRNAs levels (**Figure 6B**) in plasma samples from patients with all stage of CRC patients were higher than respectively B2M mRNA levels, TIMP-1 mRNA levels, CLU mRNA levels and sum of the plasma levels of TIMP-1 and CLU mRNAs levels in plasma samples from healthy individuals (HI).

The inventors show that the B2M mRNA levels is not higher in stage 1, 2 and 3 CRC patient plasma samples than in healthy individual plasma samples (**Figure 3A**) whereas the TIMP-1 and CLU mRNAs levels is higher in stage 1, 2 and 3 CRC patient plasma samples than in healthy individual plasma samples. (**Figures 4A and 5A**).

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CLAIMS:

1. A method for diagnosing a colorectal cancer in a subject in need thereof comprising i) determining in a sample obtained from the subject the expression levels of at least one
5 biomarker selected from the group consisting of RPS28, B2M, TIMP-1 and CLU mRNAs ii) comparing the expression level determined at step i) with its predetermined reference value and iii) concluding that the subject in need thereof has a colorectal cancer when the expression level determined at step i) is higher than its predetermined reference value, or concluding that the subject in need thereof has not a colorectal cancer
10 when the expression level determined at step i) is lower than its predetermined reference values.
2. A method for diagnosing according to the claim 1 wherein the expression level of 1, 2, 3 or 4 biomarkers selected from the group consisting of RPS28, B2M, TIMP-1 and CLU mRNAs is determined.
- 15 3. A method for diagnosing according to the claim 2 wherein the expression levels of the 3 biomarkers B2M, TIMP-1 and CLU are determined.
4. A method for diagnosing according to the claim 3 wherein if a high expression of the 3 biomarkers B2M, TIMP-1 and CLU mRNAs levels is measured, the subject is having all stage of colorectal cancer.
- 20 5. The method for diagnosing according to the claim 4 wherein if a high expression of the 3 biomarkers B2M, TIMP-1 and CLU mRNAs levels is measured, the subject is having a stage 4 of colorectal cancer.
- 25 6. A method for diagnosing according to the claims 1 to 2 comprising i) determining in a sample obtained from the subject the expression levels of the biomarkers selected from the group consisting of RPS28, B2M, TIMP-1 and CLU mRNAs ii) comparing the expression levels determined at step i) with their predetermined reference values and iii) concluding that the subject in need thereof has a colorectal cancer when the expression levels determined at step i) are higher than their predetermined reference values, or concluding that the subject in need thereof has not a colorectal cancer when

the expression levels determined at step i) are lower than their predetermined reference values.

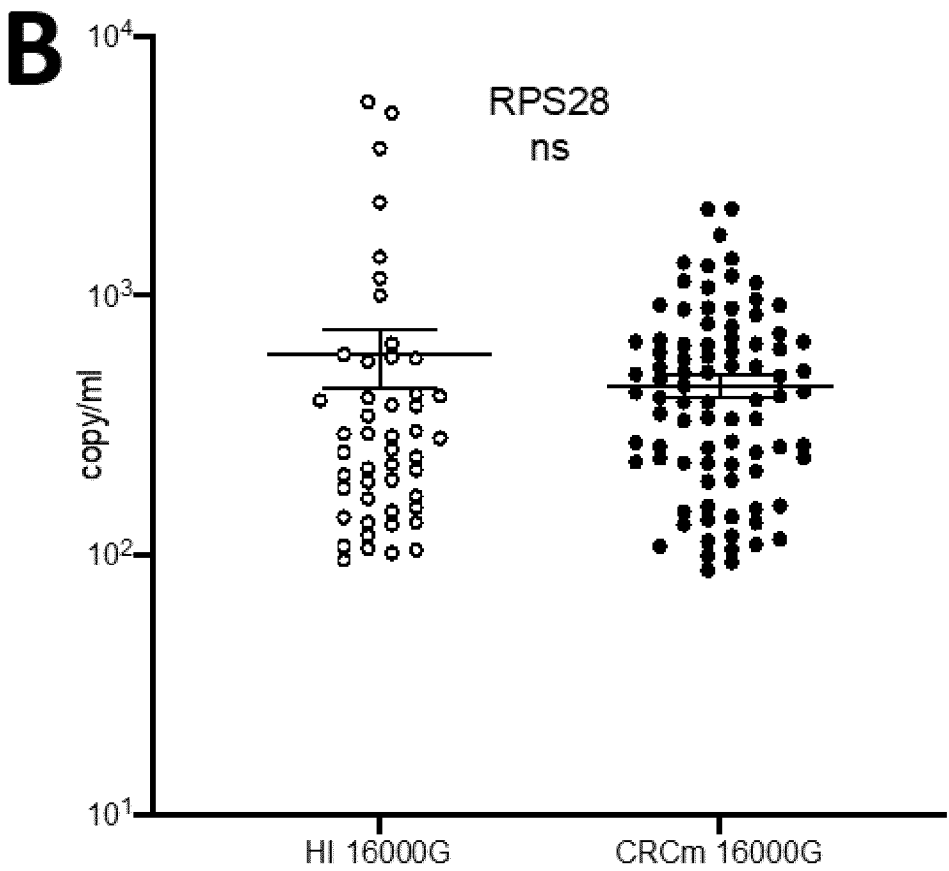
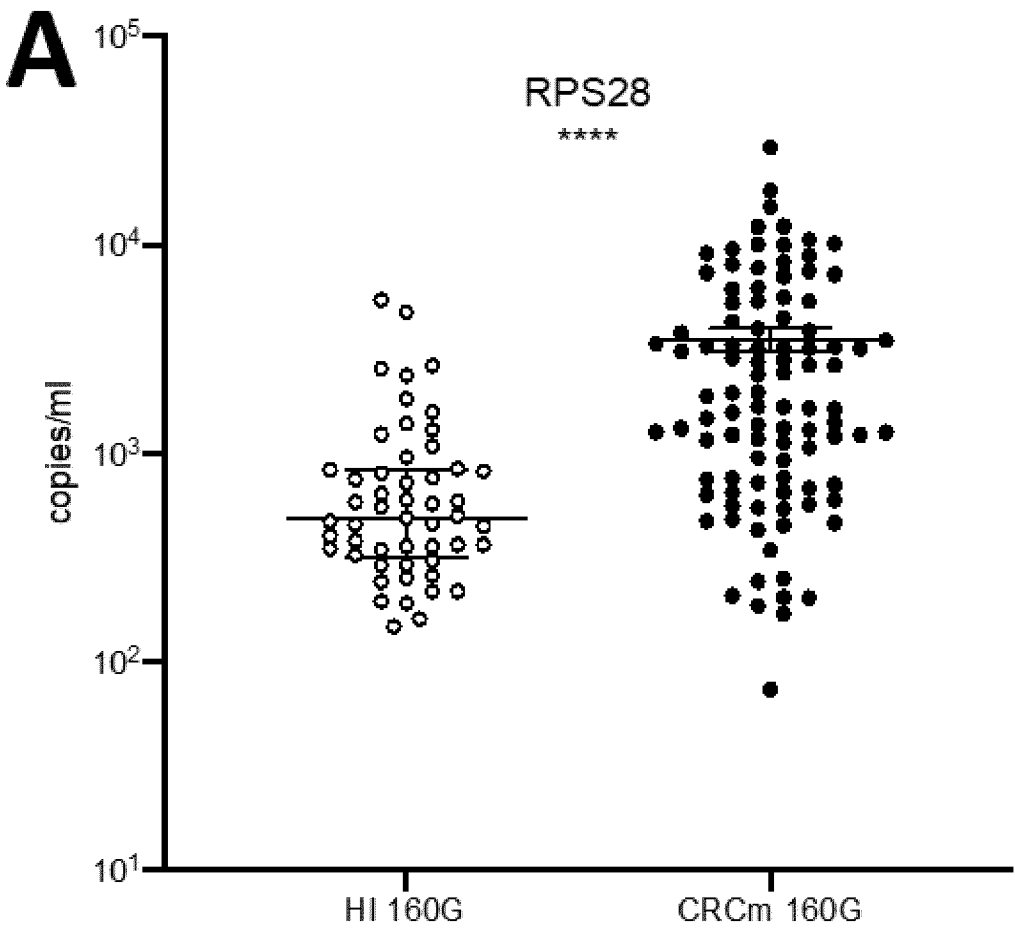
7. A method for diagnosing according to the claims 1 to 6 wherein the colorectal cancer is a metastatic colorectal cancer.

5 8. A method for diagnosing according to the claims 1 to 6 wherein the sample is plasma.

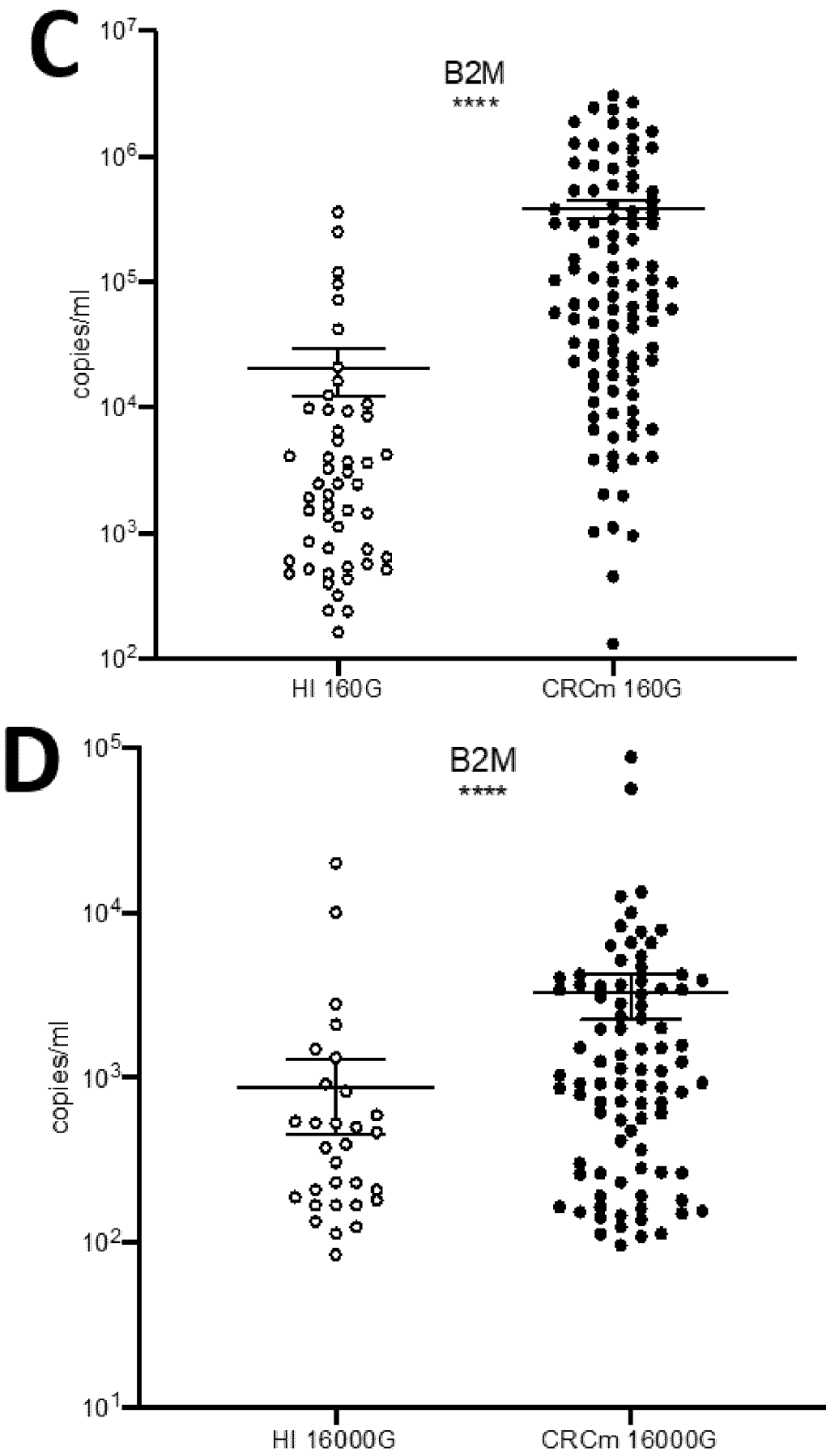
9. A method to analyse mRNA from a sample obtained from a subject comprising i) preparing a blood sample obtained from said subject to obtained plasma ii) doing a supplemental centrifugation of 160G or 16000G iii) applying an optional freezing step of the plasma obtained in ii), iv) purifying the mRNA and v) doing a PCR assay like
10 qPCR assay.

10. The method to analyse mRNA from a sample obtained from a subject according to claim 9 wherein a supplementation centrifugation of 160G or 16000G is done after the freezing.

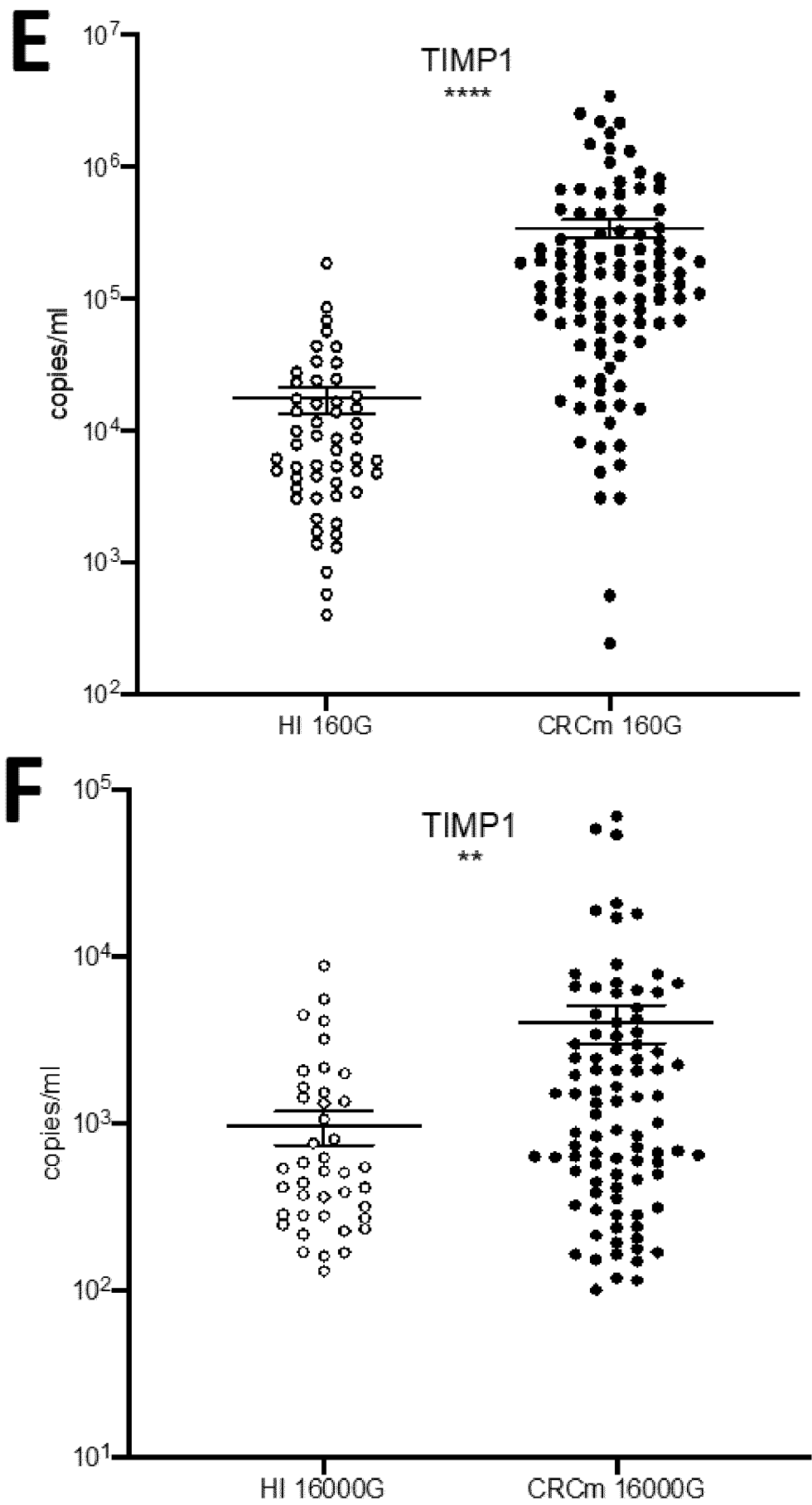
11. A method for treating a colorectal cancer in a subject diagnosed as having a colorectal
15 cancer according to the claims 1 to 8 comprising the administration to said subject of an anti-colorectal cancerous agent.



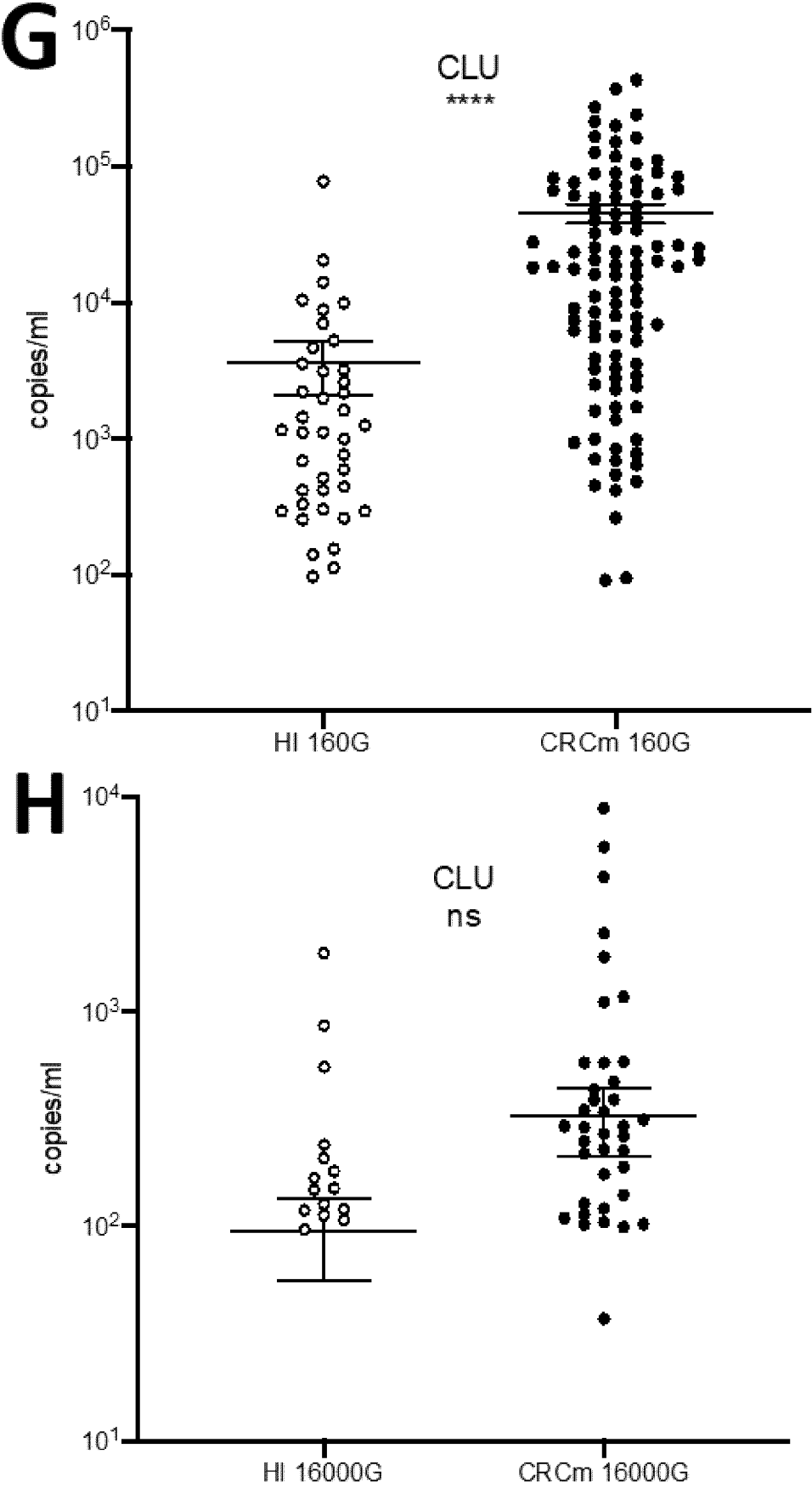
Figures 1A and 1B



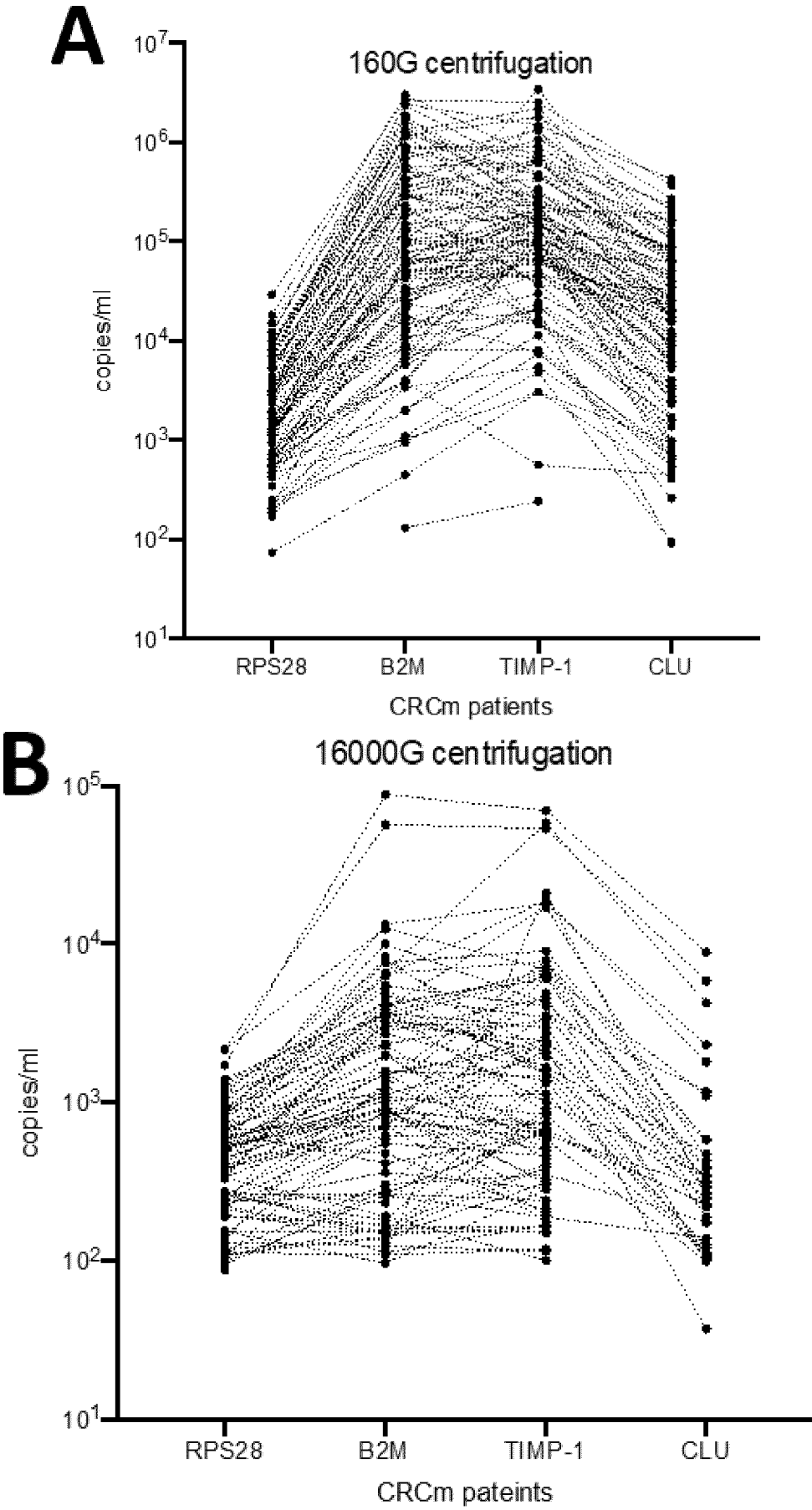
Figures 1C and 1D



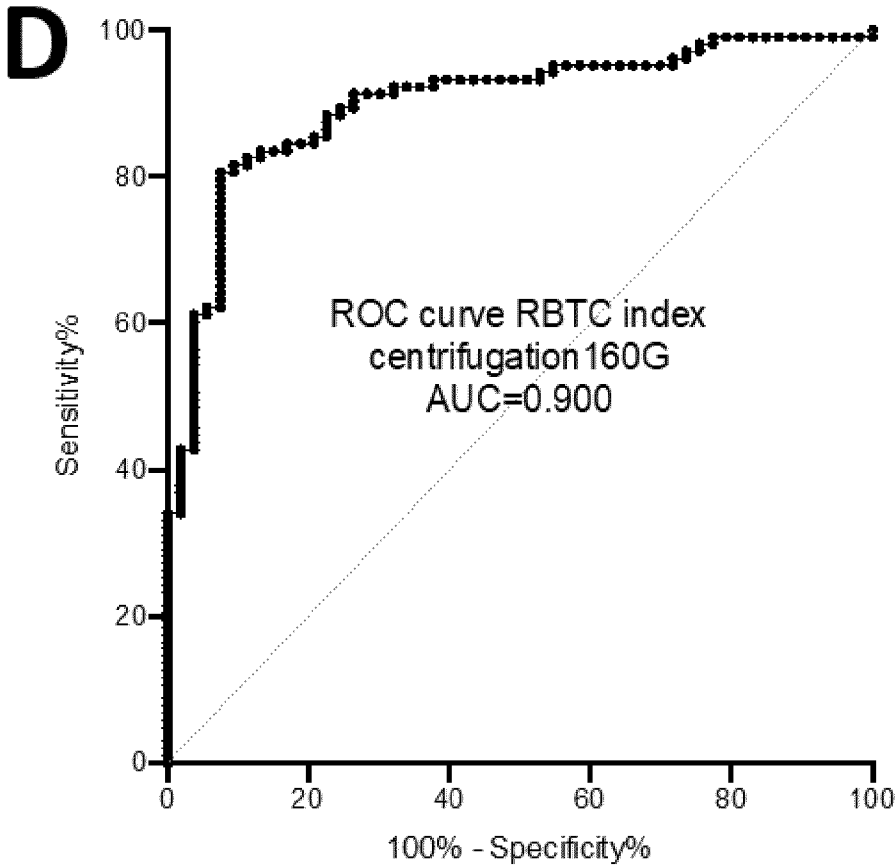
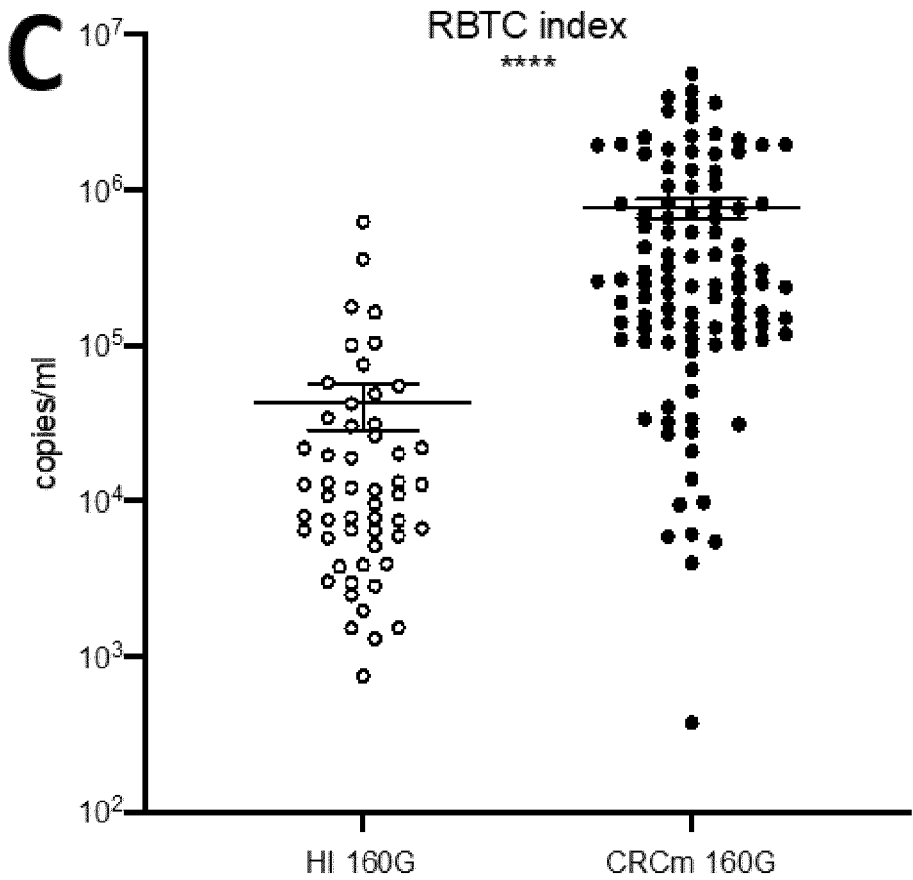
Figures 1E and 1F



Figures 1G and 1H



Figures 2A and 2B



Figures 2C and 2D

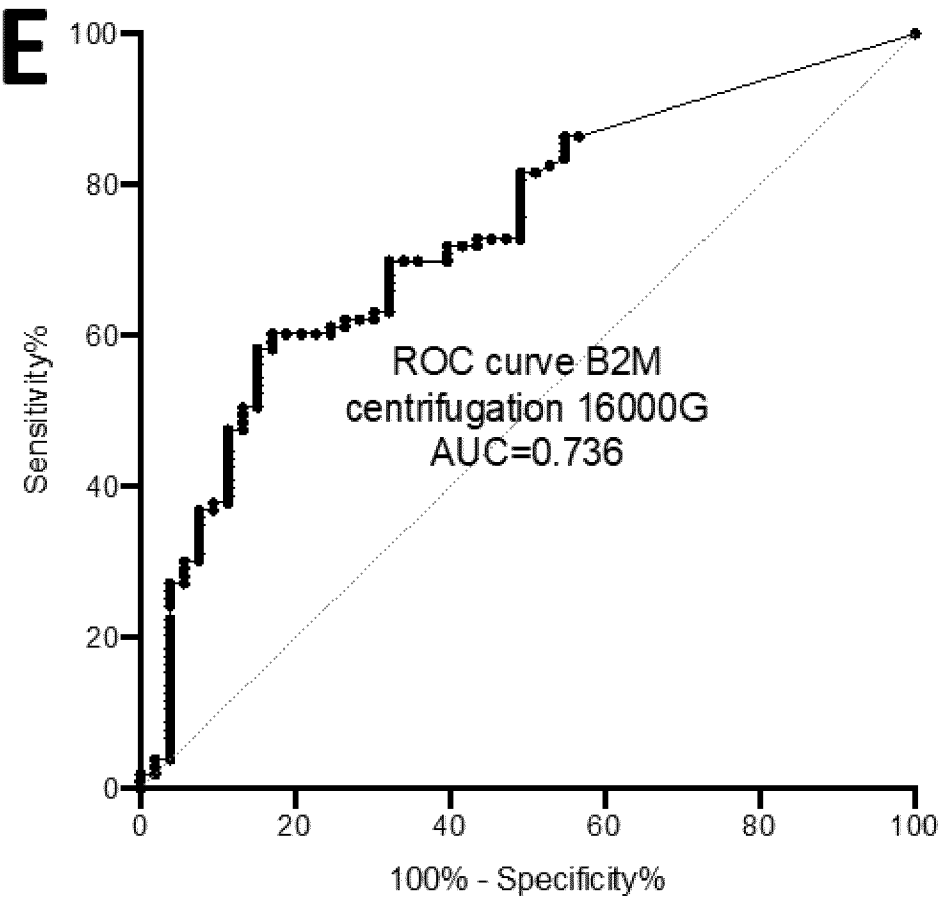
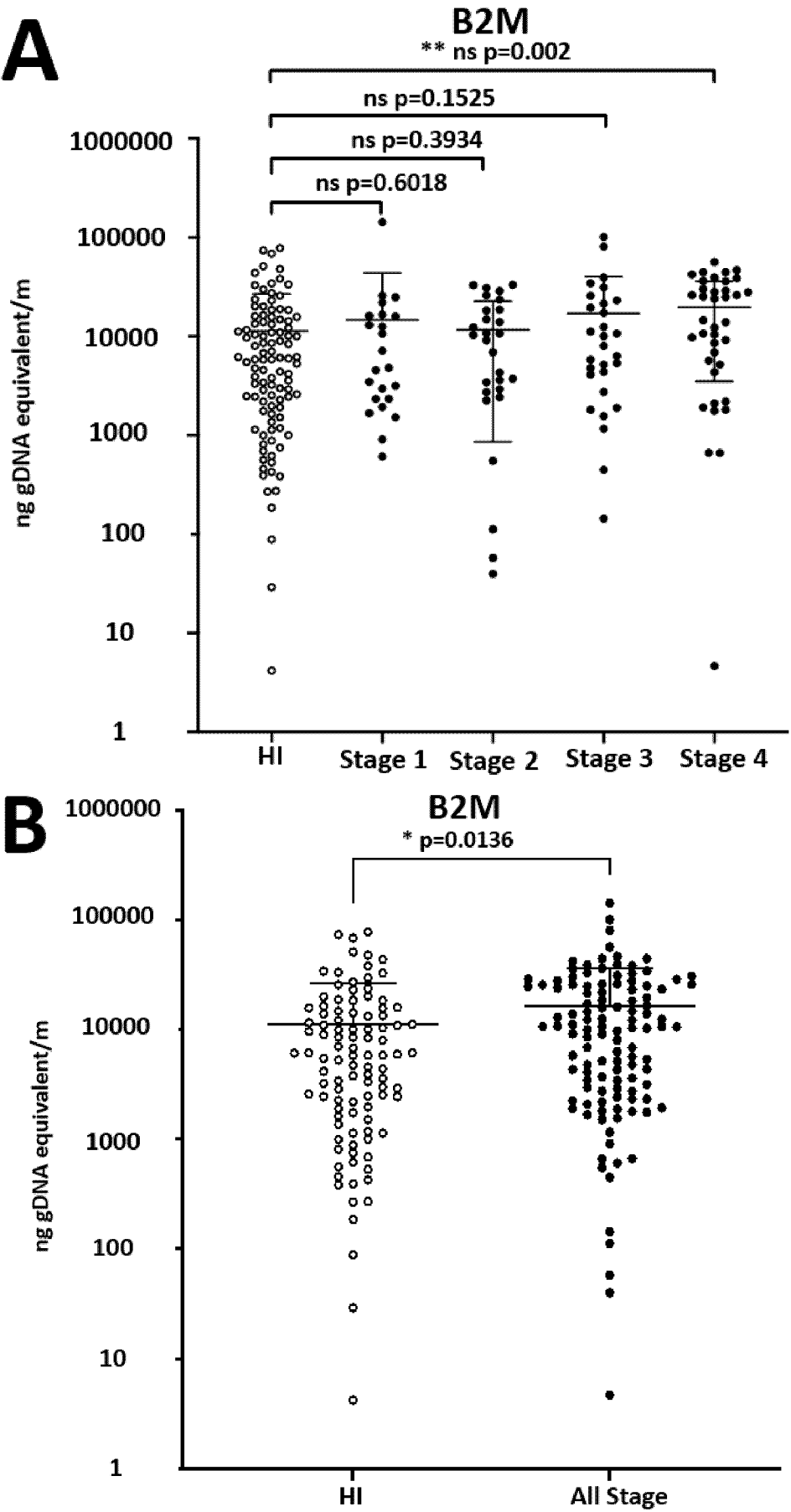
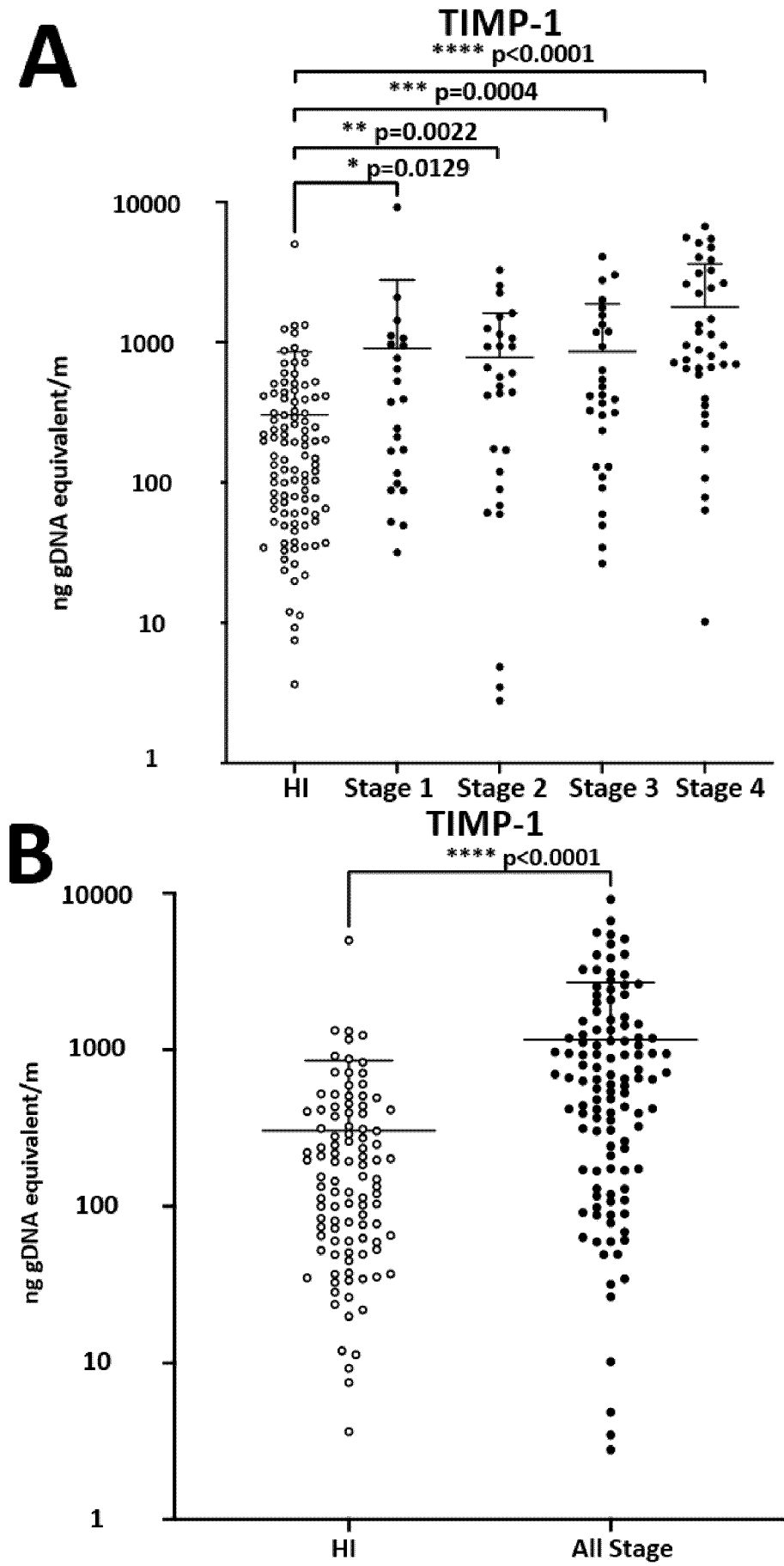


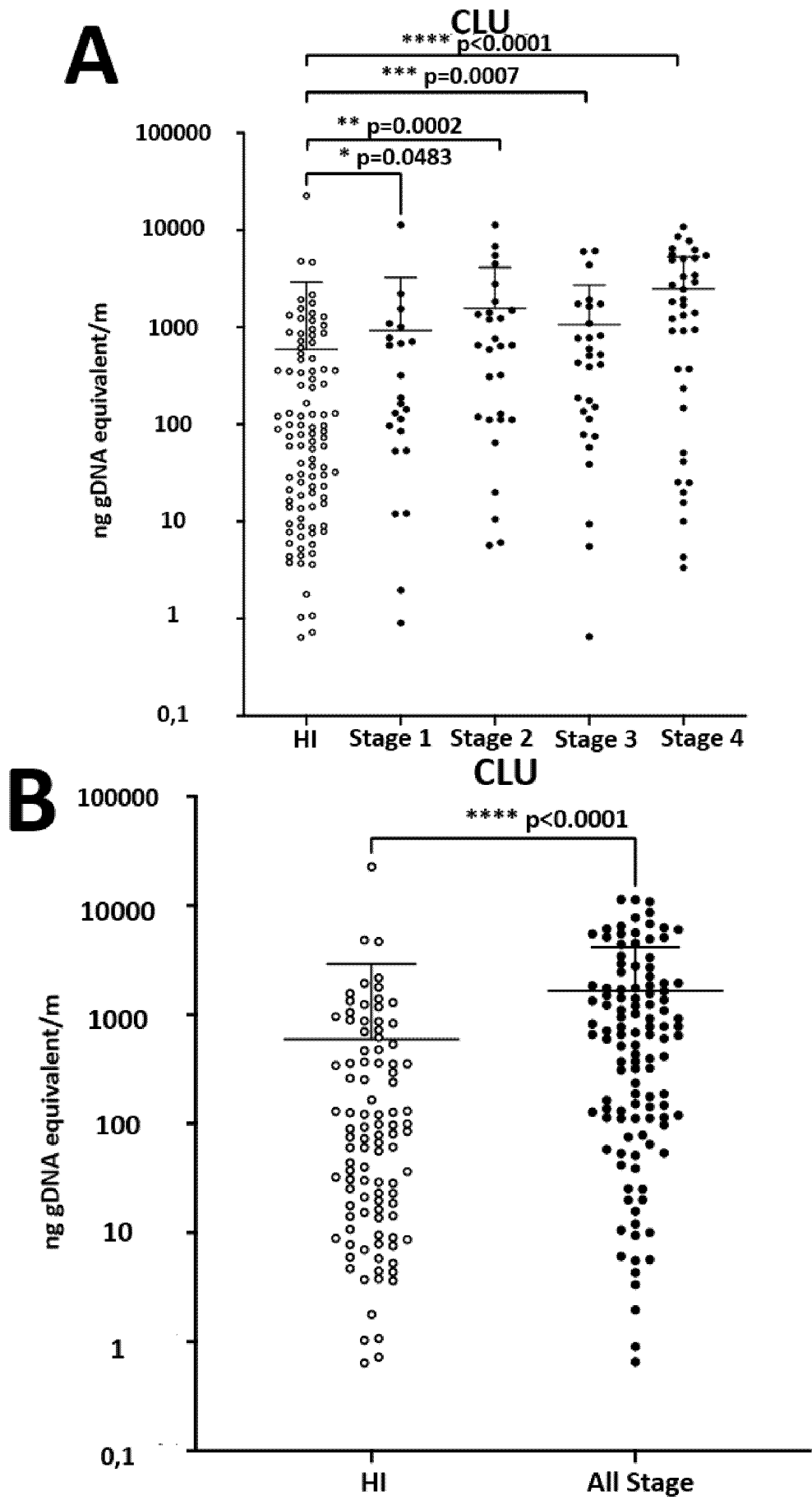
Figure 2E



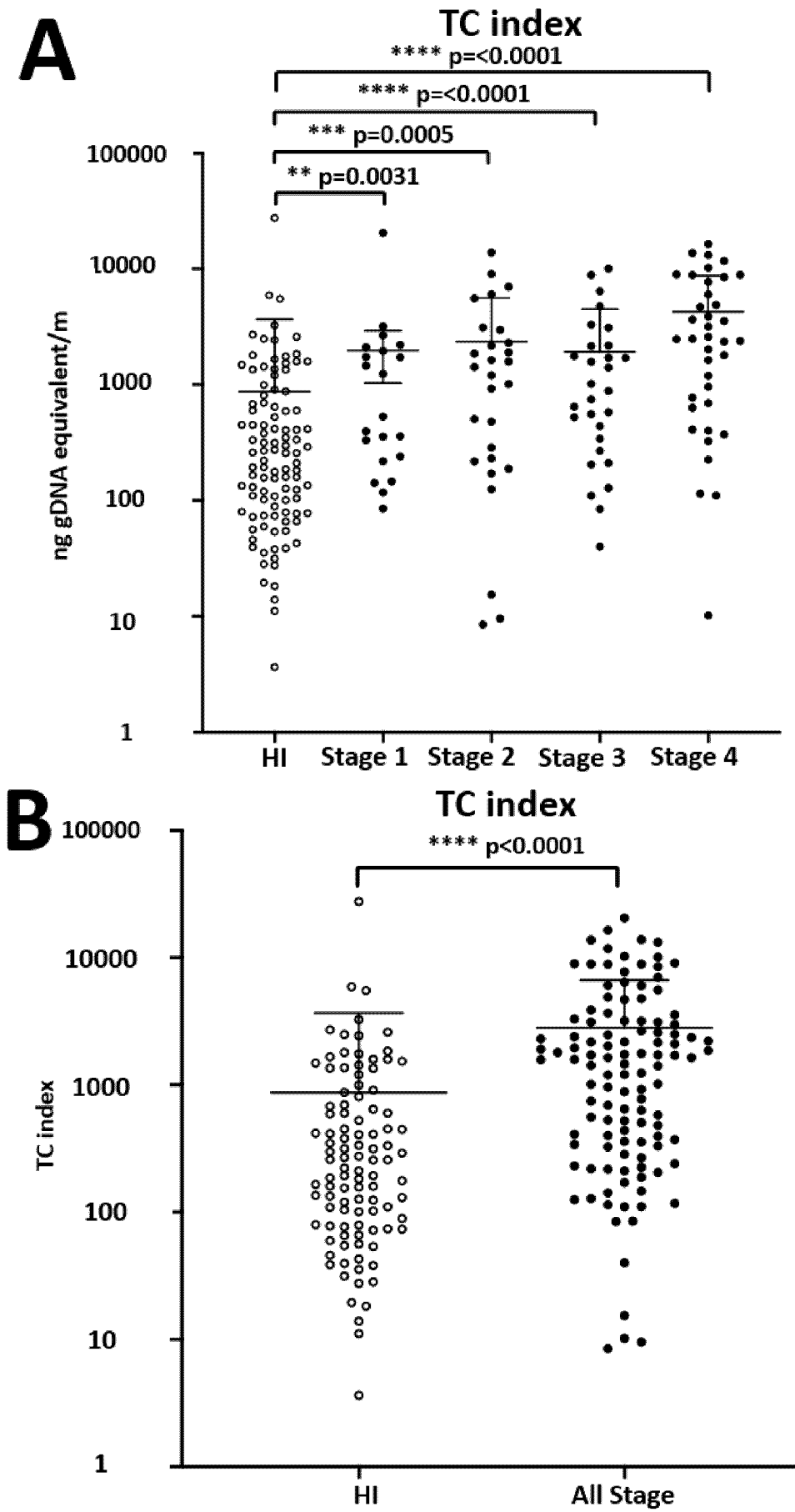
Figures 3A and 3B



Figures 4A and 4B



Figures 5A and 5B



Figures 6A and 6B

International application No
PCT/EP2023/052997

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, BIOSIS, Sequence Search, EMBASE, 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	US 2014/220580 A1 (BROWN KIRK [US] ET AL) 7 August 2014 (2014-08-07)	1-8, 11
Y	the whole document -----	9, 10
X	ARTEMAKI PINELOPI I ET AL: "High clusterin (CLU) mRNA expression levels in tumors of colorectal cancer patients predict a poor prognostic outcome", CLINICAL BIOCHEMISTRY, ELSEVIER, AMSTERDAM, NL, vol. 75, 28 October 2019 (2019-10-28), pages 62-69, XP085965161, ISSN: 0009-9120, DOI: 10.1016/J.CLINBIOCHEM.2019.10.008 [retrieved on 2019-10-28]	1-8, 11
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Date of the actual completion of the international search 5 April 2023		Date of mailing of the international search report 19/04/2023
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Botz, Jürgen

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2023/052997

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PAUL DOWLING ET AL: "Analysis of acute-phase proteins, AHSG, C3, CLI, HP and SAA, reveals distinctive expression patterns associated with breast, colorectal and lung cancer", INTERNATIONAL JOURNAL OF CANCER, JOHN WILEY & SONS, INC, US, vol. 131, no. 4, 19 November 2011 (2011-11-19), pages 911-923, XP071287719, ISSN: 0020-7136, DOI: 10.1002/IJC.26462 the whole document -----	1-11
X	EP 1 439 393 A2 (BAYER HEALTHCARE LLC [US]; MAYO FOUNDATION FOR MEDICALEDU [US]) 21 July 2004 (2004-07-21)	1-8, 11
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X	GONZÁLEZ-GONZÁLEZ MARÍA ET AL: "Tracking the Antibody Immunome in Sporadic Colorectal Cancer by Using Antigen Self-Assembled Protein Arrays", CANCERS, vol. 13, no. 11, 31 May 2021 (2021-05-31), page 2718, XP055943582, DOI: 10.3390/cancers13112718 the whole document -----	1-11
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Information on patent family members

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