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(54) **Title**: METHOD FOR PREDICTING PATIENT OUTCOME AND THERAPY RESPONSE IN METASTATIC COLORECTAL CANCER

(57) **Abstract**: Method for predicting patient outcome and therapy response in metastatic colorectal cancer. The method is based on the detection of the expression level of a panel of genes expressed in epithelial CTCs (GAPDH, VIL1, TIMP1, CLU, LOXL3, ZEB and TLN1), preferably normalized to CD45 or another gene not expressed in CTCs, and the comparison of the evolution of said levels in blood samples taken at different time points from mCRC patients undergoing therapy. The samples are classified in accordance with the expression levels of at least one of said genes or, preferably, a combination of six (GAPDH, VIL1, TIMP1, CLU, LOXL3, ZEB) or seven genes, as high- or low-CTC, the change to low or maintenance of the classification as low indicating therapy effectiveness. The method is easy to perform and can be carried out early during therapy course, allowing early decisions about continuation of therapy administration to non responding patients.

METHOD FOR PREDICTING PATIENT OUTCOME AND THERAPY RESPONSE IN METASTATIC COLORECTAL CANCER

FIELD OF THE INVENTION

5 The invention relates to the field of the diagnostic methods performed out of the body to determine if a therapy is appropriate for a particular individual. Particularly, the present invention refers to a method for determining both the outcome of patients suffering from metastatic colorectal cancer and the effectiveness of the administered therapy.

10

BACKGROUND OF THE INVENTION

Colorectal cancer (CRC) represents the fourth leading cancer-related death cause worldwide (Jemal et al., 2011), mainly due to the presence of metastasis, fact that significantly reduces survival rates. Cancer dissemination starts with 15 primary tumors shedding malignant cells into the circulation and those Circulating Tumor Cells (CTCs) can form a distant metastasis when reaching an appropriate target organ (Nguyen et al., 2009).

CTC detection and quantification has shown to be a predictive prognostic factor in different tumor types, including colorectal. CTC count is increasingly being 20 incorporated to clinical trials as a non-invasive, fast and high sensitive patient monitoring technique, what has favoured the development of several different CTC quantification methods in the last years. In fact, the American Food and Drug Administration (FDA) has given clearance to the CellSearch® system (Veridex) (https://www.cellsearchctc.com/) for enumeration of CTCs of epithelial origin 25 (CD45-, EpCAM+, and cytokeratins 8, 18+ and/or 19+) in whole blood from metastatic colorectal cancer (mCRC) patients. The CellSearch® system makes use of ferrofluid nanoparticles with antibodies that target epithelial cell adhesion antigens in order to enrich the samples in CTCs, which are magnetically separated from the bulk of other cells in the blood. Using this system, different studies have 30 shown that the presence of 3 or more CTCs per 7,5 ml of blood in mCRC patients predicted poor patient outcome at baseline, e.g. before treatment (Cohen et al., 2008; Cohen et al., 2009).

Apart from the CellSearch system, which can be regarded as the actual gold standard, other methodologies based on CTC detection have been developed, with

the aim of increasing sensitivity, due to the rare frequency of CTC appearance (Vona et al., 2000; Ozkumur et al., 2013; Hou et al., 2012). Negative leukocyte depletion or filtration methods enrich CTC without the need for specific CTC membrane antigens (like, for instance, EpCAM), possibly reducing bias (Guo et al., 5 2004).

Molecular characterization of CTCs is becoming more relevant in colorectal cancer patients, achieving similar, or even better CTC detection rates, strengthening CTC prognostic value in mCRC and opening a new way for personalized patient treatment, based on CTC characteristics (Gervasoni et al., 10 2008; Barbazan et al., 2012a; Lagoudianakis et al., 2009). For example, KRAS, BRAF, EGFR or PIK3CA mutational statuses in CTCs can be analysed, providing relevant information. Moreover, global gene expression CTC profiling has become a promising source of information, providing with hundreds of CTC markers for detection and prognosis and also potential therapeutic targets, and importantly 15 contributing to the actual knowledge of the process of metastatic dissemination (Barbazan et al., 2012b; Smirnov et al., 2005).

The group of the present inventors (Barbazan et al., 2012a), reported a method for detection of CTCs from mCRC patients that combines immune-enrichment (based on magnetic beads covered with anti-EpCAM antibodies), 20 optimal purification of RNA from very low cell numbers, and the selection of accurate PCR probes for assessing the expression level of GAPDH and VIL (as CTC detection markers) normalized to CD45 (a marker specific to leukocytes). A logistic model based on said markers is also presented, which model can be used as prognosis tool to determine progression-free survival in mCRC. The model was 25 complemented with a gene expression study of mCRC patients (Barbazan et al., 2012b), that resulted in 410 genes that characterized the CTC population. Comparison between primary carcinomas and lung and liver metastases further involved the CTC-genes in the promotion of metastasis. The correlation of CTC-gene expression with clinical parameters demonstrated detection and prognosis 30 significance. The list of validated genes with diagnostic and prognostic value included, among others, CLU and TIMP1, which are associated with cell death and anti-apoptotic activity, and TLN1, which is associated with cell adhesion. TGFβ1, APP, CD9, ITGB5, LIMS1, RSU1, VCL and BMP6 were also among the validated genes with diagnostic and prognostic value.

Prognosis of patients' outcome might be an important information, but the selection of the appropriate therapy for each individual and the assessment of the effectiveness or non-effectiveness of the administered therapy is an important goal, in order to stop it for those patients who are only suffering toxicity-derived adverse effects and to change to a more appropriate therapy as soon as possible. It is important to keep in mind that not all tumors respond to the same therapies, even though their classification and morphological appearance are similar; difficulties to choose the appropriate therapy results in more than 50% cancer patients not receiving an efficient treatment.

In order to decide the therapy to be administered, it is generally considered that classifying the tumor, as well as the patient's condition, is an important step. Cancer staging is the process of determining the extent to which a cancer has developed by spreading. Contemporary practice is to use an overall stage grouping that consists of assigning a number from I-IV to a cancer, with I being an isolated cancer and IV being a cancer which has spread to other organs, mainly if new occurrences of the disease (metastasis) have generated from the organ or part of organ of the primary tumor to another non-adjacent organ or part.

Nevertheless, the most commonly used system is the TNM staging system, a system of classification of malignant tumors that describes the extent of a person's cancer taking into account three parameters:

- T describes the size of the original (primary) tumor and whether it has invaded nearby tissue,
- N describes nearby (regional) lymph nodes that are involved
- M describes distant metastasis (spread of cancer from one part of the body to another)

TNM is developed and maintained by the Union for International Cancer Control (UICC) (https://www.uicc.org/) and by the American Joint Committee on Cancer (AJCC) (http://cancerstaging.org/About/what-is-the-ajcc/Pages/whatisajcc.aspx). UICC and AJCC staging systems were unified into a single staging system in 1987. Nevertheless, most of the common tumors have their own TNM classification system. In the case of colorectal cancer (American Joint Committee on Cancer: AJCC Cancer Staging Manual. 6th ed. New York, NY, Springer, 2002, pp 113-124), the following classification criteria are followed:

Primary tumor (T)

- TX: Primary tumor cannot be assessed
- T0: No evidence of primary tumor
- Tis: Carcinoma *in situ*: intraepithelial or invasion of the lamina propria
- T1: Tumor invades submucosa
5
- T2: Tumor invades muscularis propria
- T3: Tumor invades through the muscularis propria into the subserosa, or into nonperitonealized pericolic or perirectal tissues
- T4: Tumor directly invades other organs or structures, and/or perforates visceral peritoneum

10 *Regional lymph nodes (N)*

- NX: Regional nodes cannot be assessed
- N0: No regional lymph node metastasis
- N1: Metastasis in 1 to 3 regional lymph nodes
- N2: Metastasis in 4 or more regional lymph nodes

15 *Distant metastasis (M)*

- MX: Distant metastasis cannot be assessed
- M0: No distant metastasis
- M1: Distant metastasis

Stage IV colorectal cancer corresponds with the situation of any T, any N,
20 M1.

The patient's functional status or Performance Status (PS) scale refers to the general condition and the activities that a patient can perform. Performance Status classification is based on scales and criteria used by doctors and researchers to assess how a patient's disease is progressing, assess how the
25 disease affects the daily living abilities of the patient, and determine appropriate treatment and prognosis. It is a common practice to use the ECOG Performance Status, of the Eastern Cooperative Oncology Group, (publicly available, for instance, on the web site: http://ecog.dfci.harvard.edu/general/perf_stat.html), reported by Oken and coworkers (Oken et al., 1982). According to such
30 classification, patients are graded as follows:

0: Fully active, able to carry on all pre-disease performance without restriction

1: Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work

2: Ambulatory and capable of all selfcare but unable to carry out any work

activities. Up and about more than 50% of waking hours

3: Capable of only limited selfcare, confined to bed or chair more than 50% of waking hours

4: Completely disabled. Cannot carry on any selfcare. Totally confined to bed or chair

5: Dead

Surgery has shown to be reasonably effective when a localized tumor (Stage I) is to be treated, with a high percentage of patients (>90%) showing 5 year survival rates after treatment. Patients with localized disease (stage II and III), undergo surgery for removal of the primary tumor. Although a high number of patients are cured after surgery, recurrences appear in a subset of patients. Chemotherapy is administered to those patients, usually based on clinical criteria without prognostic value.

Stage IV colorectal cancer is the most advanced cancer stage. Also termed metastatic colorectal cancer (mCRC), stage IV CRC is characterized by the presence of tumor dissemination to nearby lymph nodes and other parts of the body, like the liver, lungs or ovaries. When the cancer has reached this stage, surgery is generally used for relieving or preventing complications as opposed to curing the patient of the disease. When disseminated disease to the liver and/or lungs is restricted enough, and after having demonstrated chemosensitiviy to adjuvant therapies, surgery might be applied to remove metastatic lesions.

After the appearance of distant metastasis, chemotherapy is considered the main treatment. For stage IV cancer that cannot be surgically removed, chemotherapy, radiation therapy, or both may be used to relieve, delay or prevent symptoms. Although it has shown to increase patient survival in this scenario, survival rates strongly decrease, with a high number of patients dying after several months due to the presence of disseminated disease.

Chemotherapy standard treatment is mainly based in the use of fluoropyrimidines (fluorouracil or capecitabine), either in monotherapy or combined with oxaliplatin/irinotecan and targeted drugs (Bevacizumab, Panitumumab or Cetuximab) (Cunningham et al., 2010). Some combinations receive specific names, such as FOLFOX (leucovorin, 5-fluorouracil and oxaliplatin), FOLFIRI (leucovorin, 5-fluorouracil and irinotecan) or FOLFOXIRI (leucovorin, 5-fluorouracil, oxaliplatin and irinotecan). Despite important improvements in mCRC therapeutic

strategies, response rates are still low (30-50%), meaning that a high number of patients will not benefit from the therapy, but will suffer toxicity-related side effects. Therefore, the identification of predictive factors is an important necessity, particularly if their predictive value can be evaluated early in the treatment course.
5 A predictive factor is a measurement that is associated with response or lack of response to a particular therapy, while response can be defined using any of the clinical endpoints commonly used in clinical trials. The necessity and difficulty of identifying suitable predictive factors for a given therapy is more clear if it is taken into account that the fact of being a prognostic factor or marker (a measurement
10 that is associated with clinical outcome in the absence of therapy) does not imply that a molecule or gene also has a predictive value.

Routinely, treatment effectiveness is assessed using sophisticated imaging methods. Computized tomographic (CT) colonography, also known as computerized tomographic colonography, virtual colonoscopy or CT scan, has
15 emerged in the last decade as an alternative to colonoscopy, due to its better safety profile, although it is not commonly accepted as the reference technique, because it is considered to be less reliable than colonoscopy. CT scan consists of a series of detailed pictures of areas inside the body, taken from different angles, created by a computer linked to an x-ray machine. Moreover, CT examination is
20 usually performed 3 months after the beginning of the treatment, which means that, when patients are non responders to therapy, a long time has been wasted administering a therapy that results finally to be not effective, increasing the risk for the patient to be aggravated the disease instead of getting an alleviation of the condition.

25 Early predictive markers such as KRAS mutation status (bearing the wild type gene or an exon 2-mutated gene) have shown to be effective for stratification of patients receiving anti-EGFR therapy (Lievre et al., 2008), and serum biomarkers like CA-125 and, specially, carcinoembrionic antigen (CEA) are also an indicative tool for treatment effectiveness in mCRC patients (Aldulaymi et al., 2010), but none
30 of them are conclusive as markers for therapy monitoring. KRAS in particular lacks prognostic value by itself.

Recently, CTCs have been proposed not only as a baseline predictive factor for patient outcome, but also as an indicator of therapy effectiveness, since changes in CTC count along treatment predicted therapy response in different

tumor types and usually earlier than current approaches (Hartkopf et al., 2011; Hayes et al., 2006; Saad et al., 2012), thus allowing the separation of patients who are benefiting from the therapy from those who do not. However, only few studies have shown the utility of CTC changes in mCRC (Matsusaka et al., 2011; Tol et al.,
5 2010), most data having emerged from breast cancer studies, as breast cancer is the tumor type most investigated in the field of CTC research.

On the other hand, the role of the epithelial to mesenchymal transition (EMT) process, has gained relevance in the last years, as several groups have shown the importance of this phenomenon in tumor dissemination (Lim et al.,
10 2012). As reviewed, for instance, by Thiery and Sleeman (Thiery and Sleeman, 2006), certain evidences support a role for EMT during tumor progression, both phenotypic and functional. Among the phenotypic evidence can be mentioned the correlation with tumor progression and poor prognosis that can be found for the expression of proteins that are characteristic of mesenchymal cells (for example,
15 vimentin, fibroblast-specific protein-1, SNAI1 (Snail) and SNAI2 (Slug), nuclear β-catenin and stromelysin-3) as well as for the lost of epithelial markers such as, for instance, E-cadherin; some functional evidences are the relapse and metastasising capacity observed in regressed tumors after spontaneous induction of SNAI1, as well as the change of phenotype and invasive behaviour resulting from the
20 manipulation of E-cadherin expression. In fact, downregulation of E-cadherin expression is one of the common endpoints of EMT-inducing signalling pathways, other common endpoint being the expression of EMT associated genes.

As Peinado and coworkers have commented in some of their reports (Peinado et al., 2005), the local invasion process is thought to constitute the first
25 event in the transition from premalignant to invasive malignant carcinomas. This step requires that tumor cells are able to disrupt the E-cadherin-mediated cell-cell adhesions and to acquire motility, and it is frequently associated with EMT. Research on the mechanism leading to the silencing of E-cadherin during tumor progression has been led to the identification of several *E-cadherin* repressors,
30 including zinc-finger factors (Snail, Slug, Zeb1 and Zeb2) and class I and II bHLH factors (E47 and Twist (Twist 1 and Twist2)), but Peinado and coworkers consider that it remains unsolved whether the different repressors may participate in silencing E-cadherin in different types of tumors or at defined stages of tumor progression (Peinado et al., 2005).

Peinado and coworkers disclosed in the above-referred publication the identification of two members of the LOX (lysyl oxidase-like) gene family, LOXL2 and LOXL3, as enzymes inducing EMT and participating in the Snail-mediated silencing of *E-cadherin in vivo*. Both LOXL2 and LOXL3 require the same domain
5 of Snail, SNAG, for interaction and collaboration with Snail in *E-cadherin* repression. However, the effect on tumor progression seems not to be equivalent for both proteins: while Peinado and coworkers comment that their data provide substantial new evidence that LOXL2 is required for efficient growth and progression of tumors with invasive/angiogenic potential, even suggesting the
10 possibility of using its expression as an additional predictive/prognosis marker for carcinoma progression, no conclusive data about the role of LOXL3 could be found by said group, which focused the last part of the study on LOXL2 due to partial and almost contradictory preliminary results found with LOXL3.

Other EMT markers have been related with cancer progression and with
15 primary colorectal cancer progression in particular, such as the ZEB (Zinc Finger E-Box-Binding Homeobox) family of transcription factors, particularly ZEB1 and ZEB2, which repress E-cadherin promoter, are often cited together in relation to EMT and cancer progression. ZEB1 has been implicated in EMT in human colorectal cancer (Xiong et al., 2012). Kahlert and coworkers, in turn, reported
20 upregulation of ZEB2 at the invasion front in primary colorectal cancer and liver metastases, as well as a significant correlation between overexpression of ZEB2 at the invasion front and tumor stage in primary colorectal cancer (Kahlert et al., 2011). ZEB2 is therefore considered a predictor of cancer-specific survival in primary colorectal cancer. Kahlert and coworkers suggest that the process of EMT
25 most probably occurs at the edge of the tumors, in connection with gene overexpression at the invasion front, which confers the invasion front the ability to break down the basement membrane and to extravasate into lymph and blood vessels in the adjacent tissue, a context wherein the overexpression of ZEB2 at the invasion front is relevant for tumor cell dissemination and progression in primary
30 colorectal cancer.

Tumor cells undergoing EMT have been shown to gain, among other attributes, drug resistance abilities. For instance, mesenchymal cell expressing low E-cadherin levels have been related to EGFR kinase inhibitors resistance in Non-Small Cell Lung Cancer (NSCLC) (Witta, 2006), or to monoclonal anti-EGFR

antibodies (Cetuximab) in the case of colorectal cancer, as mesenchymal cells can regulate AKT activation, one of the main targets for these therapies, through EGFR-independent pathways like Integrin Linked Kinase (ILK) (Laure et al., 2005).

In this same line, some researchers have not focused on individual markers, but on the status of the EMT process, in order to assess the effectiveness of therapy. International Application No. WO12149014A1 discloses a method of identifying patients with cancer who may benefit from treatment with a pharmaceutical composition comprising a compound that inhibits tumor cells from undergoing an epithelial to mesenchymal transition, which comprises measuring the expression levels of a list of 88 genes in a tumor sample, calculating what they call the EMT gene signature (EMTGS) and identifying the patients benefiting from the therapy as those with an EMTGS value similar to a reference value corresponding to cells of a mesenchymal phenotype. The list of 88 genes includes many genes well known for being related to the EMT process such as vimentin, TWIST1, SNAI1, SNAI2, ZEB1 and ZEB2.

Despite the knowledge provided by these works on the relevance of EMT in tumors, very little is known about EMT in CTCs, with some studies published but mainly focused on breast cancer.

It would be advisable to have a reliable method for identifying mCRC patients who are responding to therapy, especially to the chemotherapy standard treatment, easy to perform out of the body, preferably not requiring tumor samples but samples easier to take by non-invasive methods such as blood or urine. Preferably, the method should be able to classify the patients as responders and non-responders to the therapy prior to the current methods such as CT tomography, in order to have reliable data to consider the possibility of stopping the administration of the drugs that are only causing adverse effects to non-responding patients and, if possible, to replace it for another one as soon as possible.

The present invention provides a solution to said problem.

SUMMARY OF THE INVENTION

The present invention provides a panel of markers with predictive value for therapy effectiveness monitoring in mCRC patients, each marker individually being capable of allowing the assessment of therapy effectiveness by comparison of their levels before therapy start and after the first (or subsequent) therapy cycle and

even, in terms of overall survival, being capable of allowing the assessment of therapy effectiveness simply by determining its level at a moment of therapy cycle and comparing it with a reference value. Moreover, the present invention also provides a qPCR-based multimarker CTC detection panel for outcome prediction before treatment and for therapy effectiveness monitoring in mCRC patients, by using a combination of the above mentioned intestine-specific epithelial markers and EMT transcripts, which combination means an improvement for therapy effectiveness monitoring because diminish the probability of false positive results.

Particularly, the present invention relates to a method for determining the effectiveness of a therapy administered to a subject suffering from metastatic colorectal cancer (mCRC) and/or the outcome of said subject that comprises the steps of:

a) taking a first follow-up blood sample from the subject after having been administered at least one therapy cycle;

b) assessing the expression level of at least one gene selected from the set of genes of the group of GAPDH, VIL1, TIMP1, CLU, TLN1, LOXL3 and ZEB2 in the CTCs of the blood sample;

c) classifying the expression level of the selected gene, or of each one of the selected genes, in the CTCs of the blood sample as "high" when it is higher than a reference cutoff value of the expression level of said gene and "low" when it is equal or lower than said reference cutoff value;

d) classifying the blood sample as

i. "low-CTC", if the expression level of the selected gene in CTCs is low or, when more than one gene of the above mentioned set of genes is being analysed, if the expression level in CTCs of more than a half of said analysed genes is low;

ii. "high-CTC", if the expression level of the selected gene in CTCs is high or, when more than one gene of the above mentioned set of genes is being analysed, if the expression level in CTCs of at least one half of said analysed genes is high;

e) assessing the effectiveness of the administered therapy by classifying the subject as:

i. "non responder" to the therapy, if the subject is "high-CTC" for the analysed follow-up blood sample, and

ii. "responder" to the therapy, if the subject is "low-CTC" for the analysed follow-up blood sample;

f) optionally, taking a second or additional follow-up blood samples from the subject after having been administered a therapy cycle subsequent to the therapy cycle already administered when the first follow-up sample was taken and repeating steps b) to e) with said second or subsequent follow-up sample to confirm the previous assessment of therapy effectiveness;

g) additionally or alternatively to steps b) to f), predicting the outcome of the subject at a time point of therapy course by classifying the subject as a "high risk" subject when the blood sample taken at that time point is "high-CTC", and as a "low risk" subject when the blood sample taken at that time point is "low-CTC".

It is obvious for any skilled in the art that, if the expression level in CTCs of only one gene of set of genes of the group of GAPDH, VIL1, TIMP1, CLU, TLN1 is to be determined and used for the assessment of therapy effectiveness and/or subject (patient) outcome, step d) needs not to be carried out, being possible to classify the subject as "responder" or "non responder" according to the classification of the expression level of the gene as "low" or "high", respectively. That alternative way of classification is also encompassed by the scope of the present invention.

The gene or each one of the genes selected for carrying out the steps of the method of the invention is also referred as an "analysed gene" and the group of genes selected for carrying out the steps of the method of the present invention are also called "the set of analysed genes" or "the panel of analysed genes".

If the expression level in CTCs of only one gene of set of genes of the group of GAPDH, VIL1, TIMP1, CLU, TLN1, LOXL3 and ZEB2 is to be determined and used for carrying out the other steps of the method of the invention, it is preferred that it is selected between LOXL3 and ZEB2, more preferably LOXL3.

It is preferred that more than a gene of the group of GAPDH, VIL1, TIMP1, CLU, TLN1, LOXL3 and ZEB2 is included in the set of analysed genes, that is, it is a preferred embodiment of the present invention that one wherein the expression level in CTCs at least two, three, four, five, six or all the seven genes of the group of GADPH, VIL1, TIMP1, CLU, TLN1, LOXL3 and ZEB2 is determined and classified in step b) and c), and the analysed blood sample and, subsequently, the subject are both classified according to the expression level of said genes in the

CTCs of said blood sample in steps d) and e) and, when carried out, in step f) and/or g). Even when the expression level of more than one of said genes is determined and used for classifying the blood sample and the subject at the moment of taking that blood sample, it is preferred that LOXL3 or ZEB2 or both of
5 them are among the set of analysed genes.

When the expression level in CTCs of at least two genes is to be determined and classified, another preferred embodiment is that LOXL3 and VIL1 are among the selected genes for carrying out the method of the invention. More particular embodiments are those where LOXL3, VIL1 and CLU are selected when
10 the expression level of at least three genes is determined or where LOXL3, VIL1, CLU and GAPDH are selected when at least four genes of the group of GAPDH, VIL1, TIMP1, CLU, TLN1, LOXL3 and ZEB2 are used for determining their expression levels and carrying out with them the method of the present invention.

Another preferred embodiment of the method of the present invention is that
15 one wherein at least six genes of the group of GAPDH, VIL1, TIMP1, CLU, TLN1, LOXL3 and ZEB2 are included in the set of analysed genes, as in Examples 1 to 4 of the present application. The selected genes can be GAPDH, VIL1, TIMP1, CLU, LOXL3 and ZEB2, as in Example 2, or the seven genes of the group, GAPDH, VIL1, TIMP1, CLU, TLN1, LOXL3 and ZEB2, as in Example 3 of the present
20 application. Other possible combinations are also encompassed by the scope of the present invention, such as performing the method with a set of six genes wherein TIMP1 or CLU are replaced by TLN1, that is, the set of analysed genes can also be GAPDH, VIL1, CLU, TLN1, LOXL3 and ZEB2, or, GAPDH, VIL1, TIMP1, TLN1, LOXL3 and ZEB2.

25 In those embodiments wherein the at least six genes of the group of GAPDH, VIL1, TIMP1, CLU, TLN1, LOXL3 and ZEB2 are included in the set of analysed genes and particularly, when the set of analysed genes are GAPDH, VIL1, TIMP1, CLU, LOXL3 and ZEB2, or GAPDH, VIL1, TIMP1, CLU, TLN1, LOXL3 and ZEB2, the blood sample to be classified in step c) will be classified as
30 "low-CTC" when the expression level of at least four genes of the set of analysed genes is low, and "high-CTC" when the expression level of three or more genes of the set of analysed genes is high.

It is preferred that a previous blood sample is taken from the subject before the administration of the therapy cycle that is already administered when the first

follow-up sample is taken (for instance, when the first follow-up sample is taken after having administered therapy cycle 1, said previous blood sample will be the baseline sample, that is, a blood sample taken before the start of the therapy. A preferred embodiment of the method of the invention is that wherein steps b) to e) (assessment and classification of the expression level of selected CTC genes, classification of the blood sample as "high-CTC" or "low-CTC" and assessment of therapy effectiveness) , and optionally, step f) (repeating steps b) to e) in follow-up samples subsequent to the first follow-up blood sample) are carried out in said previous blood sample; optionally, additionally or alternatively to steps b) to e), step g) (prediction of the outcome of the subject at the time point of the therapy course when said previous blood sample has been taken) can be carried out in said blood sample. Compatible with this embodiment is another very preferred one wherein step f) of confirmation is carried out when the subject is "low-CTC" for the follow-up blood sample considered in step d) and "high-CTC" for the immediately previous sample (for instance, "low-CTC" for the sample taken before administering therapy cycle 2, the first follow-up sample, but "high-CTC" for the sample taken before therapy start, the baseline sample). That confirmatory step has the aim of confirming that the subject is indeed responding to the administered therapy and detecting a possible change in the response. Of course, this confirmatory step can be repeated as many times as it is considered advisable, to obtain a new assessment of therapy effectiveness in different time points along the therapy course. It is also compatible with any embodiment which comprises taking at least a blood sample previous to the first follow-up blood sample to use such previous blood sample to carry out step g) (alternatively to steps b) to f) or additionally to them) for predicting the outcome of the patient, which can be a particularly preferred embodiment when said previous sample is the baseline blood sample and it is use for predicting the outcome of the patient before the start of the therapy.

It is preferred that the administered therapy in evaluation is chemotherapy, particularly the most common one for mCRC patients, that one comprising the administration of at least one fluoropyrimidine (fluorouracil or capecitabine) alone or in combination with oxaliplatin or irinotecan and/or with anti-EGFR or anti-VEGF antibodies. Under such circumstances, the first follow-up sample may be the sample taken before administering therapy cycle 2, and will be normally taken 4 weeks after the start of therapy, which might be an alternative way of defining the

moment of taking the sample. Moreover, linked to that possible embodiment, wherein step f) is carried out, the second follow-up sample can be (and it is preferred to be) the sample taken before administering the next therapy cycle, before therapy cycle 3, so the time point when it is taken can be normally 5 alternatively defined as 16 weeks after the start of therapy.

Also compatible with all the embodiments above mentioned are other possible and/or preferred embodiments. Some of them are mentioned below and are compatible with each other.

For instance, in preferred embodiments of the method of the present 10 invention, the expression level of each gene is normalized with regard to the expression level of a reference gene. Preferably, the reference gene is a lymphocyte-specific gene, such as CD45, which allows to take into account possible leukocytes that may have contaminated the sample.

It is also a preferred embodiment that each blood sample is enriched in 15 CTCs, to decrease the noise of the sample and the risk of non-specific results. It is particularly preferred to separate CTCs from other components of the original sample by applying any immunoaffinity technique based on the use of specific antibodies against a membrane antigen present in CTCs, such as anti-EpCAM antibodies, since EpCAM is specific of cells of epithelial origin. The use of a system 20 that facilitates separation of CTCs from other blood components by magnetic means, such as the use of magnetic beads covered with an anti-EpCAM antibody is particularly preferred because, apart from the ease of application, a good level of purification can be obtained so that CTCs can be considered to become isolated from the rest of the original sample components.

25 The expression level of each gene in a sample can be also assessed by different techniques, all of them compatible with the above-mentioned embodiments of the method of the present invention, such as determination of the levels of the protein expressed by the gene. For the present invention it is preferred the assessment of gene expression levels in CTCs of a sample by quantifying the 30 level of the corresponding messenger RNA (mRNA) in the sample, particularly by means of quantitative Real-Time PCR.

On the other hand, the cutoff value that allows the classification of the expression level of each gene as high or low (step c)) can be selected depending on particular circumstances in the application of the method. A possibility is using

the 75% percentile value of the expression level of the gene resulting from a statistical study of blood samples taken from mCRC patients at the time point of therapy course wherein the blood sample to be classified has been taken. The particular value can be that used in the Examples of the present application or, preferably, one resulting from a broader study.

Moreover, the method of the present invention is compatible with any other method of assessment of therapy response, and can be combined, complemented or confirmed by any of said methods, such as imaging techniques as, for instance, CT tomography, or monitoring techniques based on the determination of levels of certain biomarkers in serum, such as CA-125 or CEA. The embodiments of the method wherein the assessment is complemented with the results of an imaging technique such as CT colonography and/or with a monitoring technique based on the determination of a biomarker in serum such as CA-125 or CEA are also encompassed by the scope of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig.1. Relative gene expression values for LOXL3 (left panel) and ZEB2 (right panel) transcripts obtained by qPCR, normalized to 40 cycles and this value to the 40-Cq value for CD45 [(40-Cq target)-(Cq CD45)], in whole blood samples enriched in CTCs.

Fig. 2: Kaplan Meier survival analysis for baseline (panels A and B) and 4-week follow up (panels C and D). Progression Free Survival (panels A and C) and Overall Survival (panels B and D) curves are shown, based on the number of patients at risk depending on the time from baseline blood drawn in months (X-axis). Grey lines correspond to the results of patients classified as High-CTC, whereas darker lines correspond to patients classified as Low-CTC by using the panel including 7 markers.

Fig. 3: Patient classification according to markers evolution (Panel A) and Kaplan Meier survival analysis of the patients according to their classification based on evolution of 7 markers in CTCs: Progression Free Survival (PFS) (panel B) and Overall Survival (OS) (panel C) curves for responders (R: Dark Grey lines) and non responders (NR: light grey lines).

Fig. 4: Kaplan Meier survival analysis for baseline (panels A and B) and 4-week follow up (panels C and D). Progression Free Survival (panels A and C) and

Overall Survival (panels B and D) curves are shown, based on the number of patients at risk depending on the time from baseline blood drawn in months (X-axis). Grey lines correspond to the results of patients classified as High-CTC, whereas darker lines correspond to patients classified as Low-CTC by using the panel including 7 markers.

Fig. 5: Patient classification according to markers evolution (Panel A) and Kaplan Meier survival analysis of the patients according to their classification based on evolution of 7 markers in CTCs: Progression Free Survival (PFS) (panel B) and Overall Survival (OS) (panel C) curves for responders (R: Dark Grey lines) and non responders (NR: light grey lines).

Fig. 6: Comparison of therapy response-based patient classification between CTC-marker levels variation along treatment and first computed tomography evaluation. A) Correlation between CTC-markers and imaging classification. Kaplan-Meier plots for Progression Free Survival (PFS) (B) and Overall Survival (OS) (C) for patients groups depending on their classification by CTC-markers and imaging techniques. (SD: Stable Disease; PR: Partial Response; PD: Progressive Disease; R: Responder; NR: Non-Responder).

DETAILED DESCRIPTION OF THE INVENTION

As it has been indicated above, the present invention provides a multimarker panel for CTC detection, based on gene expression quantification, that includes tissue specific (intestine-specific epithelial markers) and EMT related CTC markers, for the effective prediction of patient outcome before therapy but, more importantly, also for the early prediction of treatment effectiveness in mCRC patients. Human mCRC patients are the preferred subjects for applying the invention.

The method of the present invention is based on the results of a study, carried out by the present inventors, whose main endpoints were the evaluation of the expression levels of selected mRNA transcripts in CTCs as predictors of Progression Free Survival (PFS) and Overall Survival (OS) at baseline, and at follow-up as a therapy-response monitoring tool.

As it is discussed in Example 1, the markers selected for the study, as well as their identification numbers according to NCBI database (Entrez Gene or RefSeq Gene ID for http://www.ncbi.nlm.nih.gov/gene/) and HUGO Gene Nomenclature Committee resources (HGNC ID, for http://www.genenames.org/),

and their genome location in the human genome according to the last available version in the Ensembl Genome Browser database (http://www.ensembl.org/Homo_sapiens/Info/Index), are as follows:

- GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Housekeeping gene
5 detecting all cell types present in the sample. (RefSeq Gene ID: 2597, updated on 22 March 2014; HGNC ID: HGNC:4141; location: Chromosome 12: 6,643,093-6,647,537 forward strand, Ensembl: ENSG0000011164, version: ENSG00000111640.10).

- VIL1 (Villin 1). CTC-marker specific of intestinal origin. (RefSeq Gene ID:
10 7429, updated on 22 March 2014; HGNC ID: HGNC:12690; location: Chromosome 2: 219,283,815-219,318,018 forward strand, Ensembl: ENSG00000127831, version ENSG00000127831.6)

- TIMP1 (Tissue Inhibitor of Metalloproteinase 1). CRC specific CTC gene. (RefSeq Gene ID: 7076, updated on 24 March 2014; HGNC ID: HGNC:11820;
15 location: Chromosome X: 47,441,712-47,446,188 forward strand; Ensembl: ENSG00000102265, version: ENSG00000102265.7).

- CLU (Clusterin or Complement Lysis Inhibitor). CRC specific CTC gene. (RefSeq Gene ID: 1191, updated on 22 March 2014; HGNC ID: HGNC:2095; location: Chromosome 8: 27,454,434-27,472,548 reverse strand; Ensembl:
20 ENSG00000120885, version: ENSG00000120885.15)

- LOXL3 (lysyl oxidase-like 3). EMT marker. (RefSeq Gene ID: 84695, updated on 22 March 2014; HGNC ID: HGNC:13869; location: Chromosome 2: 74,759,541-74,782,817 reverse strand; Ensembl: ENSG00000115318, version: ENSG00000115318.7)

25 - ZEB2 (zinc finger E-box-binding homeobox 2). EMT marker. (RefSeq Gene ID: 9839, updated on 22 March 2014; HGNC ID: HGNC:14881; location: Chromosome 2: 145,141,648-145,282,147 reverse strand; Ensembl: ENSG00000169554, version: ENSG00000169554.12)

- TLN1 (talin 1). CRC specific CTC gene. (Included in late phases of the
30 study, as an optional gene) (RefSeq Gene ID: 7094, updated on 22 March 2014; HGNC ID: HGNC:11845; location: Chromosome 9: 35,696,945-35,732,392 reverse strand; Ensembl: ENSG00000137076, version: ENSG00000137076.14).

- CD45 (or PTPRC: protein tyrosine phosphatase, receptor type, C). Lymphocyte-specific gene used as reference gene. (RefSeq Gene ID: 5788,

updated on 22 March 2014; HGNC ID: HGNC:9666; location: Chromosome 1: 198,607,801-198,726,545 forward strand.; Ensembl: ENSG00000081237, version: ENSG00000081237.14)

The NCBI reference sequence of the mRNAs encoded by each gene is indicated in Table 2 (see preliminary sections of the Examples); specifically, the transcript variant or variants that can be detected by the probes used in the Examples of the present application are indicated in said Table, as well as the proteins that can be translated from said transcripts.

This is the first work that reports the combination of CTC markers, including those for EMT, for this purpose in mCRC patients. Although the group of the present inventors had previously suggested a model to determine progression-free survival in mCRC which was based on measuring the expression levels of GAPDH and VIL1, normalized to CD45 expression levels (Barbazan et al., 2012a), the model has no predictive value for assessing therapy response in mCRC patients as it had been conceived, and no keys had been provided by the present inventors about the way of improving said model for monitoring therapeutic response in metastatic colorectal cancer. The group of the present inventors had also reported the diagnostic and prognostic value of TIMP1, CLU and TLN1 (Barbazan et al., 2012b), but its combination with GAPDH and VIL1 in the previous design model had not been suggested. Moreover, no genes with predictive value for assessing the effectiveness of therapies administrated to mCRC patients had been reported by the group of the present inventors.

In the present application, however, a method is provided for indirect CTC quantification based on seven different markers, for patient outcome prediction but also for the monitoring of treatment response. The set of markers includes two genes previously associated to the induction of EMT-like aggressive cancer phenotypes, LOXL3 and ZEB2.

As can be seen in Examples 1 and 3, each of the seven markers individually has a predictive value for the response to the administered therapy, both for predicting PFS and OS for the patients undergoing the treatment to assess, particularly when the classification as responders and non responders to the therapy is made by comparing a first follow-up sample with a previous sample, specially a baseline sample taken before therapy start, and confirmed in a second follow-up sample taken in a moment of the therapy course subsequent (when at

least one more or more than one additional therapy cycle has been administered) to the moment when the first follow-up sample was taken.

Moreover, as can be seen in Example 1, all markers show a significant correlation with the other six additional markers, not only when the baseline blood sample (the sample taken before therapy start) is considered, but also for the first follow-up sample (taken approximately 4 weeks after therapy start, when the therapy cycle 1 has been administered) and the second follow-up sample (taken approximately 16 weeks after therapy start, once therapy cycle 2 has been administered and before therapy cycle 3). Therefore, it is expectable that combinations of said seven markers might also have a predictive value for the patients undergoing therapy. For that reason, the method of the present invention encompasses the assessment of therapy response by analysing the expression level in CTCs of at least one of the genes of the group of GAPDH, VIL1, TIMP1, CLU, TLN1, LOXL3 and ZEB2 and its comparison with a reference value (for instance, the 75% percentile cutoff value), considering responders to the therapy those subjects having values lower than said cutoff value in the time point of therapy course when the analysed blood sample has been taken. But it is also encompassed within the scope of the invention the assessment of therapy response by considering combinations of the values obtained for two, three, four, five, six or seven of said genes, classifying each blood sample as "low-CTC" if the expression level in CTCs of more than a half of the considered genes is low and "high-CTC" if the expression level in CTCs of at least one half of the genes included in the combination being considered is high, being "non responders" to the therapy those subject whose blood sample are classified as "high-CTC" and "responders" those subjects whose corresponding follow-up blood sample has been classified as "low-CTC". The regression analysis shown in section 3.4. below confirms that the method of the present invention can be carried out determining only one marker or determining only (or at least) two, three or four markers. Such regression analysis shows that LOXL3 is the marker whose contribution is more important in the model and, for that reason, it is the preferred marker when the method of the invention is to be carried out with only one marker, and it is one of the markers that, preferable, should be included in any combination of markers. Thus, when it is decided that it is preferable to use a minimum number of markers for carrying out the method of the present invention, and the markers are selected

according to their contribution in a model generated with such minimum number of markers, the preferred combinations of set of markers for determining and analysing their expression levels are:

- Two marker genes: LOXL3 and VIL1
- Three marker genes: LOXL3, VIL1 and CLU
- Four marker genes: LOXL3, VIL1, CLU and GAPDH

The embodiments of the method of the invention based on the combination of a set of markers in a model, instead of being based on the results obtained for an individual marker, diminish the probability of type I errors, that is, false positives. In particular, the six-gene panel based on GAPDH, VIL1, TIMP1, CLU, LOXL3 and ZEB2 considered in Examples 1 to 3 of the present invention significantly reduced the probability of type I error and increased the reliability of the analysis when working with relatively small patients cohorts. For instance, the inventors of the present invention have calculated that, for a p=0.1 as the minimum of the type I error probability for said 6 markers individually, the probability of false positive by using the multimarker model based on said 6 markers would be less than 0.01585. Therefore, the embodiments of the method of the present invention based on the combination of several markers of the group of GAPDH, VIL1, TIMP1, CLU, TLN1, LOXL3 and ZEB2 are preferred, particularly those based on six of said markers or on the seven markers. As TLN1 is a CTC-marker specific of intestin origin, just as TIMP1 and CLU, and its diagnostic and prognostic value for mCRC is similar to that of said genes according to previous works of the group of the present inventors (Barbazan et al., 2012b), in the models based on the combinations of six markers, it is considered that TIMP1 or CLU can be replaced by TLN1, particularly in the model applied in Examples 1 to 3, which is discussed below.

At baseline, a model generated by the combination of all six mentioned basic markers (GAPDH, VIL1, TIMP1, CLU, LOXL3 and ZEB2) effectively identified patients with shorter PFS and OS times, with more than 3 fold risk of disease progression than the patients classified in the low-CTC group. 26% (13 out of 50) of the patients were classified by the model as high-CTC, similar rates to the ones usually reported in CellSearch-based studies (Cohen et al., 2009; Tol et al., 2010). However, to effectively determine patients at high risk, cutoffs for our markers were set as the 75% percentile of the global gene expression for each marker, greatly reducing the number of patients classified as CTC-high but ensuring patient

classification. Similar positivity rates (24%) were found for the 4-week follow up, although prediction power was moderately reduced in this model. The cutoff values used in the present study can be found in Table 4, in the column with the heading "Q75%"; for future studies, the same values could be used, although it should be advisable to carry out a larger study, including more patients, to obtain ranges of values.

When examining the expression values for each marker separately, a different tendency was found for EMT markers compared with the rest. LOXL3 and ZEB2 were not able to significantly predict patient outcome at baseline, both for PFS and OS, while GAPDH, VIL1, TIMP1 and CLU did. Interestingly, after one chemotherapy cycle a change in markers tendency was observed, where EMT markers gained statistical significance whereas the others tended to predict prognosis worse than at baseline. Thus, parallel changes for LOXL3 and ZEB2 between baseline and after treatment suggest a potential link between patient survival and an EMT-related chemotherapy resistance.

Clinico-pathological variables like T or N stage, number or metastatic sites, carcinoembrionic antigen (CEA) levels, KRAS mutational status, among others, failed to predict outcome in univariate Cox regression analysis, with PS grade and the presence of lung metastases as the only predictive variables. Multimarker CTC detection models, both for baseline and 4-week follow up time points, showed similar (or even better) predictive behavior as the best clinical parameter (usually PS grade), becoming independent prognostic factor for PFS and OS in this type of patients, as analyzed by multivariate Cox regression.

Then, as the assay set forth in section 3.1. of Example 3 shows, the present inventors appear to have surprisingly arrived at an appropriate combination of markers that allow good patient stratification before therapy start and also therapy response monitoring. And it is particular surprising because the predictive value of the method of the present invention seems not to arise simply from the inclusion of EMT markers in the set of analysed genes.

As it is shown in the mentioned assay, not all EMT markers have predictive value when their levels are assessed in CTCs of epithelial origin, not even when the protein products of the genes under study are all factors inducing E-cadherin silencing (LOXL2, ZEB1, E47, SNAIL1, SNAIL2, TWIST1), just like LOXL3 and ZEB2. Very surprisingly, for LOXL2, SNAIL2 and TWIST1, the methodology applied

did not allow to detect any signal in most of the patients under assay; the fact is particularly surprising not only because of the close relation between Loxl3 and Loxl2 and because both proteins participate in the silencing of *E-cadherin* through its interaction with Snail, but also because a possible predictive value for carcinoma
5 progression have been previously suggested for Loxl2, a protein with an importance for efficient growth and substantial and progression of tumors with invasive/angiogenic potential appeared to have been clearly demonstrated, contrary to Loxl3 (Peinado et al., 2005).

SNAIL1, the gene encoding Snail, which is a protein involved in E-cadherin
10 silencing where several networks regulating EMT converge, did not give rise to statistically significant results in order to predict the response treatment in terms of Progression Free Survival (PFS) and Overall Survival (OS). The same can be said for E47 and even for ZEB1, a gene encoding a transcription factor closely related to ZEB2 and frequently mentioned together with ZEB2 (or encompassed by the
15 common denomination ZEB) as a factor with similar involvement in the EMT process. The result is also surprising because the contribution of ZEB1 to worse prognosis of CRC patients had been previously suggested (Xiong et al., 2012).

With this situation, one of the advantages of the method of the present invention is the short numbers of markers (six in the basic design of the method,
20 preferably normalized to a seventh one, CD45) that are enough for good monitoring of treatment response; and indeed, as commented in Example 1, each of said seven markers has predictive value by itself and can be used individually. This is in sharp contrast with previous methods such as that disclosed in WO12149014A1, wherein it is necessary to measure the expression levels of 88 genes to obtain a
25 value that indicates the global EMT gene signature of the sample, which value allows the identification benefiting from the therapy, provided that the patients are treated with a pharmaceutical composition comprising a compound that inhibits tumor cells from undergoing an epithelial to mesenchymal transition. Moreover, the sample needed for carrying out the method disclosed in WO12149014A1 must be a
30 tumor sample. The study giving rise to the method of the present invention, to the contrary, has been carried out with mCRC patients receiving the standard treatment for mCRC patients, and the samples taken to the patients are blood samples, easier to obtain and well admitted by most patients.

Another important advantage of the method of the present invention is the

early moment during therapy course when the effectiveness of therapy is evaluated. One of the main objectives in this study was to analyze how CTC-marker variations could predict therapy response. This evaluation should be done as soon as possible, as those treatment-refractory patients will not have any
5 beneficial effect but they will be affected by chemotherapy-derived toxicity. Routinely, evaluation of tumor evolution is performed approximately three months after treatment onset, and during this time some patients may progress without being detected, dying before first CT evaluation in worst cases. Variations in the analyzed CTC-markers between baseline and 4-week follow up, effectively
10 predicted PFS and OS outcome, generating a model that early classifies responding and non responding patients after only one chemotherapy cycle. Based on multimarker CTC-model results, patients were divided in the responders or non-responders group, depending on changes from low or high-CTC groups when comparing baseline and 4-weeks time points. Only patients with markers
15 reductions between both times were subjected to a third blood drawn, approximately at week 16, to confirm response to treatment. For that purpose, in the present study, said patients were classified as "non-responders"; for clarity of the definition of the method of the present invention, in its general definition, patients changing from "high-CTC" to "low-CTC" when comparing to consecutive
20 samples should be considered "responders", although it is advisable to take a subsequent sample and confirm the assessment before taking any decision about discontinuing the therapy administration, particularly when the first result (the first classification and therefore, the first therapy effectiveness assessment) is obtained by comparing the baseline sample and the first follow-up sample, in this case, the
25 4-week follow up sample.

Using this approach it is ensured that no patients with spurious early treatment responses were included in the group of responders, finally improving prediction power of the model. A possible clinical interpretation of these results can be speculated, based on therapeutic decisions. Patients becoming to the low-CTC
30 groups at both time points (baseline and 4 weeks) should continue with the same treatment. Those changing from the low-CTC group at baseline to the high-CTC one at 4-week follow up, together with patients classified as high-CTC at both times, should be considered for a new therapy line, based on clinical standard protocols. Treatment could be maintained in patients who had a CTC marker

reduction waiting for a later confirmatory evaluation. As explained in a previous section, in routinely clinical use, in those cases where therapy continues, it might be advisable to take further subsequent samples and confirming that the therapy effectiveness is maintained. Anyway, as the inclusion of TLN1 in the set of analysed genes appears to make the method more accurate and reliable, as set forth in Example 3 of the present application, the need of further confirmations can be considered innecessary or optional, depending of the practitioner's opinion.

On the other hand, the method of the present invention is compatible with any other assessment methods, particularly with imaging techniques such as CT tomography and/or with the determination of the level of other biomarkers, for instance in serum, such as CA-125 or CEA. Thus, the method of the present application can be seen as complementary to such techniques or any other similar ones, which can be used to confirm the assessment. Nevertheless, it is remarkable that the assays described in Example 4, (wherein the results obtained by the method of the present invention were compared with the results derived from CT colonographies performed on the same patients), not only confirm the validity of the method of the present invention as a reliable tool for assessing mCRC patients' response to an administered therapy well before the CT colonographies are performed, but they also indicate that the present method seems to be more reliable, because it gives rise to a better classification of a group of patients as responders or non responders than said imaging technique when their PFS and OS values were taking into account.

The study set forth in the Examples of the present application, was based on patients that were undergoing a therapy treatment based on fluoropyrimidines, the standard treatment for mCRC patients. In this sense, it is important that the method of the present invention is based on a "CTC multimarker panel", that is, a set of genes whose expression levels are measured on CTCs. For that reason, normalizing the expression levels obtained with regard to the expression levels of a non-CTC gene, such as CD45, is highly recommendable in order to avoid signals coming from other possible cells remaining after CTC enrichment, such as lymphocytes. On the other hand, metastatic colorectal cancer is said to rely on the detachment of aggressive malignant cells from the primary tumor into the bloodstream and, concordantly, the presence of this circulating tumor cells (CTCs) has been associated with poor prognosis, among other things because they are

presumptive founder in the generation of metastasis. Therefore, as measuring the expression levels of some markers in CTCs can be regarded as a measure closely related to the number of CTCs present in the blood samples, the applicability of the method of the present invention to the assessment of other kind of therapies seems
5 to be plausible, including chemotherapies different from the one applied to the patients included in the study of the present application.

Moreover, the use of epithelial CTC specific transcripts combined with EMT-markers might improve CTC detection rates, compared for example with CellSearch®, as a broader range of cellular subtypes could be identified.

10 Finally, and in connection with therapy, it must be kept in mind that the method of the present invention allows the stratification of patients prior to treatment by means of the quantification of CTC markers in the baseline sample. Then, it may allow the use of more or less aggressive therapies based on marker levels before the beginning of a systemic treatment line.

15 In conclusion, the present invention provides a method which can be regarded as an early prognostic test, useful in the clinical setting for mCRC patients undergoing therapy. In order to integrate it into the clinical routine, a multicenter study including a larger number of patients should be advisable, that would allow to obtain more accurate results about the reference cutoff values for marker
20 expression levels or possible circumstances that could make advisable to use values different from the 75% percentile value as reference value. Anyway, the data provided in the present application are useful orientative tools in that sense.

The present invention will be now explained in more detailed by means of the Examples and Figures set forth below.

25

EXAMPLES

The Examples set forth below were carried out following the techniques and design protocols that follows:

- Study design

30 50 mCRC patients were recruited at the University Clinical Hospital of Santiago de Compostela (Spain) (Table 1).

Table 1. Patients demographics

Age (years)		
Mean	SD	Range
64,5	10,3	31-84

Gender			
Male		Female	
N	%	N	%
37	74	13	26

Primary tumor location					
Colon		Rectum		Both	
N	%	N	%	N	%
34	68	14	28	2	4

KRAS status					
WildType		Mutated		Unknown	
N	%	N	%	N	%
31	62	18	36	1	2

T							
T_1-T_2		T_3		T_4		T_x	
N	%	N	%	N	%	N	%
1	2	36	72	10	20	3	6

N							
N_0		N_1		N_2		N_x	
N	%	N	%	N	%	N	%
4	8	20	40	20	40	6	12

Number of metastatic sites			
1		≥2	
N	%	N	%
23	46	27	54

Metastasis location					
Liver		Liver and other		Non liver	
N	%	N	%	N	%
18	36	25	50	7	14

ECOG PS grade							
1		2		3		Unknown	
N	%	N	%	N	%	N	%
17	34	1	2	30	60	2	4

First line chemotherapy									
Folfox		Folfiri		Capecitabine		Capecitabine-Oxaliplatin		Irinotecan	
N	%	N	%	N	%	N	%	N	%
41	82	3	6	1	2	4	8	1	2

First line combined biological therapy					
Anti-EGFR		Anti-VEGF		None	
N	%	N	%	N	%
19	38	8	16	23	46

Abbreviations:
SD: Standard deviation
ECOG: Eastern Cooperative Oncology Group
PS: Performance Status
"T" and "N" relates to TNM staging system

All patients signed informed consent approved by the correspondent ethical committee. Inclusion criteria were the presence of measurable metastatic colorectal cancer (stage IV), a Performance Status (PS) equal or less than 3, based on the Eastern Oncology Cooperative Group (ECOG) scale, and the initiation of a new systemic chemotherapy line. Patients treated with fluoropyrimidines (fluorouracil or capecitabine) alone or in combination with oxaliplatin/irinotecan and biological targeted therapies (Bevacizumab: anti-VEGF, Cetuximab: anti-EGFR) were included. Patients reporting previous cancer episodes treated or not with chemotherapy, were excluded from the study.

Tumor burden, metastasis location and therapy response were evaluated by standard imaging procedures (computed tomography, CT) by a specialized radiologist. Following RECIST 1.1 guidelines (Eisenhauer et al., 2009), disease progression was defined as an increase in the number of metastatic lesions, growth of pre-existing distant tumors in more than 20% of the initial size, or both. Patients who died during the follow-up period without being evaluated by CT, were also considered as progression events, having verified that death was disease-related. Eight healthy controls, matched for age and sex with patients, were included for the validation of LOXL3 and ZEB2 as CTC markers.

One 10ml EDTA tube was collected for all patients at baseline (day 1, cycle 1, before therapy start) and before chemotherapy cycle 2 (≈4 weeks). In some cases, a third sample was collected before cycle 5 (≈16 weeks). For controls, only one single sample was collected. Samples were processed within the same day of collection.

- CTC isolation and gene expression analysis

Sample processing procedures have been previously described (Barbazan et al., 2012a). Briefly, CTCs were enriched from 7,5ml of whole blood using anti-

EpCAM coated magnetic beads (CELLection epithelial enrich, Life Technologies) and stored in RNAlater® solution at –80ºC until further processing. RNA was extracted with a methodology optimized for low concentration samples (Qiamp Viral, Qiagen) and cDNA was synthesized using SuperScriptIII polymerase (Life
5 Technologies). To optimize target detection, samples were first preamplified (PreAmp Master Mix kit, Life Technologies). MRNA levels of CD45, GAPDH, VIL1, TIMP1, CLU, TLN1, LOXL3 and ZEB2 genes were quantified by quantitative Real-Time PCR using hydrolysis probes chemistry (Life Technologies) in a StepOne plus thermocycler (Life Technologies). Probe characteristics are detailed in Table 2, as
10 well as the mRNA reference sequence (accession in GenBank, http://www.ncbi.nlm.nih.gov/genbank/. wherein the last digit indicates the version of the referenced sequence) corresponding to the specific mRNA variant detected by each probe, and the reference sequence corresponding to the protein translated from each of said transcripts:

15 Table 2. qPCR probes characteristics

Gene Name	Probe Number (TaqMan Assay)	Reference Sequence(s) (GenBank)	Translated Protein (GenBank)	Amplicon length (base pairs)
CD45	Hs00894734_m1	NM_002838.3	NP_002829.3	70
GAPDH	Hs99999905_m1	NM_002046.3	NP_002037.2	124
VIL1	Hs00200229_m1	NM_007127.2	NP_009058.2	99
TIMP1	Hs00171558_m1	NM_003254.2	NP_003245.1	104
CLU	Hs00156548_m1	NM_001171138.1	NP_001822.3	65
LOXL3	Hs01046945_m1	NM_032603.2	NP_115992.1	72
ZEB2	Hs00207691_m1	NM_001171653.1 NM_014795.3	NP_001165124.1 NP_055610.1	67
TLN1	Hs00196775_m1	NM_006289.3	NP_006280.3	113
SNAIL1	Hs00195591_m1	NM_005985.3	NP_005976.2	66
SNAIL2	Hs00161904_m1	NM_003068.4	NP_003059.1	79
TWIST1	Hs00361186_m1	NM_000474.3	NP_000465.1	115
LOXL2	Hs00158757_m1	NM_002318.2	NP_002309.1	62
E47	Hs00413032_m1	NM_001136139.2 NM_003200.3	NP_001129611.1 NP_003191.1	74
ZEB1	Hs01566407_m1	NM_001128128.2 NM_001174093.1 NM_001174094.1 NM_001174095.1 NM_001174096.1 NM_030751.5	NP_001121600.1 NP_001167564.1 NP_001167565.1 NP_001167566.1 NP_001167567.1 NP_110378.3	67

Every sample was run in duplicate for each gene and appropriate negative controls were included in each qPCR reaction plate. Cq values (defined as the cycle number at which the fluorescence reached a fixed threshold value) for each transcript were normalized to 40 (maximum number of cycles), and this value to the 40-Cq value for CD45 [(40-Cq target)-(Cq CD45)], used as a reference gene as it detects hematopoietic cells unspecifically isolated. We previously reported the validity of CD45 as a reference gene (Barbazan et al., 2012a) as its expression levels are equal in patient or healthy donor samples. The whole set of samples (n=50) was preamplified and checked for CD45 expression in triplicate and by independent operators to ensure reproducibility of the results, finding a high correlation between runs. Table 3 shows the results of the checking.

Table 3. CD45 Spearman correlation between runs

Run1-Run2		**Run1-Run3**		**Run2-Run3**	
R^2	**Pvalue**	**R^2**	**Pvalue**	**R^2**	**Pvalue**
0.981	<0.001	0.967	<0.001	0.968	0.001

- Statistical analysis

PFS (Progression Free Survival) and OS (Overall Survival) were defined as the time elapsed between treatment day 1 and the day reporting disease progression or death, respectively.

Prognostic groups for each analysis point (baseline and follow-up) were set based on CTC marker levels, and single patients were included into low or high-CTC groups if marker levels were, respectively, below or above cutoff, defined as the 75% percentile for each independent marker. That means that, for each marker and time point, 25% of the patients were classified as high-CTC, whereas the remaining 75% were included in the low-CTC group. Kaplan-Meier (KM) and univariate and multivariate **COX** regression survival analysis were used to study associations between marker levels and PFS/OS. Differences between controls and patients for LOXL3 and ZEB2 were analyzed using Mann-Whitney non-parametric t test. Spearman correlation test was used to evaluate differences in CD45 expression between runs. All statistic tests were performed with SPSSv20.0 and GraphPad prism v5 software, and considered significant when $p \leq 0.05$.

Example 1. Marker selection. Expression levels distribution at baseline and during follow-up

1.1. Marker selection

In this study 6 markers were initially selected based on three different
5 approaches.

- CTC detection markers: First, GAPDH and VIL1 were chosen as they have been previously validated as CTC detection markers in a previous work from the group of the present inventors (Barbazan et al., 2012a). GAPDH was used as a housekeeping gene, detecting all cell types present in the sample, and VIL1 as a
10 specific intestinal origin CTC-marker.

- CRC specific CTC genes. Moreover, TIMP1 and CLU were included as two CRC specific CTC genes, coming from a global gene expression profiling study carried out by the group of the present inventors (Barbazan et al., 2012b).

- EMT markers. Finally, LOXL3 and ZEB2 (Peinado et al., 2005; Kahlert et
15 al., 2011), were included to check the EMT status on mCRC CTCs at baseline and their changes along treatment.

- CD45 gene expression (a lymphoid cell marker) was used as a reference gene to normalize CTC-markers expression levels, minimizing the noise produced by the presence of non-specifically isolated cells in the sample. The usefulness of
20 CD45 as a normalizer had been previously validated (Barbazan et al., 2012a; Barbazan et al., 2012b).

TLN1 was also added to the study as a seventh marker, also as a CRC specific CTC gene coming from the same global gene expression profiling study as TIMP1 and CLU (Barbazan et al., 2012b).

25 Gene expression analysis was carried out as explained above.

1.2. Baseline and follow-up CTC-markers gene expression levels distribution

GAPDH, VIL1, TIMP1 and CLU were found to be more expressed in mCRC patient samples than in healthy controls as referenced above, confirming the
30 results previously obtained by the group of the present inventors. The same was tested here for LOXL3 and ZEB2 in a set of 50 mCRC patients and 8 healthy controls showing statistically significant differences for both (LOXL3 $p<0.0001$, ZEB2 $p<0.001$) (Fig. 1).

Relative expression levels at baseline for each analysed CTC marker are

shown in Table 4 below. Mean, median, 25% and 75% quartiles and standard deviation (SD) were calculated and are shown in said Table 4. Markers with negative values represent global lower marker expression than CD45, used as a reference gene, and vice versa.

5 Table 4. Relative gene expression levels of CTC markers (CD45 normalized)

	Mean			Median			SD		
Gene name	**B**	**4-W**	**16-W**	**B**	**4-W**	**16-W**	**B**	**4-W**	**16-W**
GAPDH	3.55	4.18	4.29	3.42	3.85	4.06	1.86	2.03	1.89
VIL1	-3.76	-2.84	-2.16	-3.59	-2.91	-2.35	2.77	3.10	2.32
TIMP1	2.16	2.99	3.24	2.18	2.87	2.75	2.29	2.32	2.16
CLU	4.98	5.90	6.3	4.87	5.96	6.04	2.61	2.89	2.49
TLN1	3.37	4.20	4.57	3.40	3.91	3.90	2.24	2.40	2.18
LOXL3	-3.26	-2.56	-2.19	-3.11	-3.27	-2.2	2.11	2.09	1.93
ZEB2	0.89	1.43	1.72	0.96	1.15	1.63	1.49	1.54	1.41
	Q25%			**Q75%**					
Gene name	**B**	**4-W**	**16-W**	**B**	**4-W**	**16-W**			
GAPDH	2.43	2.49	2.92	4.69	5.48	5.57			
VIL1	-5.30	-4.54	-4.18	-2.27	-1.22	-0.32			
TIMP1	0.63	1.10	1.59	3.61	4.42	4.69			
CLU	3.23	3.96	4.68	6.36	7.60	8.02			
TLN1	1.71	2.43	3.31	4.81	5.36	6.16			
LOXL3	-4.28	-3.77	-3.66	-2.06	-1.61	-0.88			
ZEB2	0.02	0.48	0.55	2.11	2.46	2.60			

B: Levels at baseline 4-W: levels at 4-week follow-up 16-W: levels at 16-week follow-up
Q: quartile; SD: Standard deviation

As a general rule, all markers tended to have higher levels in follow-up samples (4 and 16 weeks) compared with baseline samples, but no statistical differences were found.

1.3. <u>CTC-markers predict patient outcome at baseline and follow-up</u>

To investigate the potential of the analysed CTC-markers as outcome predictors, Kaplan-Meier survival analyses were performed for PFS and OS for each individual marker. As described, patients were divided into two different groups based on the 75% percentile value for each marker, being "high" those patients whose expression level of the considered marker was higher than the 75%

percentile value and "low" the remaining patients. Table 5 below shows the results obtained.

Table 5. Kaplan-Meier survival analysis for individual CTC markers

Baseline							
		PFS			**OS**		
Marker		Mean	95% CI	Pvalue	Mean	95% CI	Pvalue
GAPDH	Low	12.42	9.93-14.91	**0.001**	23.78	19.98-27.56	**0.006**
	High	6.71	4.86-8.57		12.97	6.69-19.24	
VIL1	Low	12.54	10.07-15.00	**<0.001**	24.38	20.71-28.05	**<0.001**
	High	6.33	4.56-8.11		10.34	6.41-14.27	
TIMP1	Low	12.69	10.25-15.12	**<0.001**	24.50	20.72-28.28	**<0.001**
	High	6.86	4.13-7.60		10.76	5.94-15.59	
CLU	Low	12.60	10.15-15.05	**<0.001**	23.68	19.88-27.48	**0.008**
	High	6.13	4.33-7.93		13.12	6.88-19.35	
TLN1	Low	12.42	9.93-14.82	**0.004**	24.60	20.82-28.38	**<0.001**
	High	6.91	4.43-9.40		10.56	6.09-15.02	
LOXL3	Low	11.82	9.29-14.34	0.088	23.10	19.29-27.00	0.098
	High	8.61	5.88-11.34		15.19	8.15-22.22	
ZEB2	Low	11.91	9.44-14.38	0.128	23.16	19.27-27.05	0.086
	High	8.33	5.29-11.46		14.99	7.95-22.03	
4-weeks follow up							
		PFS			**OS**		
Marker		Mean	95% CI	Pvalue	Mean	95% CI	Pvalue
GAPDH	Low	12.05	9.74-14.36	**0.044**	23.63	19.92-27.36	**0.007**
	High	7.32	4.44-10.19		12.46	7.33-17.60	
VIL1	Low	12.14	9.92-14.36	**0.017**	23.85	20.13-27.57	**<0.001**
	High	6.58	3.47-9.70		11.2	6.48-15.92	
TIMP1	Low	11.96	9.79-14.22	0.066	23.29	19.62-26.96	**0.015**
	High	7.21	4.07-10.34		12.65	7.07-18.23	
CLU	Low	11.96	9.76-14.17	**0.034**	23.60	19.96-27.25	**0.001**
	High	6.71	3.42-10.00		10.93	5.59-16.28	
TLN1	Low	11.87	9.60-14.15	0.090	23.16	19.43-26.88	**0.022**
	High	7.51	4.39-10.62		13.12	7.69-18.55	
LOXL3	Low	12.45	10.04-14.86	**0.004**	23.85	20.12-27.58	**0.006**
	High	6.41	3.90-9.32		12.93	6.45-19.41	
ZEB2	Low	12.23	9.96-14.49	**0.015**	20.09	20.53-27.65	**0.001**
	High	6.75	3.83-9.67		10.95	5.99-15.90	

PFS: Progression Free Survival; OS: Overall survival; CI: confidence interval

5 *Marker high and low levels are calculated based on 75% percentile for each marker

As shown in Table 5, at baseline, GAPDH, VIL1, TIMP1 and CLU displayed a significant association both for PFS and OS, with patients whose marker levels were above cutoff showing shorter PFS and OS times. There was no significant association between baseline LOXL3 and ZEB2 expression levels and patient outcome (PFS and OS).

Patients with GAPDH, VIL1 and CLU gene expression levels above cutoff at 4 weeks follow-up time point, showed significant shorter PFS and OS times compared with patients below cutoff, irrespective of status at baseline. Only differences for OS were found in the case of TIMP1. Interestingly, EMT markers LOXL3 and ZEB2 became significantly both for PFS and OS at this follow-up point.

1.4. Model design

In order to simplify data analysis and results interpretation, the present inventors generated a simple model for the combination of markers in one single value for each analysed patient. In order to simplify the analysis, initially, only six markers were selected, namely GAPDH, VIL1, TIMP1, CLU, LOXL3, ZEB2. For this, patients having at least 4 markers below cutoffs were classified as low-CTC, whereas those with 3 or more markers above cutoffs were included in the high-CTC group. Predictive models were generated both for baseline and 4 weeks follow-up sampling points.

Using this marker combination, after KM survival analysis, 37 patients (74%) were included in the low-CTC group, displaying significantly longer PFS and OS times at baseline, compared with patients classified as high-CTC (13, 26%) (in months; PFS: 12.7 vs 6.3; p=0.0003; OS: 24.2 vs 12.7; p=0.002) (Fig. 2A and Fig. 2B). Although with smaller differences, this model also significantly predicted patient PFS and OS outcomes at 4 weeks follow-up (Low-CTC: n=38, 76%; High-CTC: n=12, 24%) (PFS: 12.1 vs 7.3; p=0.044; OS: 23.6 vs 12.4; p=0.007) (Fig. 2C and Fig. 2D). The results obtained are summarized in Table 6 below.

Table 6: Kaplan Meier survival analysis for baseline and 4-weeks follow-up

Time from baseline blood drawn	0	5	10	15	20	25	30	35	40
Progression-Free Survival: Number of patients at risk:									
CTC markers (baseline)									
High-CTC:	13	7	2	0	0	0	0		
Low-CTC:	37	34	19	11	6	1	0		
CTC markers (4 weeks)									
High-CTC:	12	8	2	0	0	0	0		
Low-CTC:	38	34	19	10	5	1	0		
Overall survival: Number of patients at risk:									
CTC markers (baseline)									
High-CTC:	13	12	6	4	3	3	2	1	0
Low-CTC:	37	36	32	29	21	11	8	2	1
CTC markers (4 weeks)									
High-CTC:	12	8	5	4	3	1	0	0	0
Low-CTC:	38	37	33	29	21	13	10	3	1

Univariate and multivariate Cox regression analysis results of selected clinical parameters are indicated in Table 7 below.

Table 7: Univariate and multivariate Cox proportional hazard regression analysis

Univariate					
Covariate		**PFS**		**OS**	
	N	**HR (95%CI)**	**Pvalue**	**HR (95%CI)**	**Pvalue**
Age (≥65 *vs* <65 years)	50	1.00 (0.98-1.03)	0.870	1.03 (0.98-1.05)	0.428
Sex (male *vs* female)	50	1.57 (0.77-3.19)	0.212	2.09 (0.86-5-10)	0.104
T stage (4 *vs* ≤3)	47	0.84 (0.38-1.82)	0.653	0.81 (0.33-1.97)	0.637
N stage (2 *vs* ≤1)	44	1.22 (0.65-2.29)	0.545	0.71 (0.34-1.48)	0.363
Hepatic mets. (yes *vs* no)	50	0.75 (0.33-1.70)	0.492	0.81 (0.33-1.96)	0.640
Lung mets. (yes *vs* no)	50	2.79 (1.45-5.36)	**0.002**	2.12 (1.06-4-26)	**0.034**
Peritoneal mets. (yes *vs* no)	50	1.32 (0.61-2.87)	0.477	1.37 (0.61-3.10)	0.450
Nº of metastatic sites (>2 *vs* 1)	50	1.30 (0.72-2.34)	0.390	0.94 (0.48-1.83)	0.843
KRAS (mut. *vs* WT)	49	1.16 (0.62-2.17)	0.649	0.70 (0.34-1.41)	0.313
Baseline CEA* (≥75ng/ml vs <75ng/ml)	47	1.25 (0.67-2.31)	0.482	1.57 (0.83-3.35)	0.147
ECOG PS (3 *vs* ≤2)	48	2.47 (1.25-4.90)	**0.010**	8.21 (3.12-21.62)	**<0.001**
Baseline CTC-marker model (high CTC *vs* low CTC)	50	3.39 (1.68-6.83)	**0.001**	2.96 (1.44-6.09)	**0.003**
4-week CTC-marker model (high CTC *vs* low CTC)	50	2.00 (1.00-4.00)	**0.049**	2.74 (1.28-5.87)	**0.009**

Multivariate							
Covariates		**PFS**			**OS**		
	N	**HR (95%CI)**	**Pvalue**	χ^2(**)	**HR (95%CI)**	**Pvalue**	χ^2(**)
ECOG PS (3 *vs* ≤2)		2.02 (1.00-4.09)	0.051		11.18* (3.95-31.67)	**<0.001**	
Lung mets. (yes *vs* no)		2.95 (1.49-5.83)	**0.002**	**<0.001**	2.53 (1.18-5.40)	**0.016**	**<0.001**
Baseline CTC-marker model (high CTC *vs* low CTC)	48	3.49* (1.65-7.36)	**0.001**		5.10 (2.11-12.32)	**<0.001**	
ECOG PS (3 *vs* ≤2)		2.58* (1.25-5.34)	**0.010**		9.36* (3.46-25-31)	**<0.001**	
Lung mets. (yes *vs* no)		2.41 (1.21-4.79)	**0.012**		1.71 (0.77-3.78)	**0.189**	
4-week CTC-marker model (high CTC *vs* low CTC)	48	2.46 (1.15-5.27)	**0.021**	**<0.001**	3.17 (1.31-7.66)	**0.010**	**<0.001**

CTC, Circulating Tumor Cells; PFS, Progression Free Survival; OS, Overall Survival; HR, Hazard Ratio; CI,Confidence Interval; CEA, Carcinoembrionic antigen; ECOG, Eastern Cooperative Oncology Group; PS, Performance Status

*Most significant prognostic factor in Cox multivariate analysis

(**) Significance of a Chi-square test for multivariate models.

Table 7, upper panel, shows that only PS grade, presence of lung metastases and baseline and 4-week follow-up CTC-marker models were

independent predictors for PFS and OS. At baseline, patients with 2 or more markers above cutoff (high-CTC) showed Hazard Ratios (HR) of 3.39 (PFS) (95%CI: 1.68-6.83, p=0.001) and 2.96 (OS) (95%CI: 1.44-6.809 p=0.003). After four weeks, HR for those patients were 2.00 (PFS) (95%CI: 1.00-3.99, p=0.049) and 2.74 for OS (95%CI: 1.28-5.87, p=0.009). In multivariate analyses including PS grade, lung metastases and baseline CTC-marker model, we found our model to be the strongest predictor for PFS, and one of the best for OS. The same analysis was applied for the 4-week follow-up model, with significant results both for PFS and OS. However, predictive power based on HR was reduced when compared with baseline model, and PS grade and lung metastases showed to be better indicators of patient outcome (Table 7, lower panel).

Example 2. CTC-markers variations along treatment predict therapy response

To analyse CTC-markers variations between baseline and 4 weeks follow-up time points, patients were classified in accordance with the following Table 8, also shown in Fig. 3A.

Table 8. Patient classification according to markers evolution

Blood drawn time point			
Baseline	4 weeks	16 weeks	Patient classification
High-CTC →	High-CTC →	→	Non responder (NR)
High-CTC →	Low-CTC →	Low-CTC →	Responder (R)
		High-CTC →	Non responder (NR)
Low-CTC →	High-CTC →	→	Non responder (NR)
Low-CTC →	Low-CTC →	→	Responder (R)

Responders group included patients that had been previously included in the low-CTC group both at baseline and at 4 weeks, based on marker levels. Patients that changed from the low-CTC group at baseline to the high-CTC one at 4 weeks, and those being at the high-CTC one in both time points, were classified as non-responders. Patients changing from the high-CTC group at baseline to the low-CTC one at 4 weeks, were preventively included in the group of non responders and a third confirmatory sample was drawn before cycle 5 of chemotherapy (approximately 16 weeks from baseline). If those patients were corroborated as low-CTC by the CTC-marker model, they were reclassified as

responders. They continued as non-responders if they came back to the high-CTC group at the 16-week follow-up.

KM survival analysis showed that patients classified as responders (n=35, 70%) had a mean PFS of 12.70 months vs 6.75 for non-responders (n=15, 30%)
5 (p=0.004), and an OS of 24.26 vs 13.05 (p=0.007) (Fig. 3B and 3C). Table 9 shows the data represented in Fig. 3.

Table 9: Kaplan Meier analysis for responders and non-responders

Number of patients at risk									
Time from baseline blood drawn	0	5	10	15	20	25	30	35	40
Number of patients at risk:									
Progression-Free Survival									
High risk	15	9	2	1	1	0	0		
Low risk	35	33	19	10	5	1	0		
Overall survival									
High risk	15	11	6	5	4	2	1	0	0
Low risk	35	35	31	26	19	11	8	3	1

Cox regression analysis was also performed, as shown in Table 10.

10 Table 10: Univariate and multivariate Cox proportional hazard regression analysis

Univariate					
Covariate		**PFS**		**OS**	
	N	**HR (95%CI)**	**Pvalue**	**HR (95%CI)**	**Pvalue**
CTC-marker response model (NR vs R)	50	2.53 (1.32-4.83)	**0.005**	2.61 (1.27-5.39)	**0.009**

Multivariate							
Covariates		**PFS**			**OS**		
	N	**HR (95%CI)**	**Pvalue**	χ^2(**)	**HR (95%CI)**	**Pvalue**	χ^2(**)
ECOG PS (3 *vs* ≤2)	48	3.00 (1.42-6.38)	**0.004**	**<0.001**	11.22*(4.01-31.40)	**<0.001**	**<0.001**
Lung mets. (yes *vs* no)		2.23 (1.11-4.51)	**0.025**		1.46 (0.63-3.38)	0.379	
CTC-marker response model (NR vs R)		3.41* (1.61-7.20)	**0.001**		3.73 (1.51-9.26)	**<0.004**	

CTC, Circulating Tumor Cells; PFS, Progression Free Survival; OS, Overall Survival; HR, Hazard Ratio; CI,Confidence Interval; CEA, Carcinoembrionic antigen; ECOG, Eastern Cooperative Oncology Group; PS, Performance Status; mets: metastasis; R: Responder patient group; NR: Non responders patient group
*Most significant prognostic factor in Cox multivariate analysis
(**) Significance of a Chi-square test for multivariate models.

HR for the non-responders group (univariate Cox regression) was 2.53 for PFS (95%CI: 1.32-4.83, p=0.005) and 2.61 for OS (95%CI: 1.27-5.39, p=0.009) (Table 10, upper section). CTC-marker model for variations between baseline and follow-up points was found to be the best PFS outcome predictor in multivariate
5 Cox analyses, including PS grade and the presence of lung metastases as clinical parameters. Significant results were also obtained for OS (Table 10, lower section).

Example 3. Evaluation of possible additional markers and invidual markers

3.1. Possible association between EMT and prognostic or predictive value

10 In order to check if other EMT markers could be also used, the assays described in Examples 1 and 2 were performed with a group of six well-known EMT markers (LOXL2, ZEB1, E47, SNAIL1, SNAIL2, TWIST1) in 20 patients with mCRC (a random subset of the 50 patients whose demographic data are shown in Table 1), in the samples taken from said patients before and during the treatment (4
15 weeks and 16 weeks after beginning the treatment). A possible correlation between the expression levels of said genes and response to treatment was evaluated.

The results can be summarized as follows:

- For LOXL2, SNAIL2 and TWIST1, no statistical analysis could be performed because the methodology used for the other genes did not allow to
20 detect any signal for any of these three genes in most of the 20 patients under assay. Therefore, they cannot be considered prognostic or predictive markers.

- For ZEB1, SNAIL1 and E47, it was possible to obtain a detectable signal, but none of them gave rise to statistically significant results in order to predict the response to treatment in terms of Progression Free Survival (PFS) and
25 Overall Survival (OS). As can be seen in Table 11 below, (where the results obtained with the markers analysed in Examples 1 and 2, as well as the result corresponding to the marker TLN1 discussed in section 3.2. below, have been included in order to facilitate the comparison), for the same 20 patients, *p*values corresponding to all the markers of the group of GAPDH, VIL1, TIMP1, CLU,
30 LOXL3 and ZEB2 were all $p<0.05$, while *P*values corresponding to ZEB1, SNAIL1 or E47 were clearly higher than 0.05.

Table 11: Kaplan-Meier survival analysis for individual CTC markers variations along treatment

Marker	***N***	**PFS *Pvalue***
ZEB1	20	0.117
LOXL2	20	0.135
E47	20	0.475
ZEB2	20	***0.012***
LOXL3	20	***0.012***
GAPDH	20	***0.001***
VIL1	20	***0.000006***
CLU	20	***0.001***
TIMP1	20	***0.012***
TLN1	20	***0.008***

3.2. Possible additional markers improving reliability: TLN1

5 In order to see whether the inclusion of additional markers could improve the reliability of the classification of patients into responders or non-responders, an additional marker, talin1 (TLN1) was included in the model design. Like TIMP1 and CLU, this marker had been previously assayed as a part of a global gene expression profiling study carried out by the group of the present inventors 10 (Barbazan et al., 2012b).

The Kaplan-Meier survival analysis for TLN1 as individual CTC marker was previously included in Table 5. In said Table, it can be seen that TLN1 gives rise to results analogous to TIMP1.

The criteria used for the model design described in Section 1.4 were 15 maintained and, with the new added marker, patient having at least 4 markers below cutoffs were classified as low-CTC, whereas those with at least 4 markers above cutoffs were included in the high CTC group. Predictive models were generated again both for baseline and 4 weeks follow-up sampling points. Under KM survival analysis, 39 patients (78%) were included in Low-CTC group, 20 displaying longer PFS and OS times at baseline (Fig. 4A and Fig. 4B), and also at 4 weeks follow-up (Fig. 4C and Fig. 4D).

The corresponding univariate and multivariate Cox regression analysis for the same clinical parameters considered in Example 1 were also performed. The corresponding results are shown in Table 12.

Table 12. Univariate and multivariate Cox proportional hazard regression analysis (7 markers analysed)

Covariate		Univariate			
		PFS		OS	
	N	HR (95%CI)	*Pvalue*	HR (95%CI)	*Pvalue*
Age (≥65 *vs* <65 years)	50	1.00 (0.98-1.03)	0.870	1.03 (0.98-1.05)	0.428
Sex (male *vs* female)	50	1.57 (0.77-3.19)	0.212	2.09 (0.86-5-10)	0.104
T stage (4 *vs* ≤3)	47	0.84 (0.38-1.82)	0.653	0.81 (0.33-1.97)	0.637
N stage (2 *vs* ≤1)	44	1.22 (0.65-2.29)	0.545	0.71 (0.34-1.48)	0.363
Hepatic mets. (yes *vs* no)	50	0.75 (0.33-1.70)	0.492	0.81 (0.33-1.96)	0.640
Lung mets. (yes *vs* no)	50	2.79 (1.45-5.36)	**0.002**	2.12 (1.06-4-26)	**0.034**
Peritoneal mets. (yes *vs* no)	50	1.32 (0.61-2.87)	0.477	1.37 (0.61-3.10)	0.450
Nº of metastatic sites (≥2 *vs* 1)	50	1.30 (0.72-2.34)	0.390	0.94 (0.48-1.83)	0.843
KRAS (mut. *Vs* WT)	49	1.16 (0.62-2.17)	0.649	0.70 (0.34-1.41)	0.313
Baseline CEA (≥75ng/ml *vs* <75ng/ml)	47	1.25 (0.67-2.31)	0.482	1.57 (0.83-3.35)	0.147
ECOG PS (2 *vs* ≤1)	50	2.62 (1.21-5.66)	**0.014**	2.38 (1.10-5.15)	**0.027**
Baseline CTC-marker model (high CTC *vs* low CTC)	50	3.78 (1.80-7.98)	**0.001**	10.15 (3.89-26.48)	**<0.001**
4-week CTC-marker model (high CTC *vs* low CTC)	50	1.83 (0.90-3.73)	0.114	2.42 (1.10-5.28)	**0.039**

Covariates		Multivariate					
		PFS			OS		
	N	HR (95%CI)	*Pvalue*	χ^2(**)	HR (95%CI)	*Pvalue*	χ^2(**)
ECOG PS (2 *vs* ≤1)	50	1.66 (0.76-3.65)	0.204	**<0.001**	1,68 (0,75-3,73)	0.204	**<0.001**
Lung mets. (yes *vs* no)		2.95 (1.52-5.75)	**0.001**		2,24 (1,09-4,58)	**0.027**	
Baseline CTC-marker model (high CTC *vs* low CTC)		3.87* (1.79-8.40)	**0.001**		9,67* (3,56-26,25)	**<0.001**	
ECOG PS (2 *vs* ≤1)	50	2.39 (1.06-5.34)	**0.034**	**0.001**	2,81 (1,25-6,32)	**0.009**	**0.002**
Lung mets. (yes *vs* no)		2.63* (1.33-5.18)	**0.005**		2,11 (1,04-4,30)	**0.040**	
4-week CTC-marker model (high CTC *vs* low CTC)		2.30 (1.10-4.80)	**0.026**		3,11* (1,36-7,09)	**0.002**	

CTC, Circulating Tumor Cells; PFS, Progression Free Survival; OS, Overall Survival; HR, Hazard Ratio; CI,Confidence Interval; CEA, Carcinoembrionic antigen; ECOG, Eastern Cooperative Oncology Group; PS, Performance Status

*Most significant prognostic factor in Cox multivariate analysis

(**) Significance of a Chi-square test for multivariate models

As represented in Fig. 5A, patients were classified as responders and non-responders following the same criteria used in Example 2 (see Table 8). Using the

model including 7 markers (TLN1 included), KM survival analysis for responders and non-responders was performed. Figs. 5B and 5C, respectively, show the results obtained for PFS and OS.

Cox regression analysis results are shown in Table 13.

Table 13. Univariate and multivariate Cox proportional hazard regression analysis for treatment response CTC-7-marker model

Univariate					
Covariate		**PFS**		**OS**	
	N	**HR (95%CI)**	**Pvalue**	**HR (95%CI)**	**Pvalue**
CTC-marker response model (NR vs R)	50	2.47 (1.28-4.78)	**0.007**	3.46 (1.66-7.24)	**0.002**

Multivariate							
Covariates		**PFS**			**OS**		
	N	**HR (95%CI)**	**Pvalue**	χ^2(**)	**HR (95%CI)**	**Pvalue**	χ^2(**)
ECOG PS (3 *vs* ≤2)		2.57 (1.41-5.81)	**0.023**		2.72 (1.22-6.08)	**<0.015**	
Lung mets. (yes *vs* no)	50	2.63 (1.33-5.22)	**0.005**	**<0.001**	2.02 (0.98-4.14)	0.055	**<0.001**
CTC-marker response model (NR vs R)		3.08* (1.55-6.15)	**0.001**		4.03* (1.87-8.73)	**<0.001**	

CTC, Circulating Tumor Cells; PFS, Progression Free Survival; OS, Overall Survival; HR, Hazard Ratio; CI,Confidence Interval; CEA, Carcinoembrionic antigen; ECOG, Eastern Cooperative Oncology Group; PS, Performance Status; mets: metastasis; R: Responder patient group; NR: Non responders patient group
*Most significant prognostic factor in Cox multivariate analysis
(**) Significance of a Chi-square test for multivariate models.

Summarizing, it can be concluded that including TLN1 as a seventh marker in the model does not change the tendencies and variation directions shown with the 6-marker model, but the difference between the groups are considerably increased. Adding TLN1 to the six marker model initially tested improves the effectiveness of the classification method. For instance, if the multivariate Cox proportional harzard regression (HR) analyses corresponding to the six-marker (see Table 7) and seven-marker (see Table 13) models are compared, it can be observed that the HR value for the OS changes from 5.10 in the six marker model (without TLN1) to 9.67 in the seven-marker model (with TLN1). In fact, in the six-marker model without TLN1, PS ECOG is the most important variable, the most significant factor; however, once TLN1 is included in the model, the model itself becomes the most significant variable. And that is the case when the model is

prepared with the data obtained both from the baseline sample and from the 4-week follow-up sample.

Then, the 6-marker model can be regarded as a useful tool to predict therapy response and to take decisions in connection with the maintenance of the administered therapy or the change to a different one; the 7-marker model, in turn, increases the reliability of the method.

3.3. Predictive value of individual markers

Once established the convenience of taking an additional follow-up sample in order to confirm the assessment of therapy response, the possible predictive value of each individual marker in said version of the model was assessed, that is, when the classification as responder or non responder is taken after considering the gene expression levels not only in the CTCs of the first follow-up sample but also in at least a second follow-up sample (in this case, the 16-weeks follow-up sample), particularly for those patients that were high-CTC at baseline and low-CTC after administering the first therapy cycle (4 weeks after therapy start).

Thus, for each particular gene, patients were classified as responders (R) and non responders (NR) in accordance to Fig. 3A (see also Table 8), that is, when the expression level was low or high, respectively, for that gene, in the 4-weeks follow-up sample, and, in case of having a classification as high-CTC for the baseline sample and as low-CTC for the 4-week follow-up sample, patients were finally classified as responders only if the 16-week follow-up sample confirmed the classification as low-CTC obtained in the 4-week sample.

As can be seen in the following table, each marker showed to have predictive value for the therapy response, both for predicting PFS and OS.

Table 14. Predictive value of CTC-markers individually

		PFS		**OS**	
		Mean (95% CI)	**Pvalue**	**Mean (95% CI)**	**Pvalue**
GAPDH	**R**	12.8 (10.4-15.3)	0.002	25.1 (21.5-28.7)	0.0001
	NR	6.5 (4.1-9.0)		11.2 (6.9-15.5)	
VIL1	**R**	12.7 (10.4-15.0)	0.001	24.6 (20.9-28.2)	0.001
	NR	6.4 (3.8-8.9)		11.7 (6.9-16.6)	
TIMP1	**R**	12.6 (10.3-14.9)	0.005	24.6 (20.9-28.2)	0.0004
	NR	6.6 (3.9-9.2)		11.5 (6.9-16.1)	
CLU	**R**	12.7 (10.4-15.0)	0.001	24.6 (20.9-28.2)	0.001
	NR	6.4 (3.8-8.9)		11.7 (6.9-16.6)	

		PFS		OS	
		Mean (95% CI)	**Pvalue**	**Mean (95% CI)**	**Pvalue**
TLN1	**R**	12.6 (10.3-14.9)	0.005	24.6 (20.9-28.2)	0.0004
	NR	6.6 (4.0-9.2)		11.5 (6.9-16.1)	
LOXL3	**R**	12.8 (10.5-15.2)	0.0005	25.1 (21.5-28.7)	0.0001
	NR	6.3 (3.9-8.7)		11.2 (6.9-15.5)	
ZEB2	**R**	12.2 (10.0-14.5)	0.015	24.1 (20.5-27.6)	0.001
	NR	6.8 (3.8-9.7)		10.9 (6.0-15.9)	

R: Patients classified as responders by CTC marker
NR: Patients classified as non-responders by CTC-marker
PFS: Progression Free Survival; **OS:** Overall Survival

5 Once verified that each CTC-marker, individually, has predictive value, both for PFS and for OS, the present inventors verified the correlation between individual markers, both in the baseline samples and in the 4-weeks follow-up samples, as well as in the 16-weeks follow-up sample. As can be seen in Table 15, all markers show a very significant correlation among them, for the baseline 10 samples, as well as for the follow-up samples taken at 4 or 16 weeks after the start of therapy.

Table 15. Individual markers correlation

Individual markers correlation (baseline sample)								
		GAPDH	**VIL1**	**TIMP1**	**TLN1**	**CLU**	**LOXL3**	**ZEB2**
GAPDH	Pearson correlation	1	0.714	0.964	0.960	0.883	0.845	0.901
	Pvalue		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
VIL1	Pearson correlation	0.714	1	0.802	0.801	0.912	0.742	0.659
	Pvalue	<0.001		<0.001	<0.001	<0.001	<0.001	<0.001
TIMP1	Pearson correlation	0.964	0.801	1	0.958	0.923	0.856	0.871
	Pvalue	<0.001	<0.001		<0.001	<0.001	<0.001	<0.001
TLN1	Pearson correlation	0.960	0.801	0.958	1	0.929	0.863	0.874
	Pvalue	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
CLU	Pearson correlation	0.883	0.912	0.923	0.929	1	0.800	0.773
	Pvalue	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001
LOXL3	Pearson correlation	0.845	0.742	0.856	0.863	0.800	1	0.838
	Pvalue	<0.001	<0.001	<0.001	<0.001	<0.001		<0.001
ZEB2	Pearson correlation	0.901	0.659	0.871	0.874	0.773	0.838	1
	Pvalue	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	

Individual markers correlation (4-weeks sample)								
		GAPDH	**VIL1**	**TIMP1**	**TLN1**	**CLU**	**LOXL3**	**ZEB2**
GAPDH	Pearson correlation	1	0.800	0.962	0.970	0.877	0.880	0.923
	Pvalue		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
VIL1	Pearson correlation	0.800	1	0.862	0.864	0.952	0.694	0.716
	Pvalue	<0.001		<0.001	<0.001	<0.001	<0.001	<0.001
TIMP1	Pearson correlation	0.962	0.862	1	0.972	0.933	0.846	0.867
	Pvalue	<0.001	<0.001		<0.001	<0.001	<0.001	<0.001
TLN1	Pearson correlation	0.970	0.864	0.972	1	0.932	0.878	0.898
	Pvalue	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
CLU	Pearson correlation	0.877	0.952	0.933	0.932	1	0.755	0.758
	Pvalue	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001
LOXL3	Pearson correlation	0.880	0.694	0.846	0.878	0.758	1	0.880
	Pvalue	<0.001	<0.001	<0.001	<0.001	<0.001		<0.001
ZEB2	Pearson correlation	0.923	0.716	0.867	0.898	0.758	0.880	1
	Pvalue	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	
Individual markers correlation (16-weeks sample)								
		GAPDH	**VIL1**	**TIMP1**	**TLN1**	**CLU**	**LOXL3**	**ZEB2**
GAPDH	Pearson correlation	1	0.926	0.959	0.957	0.930	0.911	0.913
	Pvalue		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
VIL1	Pearson correlation	0.926	1	0.957	0.969	0.966	0.857	0.825
	Pvalue	<0.001		<0.001	<0.001	<0.001	<0.001	<0.001
TIMP1	Pearson correlation	0.959	0.957	1	0.973	0.958	0.909	0.889
	Pvalue	<0.001	<0.001		<0.001	<0.001	<0.001	<0.001
TLN1	Pearson correlation	0.957	0.969	0.973	1	0.969	0.915	0.867
	Pvalue	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
CLU	Pearson correlation	0.930	0.966	0.958	0.969	1	0.852	0.818
	Pvalue	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001
LOXL3	Pearson correlation	0.911	0.857	0.909	0.915	0.852	1	0.896
	Pvalue	<0.001	<0.001	<0.001	<0.001	<0.001		<0.001
ZEB2	Pearson correlation	0.913	0.825	0.889	0.867	0.818	0.896	1
	Pvalue	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	

Such correlation data make expectable that any combination of the seven markers (combinations of two, three, four, five, six or the seven markers) provides

a predictive value for therapy response, at least from an experimental and statistical point of view. Therefore, apart from the six- and seven-marker models discussed above, other alternative models for assessing therapy response based on different combinations of markers (two, three, four, five, or six of the seven
5 markers of the method of the present invention) may also be used for assessing therapy effectiveness and can be considered comprised within the scope of the present invention. And this is so especially when similar criteria for classifying blood samples are applied, that is: classifying the blood sample as "low-CTC" if the expression level in CTCs of more than a half of the selected analysed genes is low,
10 and "high-CTC" if the expression level in CTCs of at least one half of the selected analysed genes is high.

3.4. Models of combinations of a reduced number of markers

In order to verify if valid models for assessing therapy response could be
15 obtained based on combinations of less than six markers, a model was generated with the four more significant markers (GAPDH, VIL1, CLU, LOXL3). This markers were selected based on their individual therapy response predictive abilities, taking their statistical significance (p) as the reference value to discriminate between markers. That is, those that, individually, showed the lowest *P*value for the
20 prediction of the PFS (see Table 14), following an approach similar to that explained in section 1.4 above. Thus, patients samples taken at baseline and 4-weeks after beginning of the treatment were classified as “low-CTC” when the expression level of at least three (that is, more than one half) of the selected genes was low and as “high-CTC” when at least two of the selected genes showed high
25 expression levels. The same was done for the 16-week sample in the cases where it had been taken. Patients were classified as responders and non-responders in accordance with the classification of their blood samples and the criteria explained in Panel A of Fig. 5. A regression analysis was performed with the data obtained, as it is shown in the first part of Table 16.
30 For model building, the variable with the lowest p value (Sig.) obtained using a Wald statistic (Chi-square based), was removed from the set of selected combined genes and subsequent models were generated, repeating the analysis until only the marker with more importance for the analysis remained. Such marker resulted to be LOXL3, as can be shown in Table 16 below, where the results of the

regression analyses performed for the four models generated can be seen. Other statistical terms are represented in the table, although they were not taken into account for model elaboration: B. Logistic Coefficient, S.E. Standard Error, df, Degrees of freedom, CI, Confidence Interval.

5

Table 16. Regression analysis of models combining different numbers of markers

4-marker model								
							95.0% CI for Exp(B)	
	B	ET	Wald	df	Sig.	Exp(B)	Lowest	Highest
GAPDH	-0.301	0.729	0.170	1	**0.680**	**0.740**	**0.177**	**3.090**
VIL1	1.452	1.276	1.295	1	**0.255**	**4.272**	**0.350**	**52.089**
CLU	-0.582	1.104	0.278	1	**0.598**	**0.559**	**0.064**	**4.865**
LOXL3	0.857	0.452	3.595	1	**0.058**	**2.356**	**0.972**	**5.715**
3-marker model								
							95.0% CI for Exp(B)	
	B	ET	Wald	df	Sig.	Exp(B)	Lowest	Highest
VIL1	1.148	1.041	1.218	1	**0.270**	**3.153**	**0.410**	**24.233**
CLU	-0.495	1.084	0.209	1	**0.648**	**0.609**	**0.073**	**5.098**
LOXL3	0.759	0.388	3.814	1	**0.051**	**2.135**	**0.997**	**4.572**
2-marker model								
							95.0% CI for Exp(B)	
	B	ET	Wald	df	Sig.	Exp(B)	Lowest	Highest
VIL1	0.697	0.383	3.313	1	**0.069**	**2.007**	**0.948**	**4.249**
LOXL3	0.724	0.381	3.607	1	**0.058**	**2.063**	**0.977**	**4.357**
1-marker model								
							95.0% CI for Exp(B)	
	B	ET	Wald	df	Sig.	Exp(B)	Lowest	Highest
LOXL3	0.970	0.348	7.786	1	0.005	2.637	1.334	5.211

Therefore, when the method of the invention is to be carried out with only one marker, LOXL3 is one of the preferable options. For the combination of two,

10 three of four markers, good combinations could be:

- Two marker combination: LOXL + VIL1
- Three marker combination: LOXL3 + VIL1 + CLU
- Four marker combination: LOXL3 + CIL1 + CLU + GAPDH

Example 4. Comparison with CT colonography

For 49 of the initial 50 patients of the present study, CT colonography was performed three months after the beginning of the therapy. The assessment of the response obtained from the CT colonography images and with the seven CTC-marker model was compared.

As can be seen in the correlation of results shown in Fig. 6A:

- 35 (34 según el texto inicial enviado) patients are classified as responders by means of both techniques
- 3 patients are classified as non-responders by both techniques
- 11 patients (12 según el texto recibido por correo-e) are classified as responders according to the CT results and as non-responders according to the CTC-marker model.

Correlation is significant (P=0.020, Chi-square test), but the groups are not identical.

Fig. 6B and Fig. 6C show the results of a survival analysis performed in order to compare the terms corresponding to PFS (Fig. 6B) and OS (Fig. 6C) for the three groups of patients. Both survival studies are especially meaningful.

The members of the group classified as non-responders by both techniques are those with the worst prognosis, and those classified as responders by both techniques are those with best prognosis. However, those patients classified as responders according to the CT results and as non responders using the CTC marker model exhibit significantly lower PFS and OS values (approximately 5 months of PFS and 10 of OS) than the members of the group classified as responders by both techniques.

This comparative analysis not only confirms the validity of the CTC marker model as a reliable tool for assessing mCRC patients' response to an administered therapy, but also shows that the use of the method based on the classification of patients from the CTC marker level might advantageous for that purpose, not only because it appears to be more accurate and reliable, classifying as non-responders patients with bad PFS and OS values instead of responders as the CT colonography indicates, but because the CTC-marker technique allows to obtain reliable results well before the CT colonography is applied.

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CLAIMS

1. A method for determining the outcome of a subject suffering from metastatic colorectal cancer (mCRC) and/or the effectiveness of a therapy administered to said subject that comprises the steps of:

a) taking a first follow-up blood sample from the subject after having been administered at least one therapy cycle;

b) assessing the expression level of at least one gene selected from the set of genes of the group of LOXL3, ZEB2, GAPDH, VIL1, TIMP1, CLU and TLN1 in the CTCs of the blood sample;

c) classifying the expression level of the selected gene, or of each one of the set of genes selected for being analysed, in the CTCs of the blood sample as "high" when it is higher than a reference cutoff value of the expression level of said gene and "low" when it is equal or lower than said reference cutoff value;

d) classifying the blood sample as

i. "low-CTC", if the expression level of the selected gene in CTCs is low or, when more than one gene of the above mentioned set of genes is being analysed, if the expression level in CTCs of more than a half of said analysed genes is low;

ii. "high-CTC", if the expression level of the selected gene in CTCs is high or, when more than one gene of the above mentioned set of genes is being analysed, if the expression level in CTCs of at least one half of said analysed genes is high;

e) assessing the effectiveness of the administered therapy by classifying the subject as:

i. "non responder" to the therapy, if the subject is "high-CTC" for the analysed follow-up blood sample, and

ii. "responder" to the therapy, if the subject is "low-CTC" for the analysed follow-up blood sample;

f) optionally, taking a second or additional follow-up blood samples from the subject after having been administered a therapy cycle subsequent to the therapy cycle already administered when the first follow-up sample was taken and repeating steps b) to e) with said second or subsequent follow-up sample to confirm the previous assessment of therapy effectiveness;

g) additionally, or alternatively to steps e) and f), predicting the outcome of the subject at a time point of therapy course by classifying the subject as a "high risk" subject when the blood sample taken at that time point is "high-CTC", and as a "low risk" subject when the blood sample taken at that time point is "low-CTC".

5

2. The method according to claim 1, wherein the expression level of at least two, three, four, five, six or all the seven genes of the group of LOXL3, ZEB2, GAPDH, VIL1, TIMP1, CLU and TLN1 is determined and classified in steps a) and b) and used for classifying the blood sample and the patients in step c) and d) and
10 e) and, when carried out, in step e) and/or f).

3. The method according to claim 1 or 2, wherein LOXL3 and/or ZEB2 is selected for determining and classifying its expression level in steps a) and b) and used for classifying the blood sample and the patients in step c) and d) and,
15 optionally, in step e) and, when carried out, in step e) and/or f).

4. The method according to claim 2 and 3, wherein the expression level of at least the two genes LOXL3 and VIL1 is determined and classified in steps a) and b) and used for classifying the blood sample and the patients in step c) and d) and,
20 when carried out, in step e) and/or f).

5. The method according to claim 4, wherein the expression level of at least the three genes LOXL3, VIL1 and CLU is determined and classified in steps a) and b) and used for classifying the blood sample and the patients in step c) and
25 d) and, when carried out, in step e) and/or f).

6. The method according to claim 5, wherein the expression level of at least the four genes LOXL3, VIL1, CLU and GAPDH is determined and classified in steps a) and b) and used for classifying the blood sample and the patients in step
30 c) and d) and, when carried out, in step e) and/or f).

7. The method according to any one of claims 1 to 3, wherein six genes of the group of GAPDH, VIL1, TIMP1, CLU, TLN1, LOXL3 and ZEB2 are selected for carrying out the steps of the method and the six genes are:
35 GAPDH, VIL1, TIMP1, CLU, LOXL3 and ZEB2, or

GAPDH, VIL1, CLU, TLN1, LOXL3 and ZEB2, or,
GAPDH, VIL1, TIMP1, TLN1, LOXL3 and ZEB2,

and wherein the blood sample to be classified in step c) is classified as "low-CTC" when the expression level of at least four genes of the set of analysed genes is low, and "high-CTC" when the expression level of three or more genes of the set of analysed genes is high.

8. The method according to any one of claims 1 to 3, wherein the seven genes of the group of GAPDH, VIL1, TIMP1, CLU, TLN1, LOXL3 and ZEB2 are selected for carrying out the steps of the method, and wherein the blood sample to be classified in step c) is classified as "low-CTC" when the expression level of at least four genes of the set of analysed genes is low, and "high-CTC" when the expression level of three or more genes of the set of analysed genes is high.

9. The method according to any one of claims 1 to 8, wherein a previous blood sample is taken from the subject before having administered the therapy cycle that has been administered when the first follow-up sample is taken, and steps b) to e) and, optionally, f), and, optionally or alternatively to steps e) and f), step g), are also performed in said previous blood sample.

10. The method according to claim 9, wherein step f) of confirmation is carried out when the subject is "low-CTC" for the follow-up blood sample considered in step d) and "high-CTC" for the immediately previous sample.

11. The method according to claim 10, wherein the follow-up sample considered in step d) is the first follow-up blood sample and the immediately previous sample is the baseline sample taken before the start of the therapy.

12. The method according to any one of claims 9 to 11, wherein step g) is carried out for predicting the outcome of the patient before therapy, on a blood sample which is the baseline sample taken before the start of the therapy.

13. The method according to any one of claims 1 to 11, wherein the administered therapy to be assessed is chemotherapy

14. The method according to claim 13, wherein the chemotherapy to be assessed comprised the administration of at least one fluoropyrimidine (fluorouracil or capecitabine) alone or in combination with oxaliplatin or irinotecan and/or with
5 anti-EGFR or anti-VEGF antibodies.

15. The method according to claim 13 or 14, wherein the first follow-up blood sample is taken before therapy cycle 2 or 4 weeks after the start of therapy.

10 16. The method according to claim 14 or 15, wherein step f) is carried out and a second follow-up blood sample is taken before therapy cycle 3 or 16 weeks after the start of therapy.

17. The method according to any one of the preceding claims, wherein the
15 expression level of each analysed gene is normalized with regard to the expression level of a reference gene.

18. The method according to claim 17, wherein the reference gene is CD45.

20 19. The method according to any one of the preceding claims, wherein the blood samples are enriched in CTCs by using a immunoaffinity technique based on anti-EpCAM antibodies.

20. The method according to any one of the preceding claims, wherein the
25 expression level of each gene in each sample is assessed by quantifying the level of its corresponding mRNA in said sample.

21. The method according to any one of the preceding claims, wherein each reference cutoff value that is used in step c) has been determined in a
30 statistical study with mCRC patients as the 75% percentile value of the expression level of the corresponding gene at the time point of the therapy course wherein the blood sample to be classified has been taken.

22. The method according to any one of the preceding claims, wherein the assessment is complemented with the results of an imaging technique such a CT colonography and/or with a monitoring technique based on the determination of a biomarker in serum such as CA-125 or CEA.

5

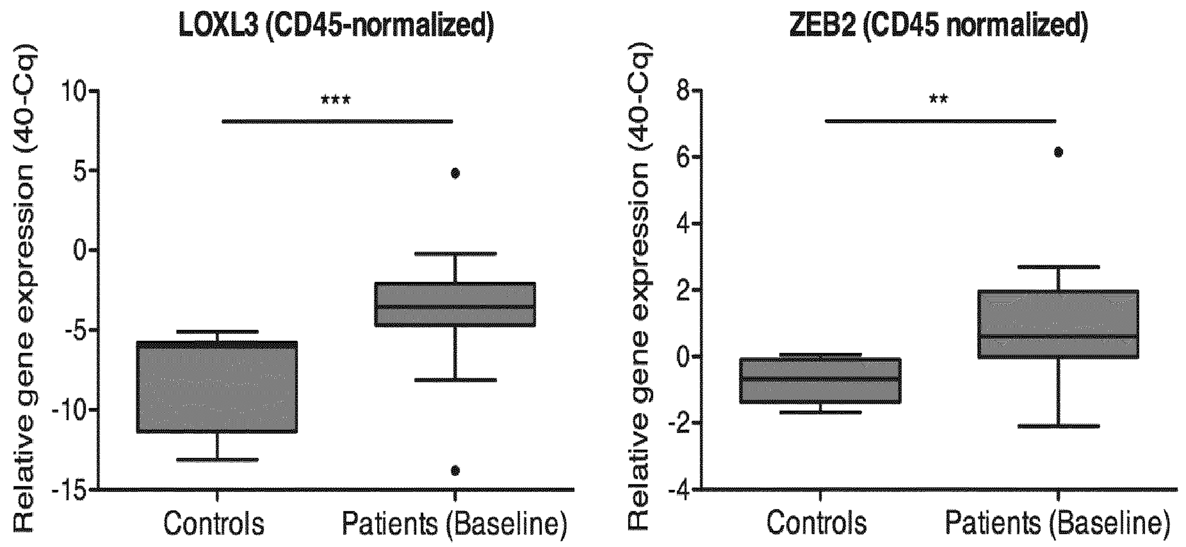
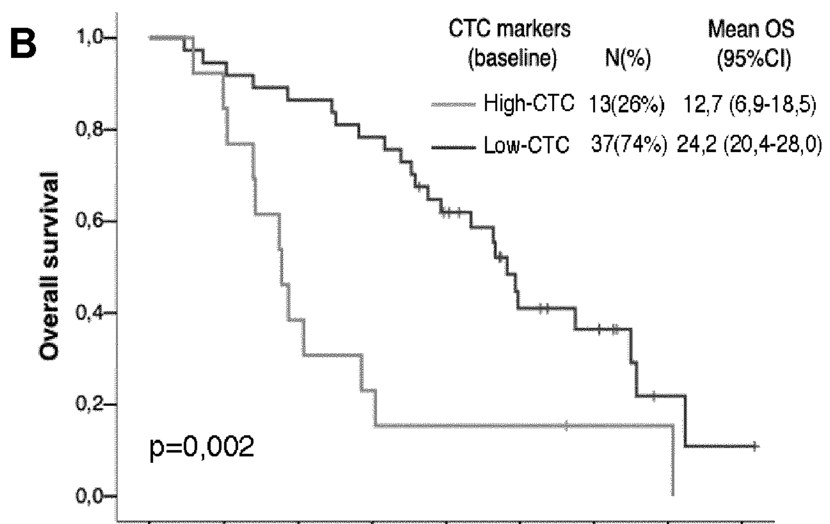
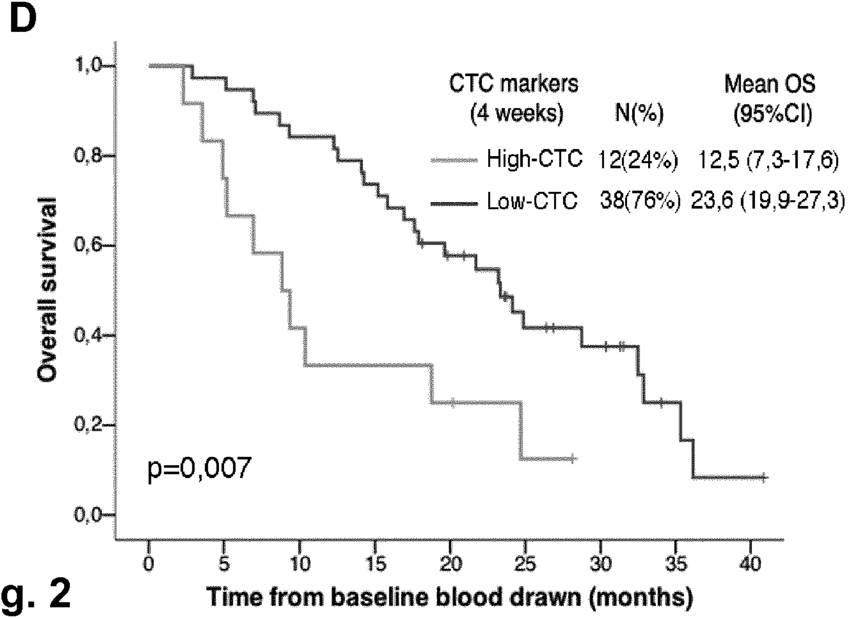
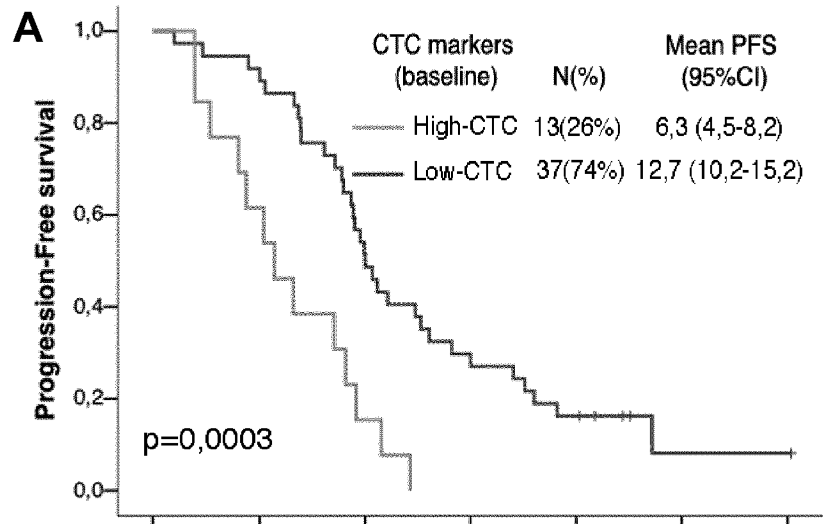


Fig. 1







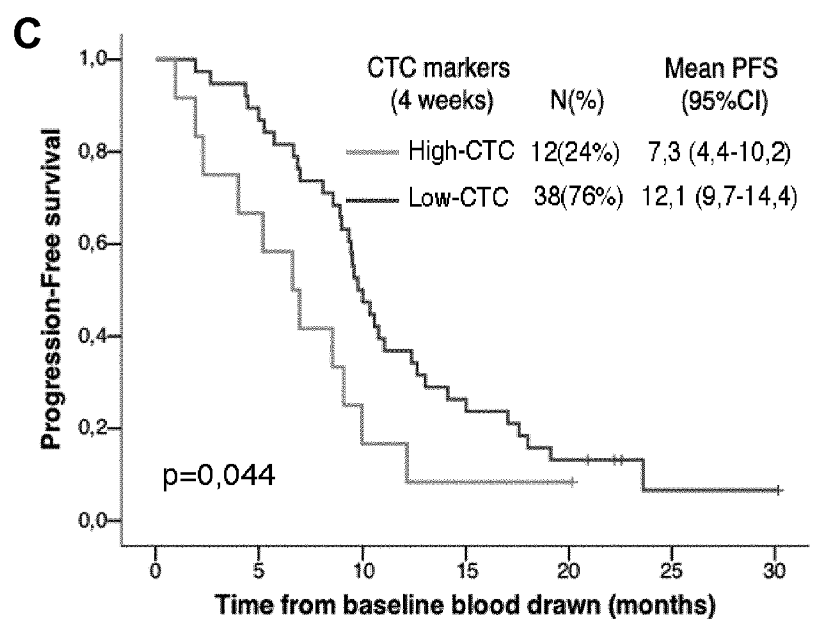
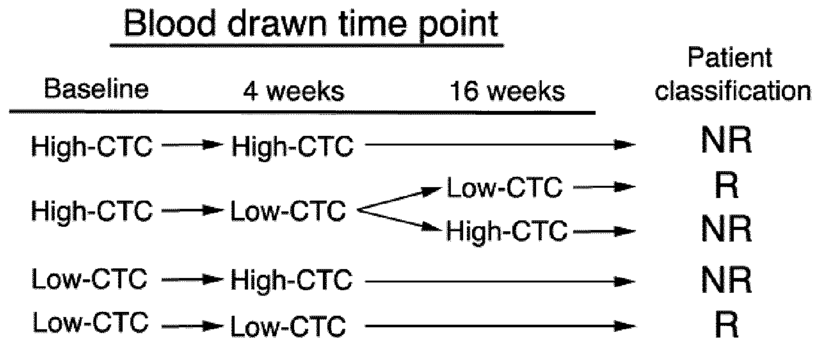


Fig. 2

A



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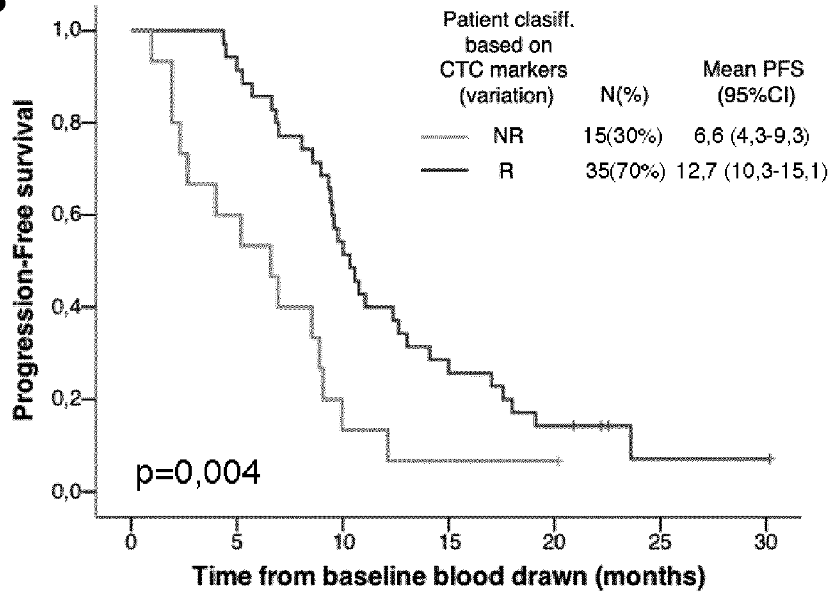

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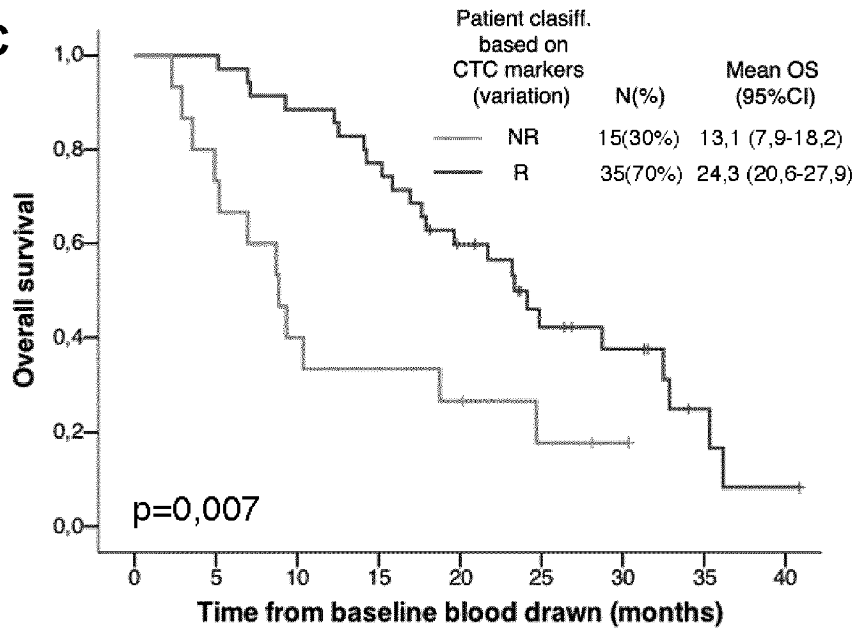


Fig. 3

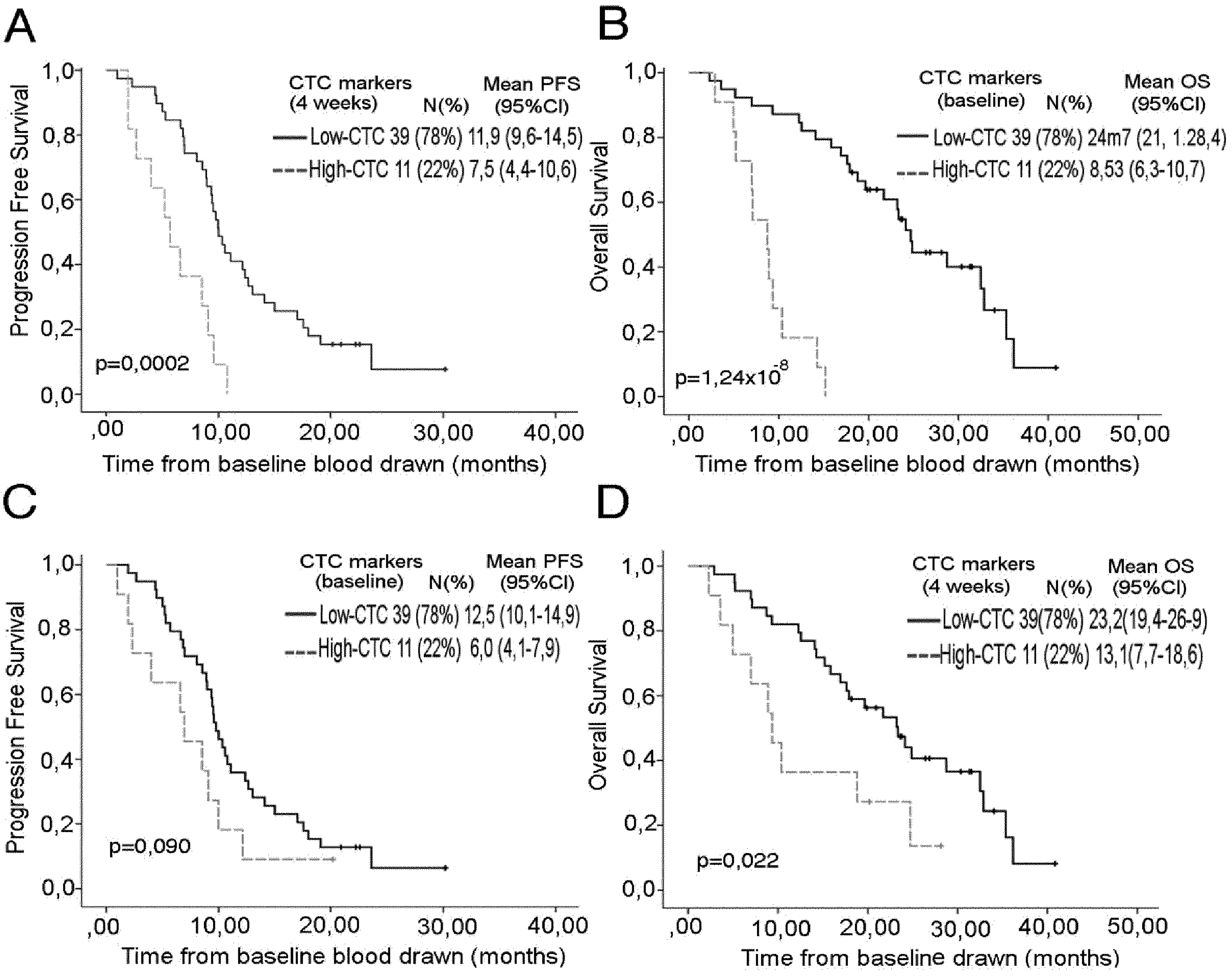
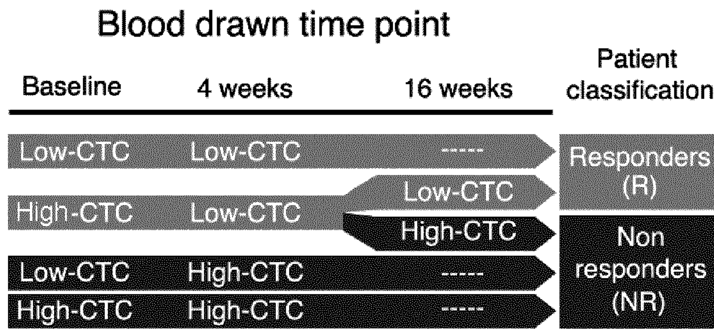


Fig. 4

A



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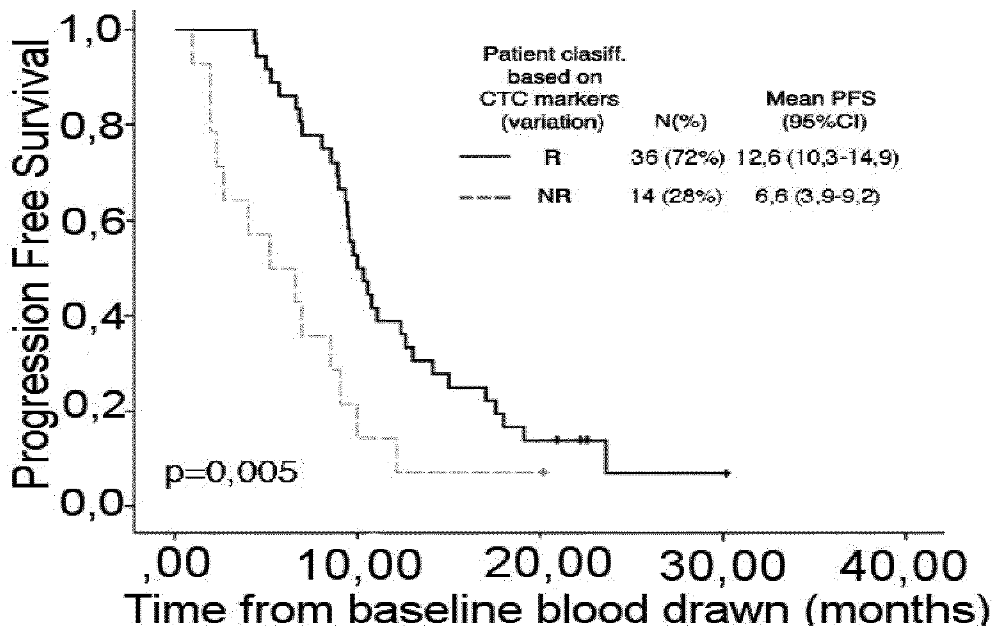

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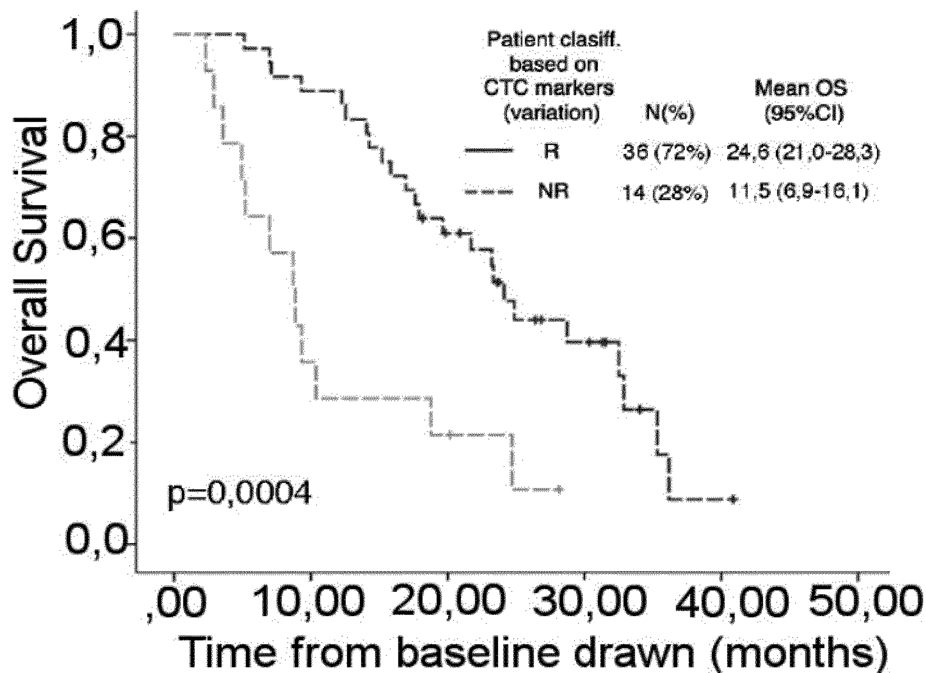


Fig. 5

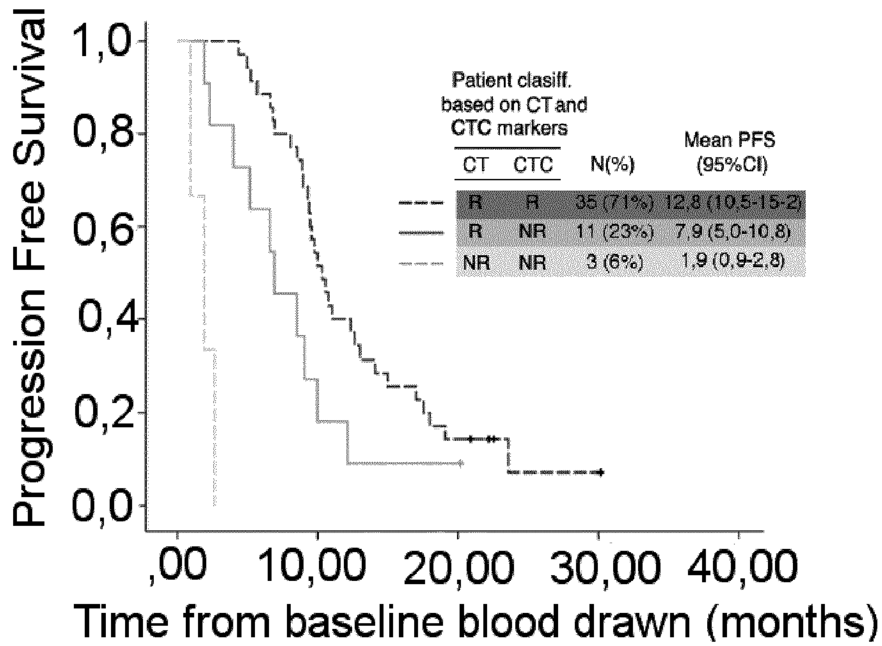
A

CTC markers classification

First CT evaluation	(R)	(NR)
SD or PR	35	11
PD	0	3

Chi-Square Pvalue: 0,020

B



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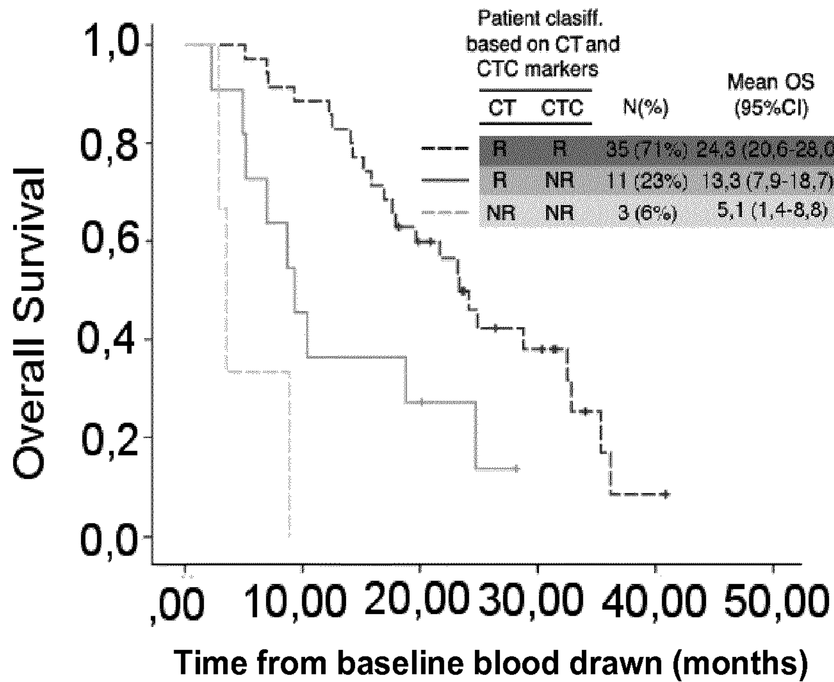

Fig. 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/056649

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
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, 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,P	JORGE BARBAZÁN ET AL: "A multimarker panel for circulating tumor cells detection predicts patient outcome and therapy response in metastatic colorectal cancer", INTERNATIONAL JOURNAL OF CANCER, vol. 135, no. 11, 29 April 2014 (2014-04-29), pages 2633-2643, XP055195472, ISSN: 0020-7136, DOI: 10.1002/ijc.28910 the whole document tables 2,3 figures 1-2 page 2640, column 1, paragraph 2 - page 2642 -----	1-22

-/--

[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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 June 2015	08/09/2015

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	Bruma, Anja

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2010/096734 A2 (WAYNE JOHN CANCER INST [US]; HOON DAVE S B [US]) 26 August 2010 (2010-08-26) claims 1,6-9 paragraphs [0015], [0017], [0031], [0032] -----	1-6,9-22
Y	WO 2012/149014 A1 (OSI PHARMACEUTICALS LLC [US]; AVEO PHARMACEUTICALS INC [US]; KAN JULIE) 1 November 2012 (2012-11-01) cited in the application claims 2,3,4-6,9,10 paragraphs [0115], [0162] table 13 -----	1-6,9-22
X	JORGE BARBAZÁN ET AL: "A logistic model for the detection of circulating tumour cells in human metastatic colorectal cancer", JOURNAL OF CELLULAR AND MOLECULAR MEDICINE, vol. 16, no. 10, 26 September 2012 (2012-09-26), pages 2342-2349, XP055129071, ISSN: 1582-1838, DOI: 10.1111/j.1582-4934.2012.01544.x cited in the application	1-6,9-22
A	the whole document abstract; figure 1 page 2345, column 2, paragraph 3 - page 2348 -----	7,8
Y	JORGE BARBAZÁN ET AL: "Molecular Characterization of Circulating Tumor Cells in Human Metastatic Colorectal Cancer", PLOS ONE, vol. 7, no. 7, 10 July 2012 (2012-07-10), page e40476, XP055129285, ISSN: 1932-6203, DOI: 10.1371/journal.pone.0040476 the whole document abstract page 2, column 2, last paragraph figure 2 page 4, column 2, paragraph 2 page 5 -----	1-6,9-22

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SHIU-RU LIN ET AL: "Molecular Detection of Circulating Tumor Cells With Multiple mRNA Markers by Genechip for Colorectal Cancer Early Diagnosis and Prognosis Prediction", GENOMIC MEDICINE, BIOMARKERS, AND HEALTH SCIENCES, vol. 3, no. 1, 1 March 2011 (2011-03-01), pages 9-16, XP055129295, ISSN: 2211-4254, DOI: 10.1016/S2211-4254(11)60003-4 the whole document abstract page 13, column 1, paragraph 1 - page 15, column 1, paragraph 2 -----	1-6,9-22
Y	QIAN XIAO ET AL: "Lysyl Oxidase, Extracellular Matrix Remodeling and Cancer Metastasis", CANCER MICROENVIRONMENT ; OFFICIAL JOURNAL OF THE INTERNATIONAL CANCER MICROENVIRONMENT SOCIETY, SPRINGER NETHERLANDS, DORDRECHT, vol. 5, no. 3, 13 April 2012 (2012-04-13), pages 261-273, XP035118671, ISSN: 1875-2284, DOI: 10.1007/S12307-012-0105-Z the whole document table 1 -----	1-6,9-22
Y	SCENEAY JACLYN ET AL: "The pre-metastatic niche: finding common ground", CANCER METASTASIS, KLUWER ACADEMIC PUBLISHERS, DORDRECHT, NL, vol. 32, no. 3, 1 May 2013 (2013-05-01), pages 449-464, XP035326412, ISSN: 0167-7659, DOI: 10.1007/S10555-013-9420-1 [retrieved on 2013-05-01] the whole document page 460, column 2, paragraph 1 -----	1-6,9-22
Y	Hé Ctor Peinado ET AL: "A molecular role for lysyl oxidase-like 2 enzyme in Snail regulation and tumor progression", , 18 August 2005 (2005-08-18), pages 3446-3458, XP055195703, Retrieved from the Internet: URL:http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1276164/pdf/7600781a.pdf [retrieved on 2015-06-15] the whole document page 3454 - page 3456 -----	1-6,9-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2015/056649

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-22(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-22(partially)

   Method for determining the outcome of a subject suffering from metastatic colorectal cancer (mCRC) and/or the effectiveness of a therapy administered to said subject using LOXL3 as marker for poor prognosis and non-response to therapy.

   ---

2. claims: 1-22(partially)

   Method for determining the outcome of a subject suffering from metastatic colorectal cancer (mCRC) and/or the effectiveness of a therapy administered to said subject using ZEB2 as marker for poor prognosis and non-response to therapy.

3-7. claims: 1, 2, 4-22(all partially)

   Method for determining the outcome of a subject suffering from metastatic colorectal cancer (mCRC) and/or the effectiveness of a therapy administered to said subject using GAPDH,VIL1, TIMP1, CLU and TLN1 respectively, as marker for poor prognosis and non-response to therapy.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2015/056649

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010096734 A2	26-08-2010	AU 2010215838 A1	26-08-2010
		CA 2729593 A1	26-08-2010
		EP 2399130 A2	28-12-2011
		US 2011206705 A1	25-08-2011
		WO 2010096734 A2	26-08-2010
WO 2012149014 A1	01-11-2012	EP 2702173 A1	05-03-2014
		JP 2014519813 A	21-08-2014
		US 2012302572 A1	29-11-2012
		WO 2012149014 A1	01-11-2012