

"IN-VITRO METHOD FOR DIAGNOSING OR ASSESSING PROGRESSION OR
RESPONSE TO TREATMENT OF A DISORDER BY A CIRCULATING
DISEASE LOAD PARAMETER"

5 Cross-Reference to Related Applications

This Patent Application claims priority from Italian Patent Application No. 102023000019227 filed on September 19, 2023, the entire disclosure of which is incorporated herein by reference.

10 Technical Field of the Invention

The present invention relates to an in-vitro method for diagnosing or assessing the progression of a disorder in an individual or assessing the response/responsiveness to treatment in the individual or assessing the presence of
15 a treatment target in a sample of organic fluid and determining a circulating disease load (CDL) parameter that is a function of the amount of cells of interest, cellular fragments of interest, circulating proteins, RNA and DNA, the latter three being collectively designated as
20 circulating biomarkers.

Prior Art

Carcinomas start from a primary tumor in the body. They grow locally and start to spread to more distant locations after some time. In this evolutionary process,
25 cancer cells acquire or lose genes that allow migration and growth in distant organs. Most patients die of metastases, either because the metastasis suppresses vital functions, or because the growth of the carcinoma throughout the body leads to exhaustion. At various stages in this process,
30 there are different issues to be dealt with to take the

best treatment decisions.

First, cancer must be diagnosed, then a treatment should be selected, then the success of the selected treatment should be assessed. In the selection of the treatment, an account should be taken of whether the risk of disease aggravating is sufficiently high that treatments with severe side effects are justified. In addition, there should be an assessment of whether targeted therapy has chance of being effective.

In this decisional process, information obtained from circulating tumor cells (CTCs) can be very helpful. However, CTCs are very rare cells and counts can be zero even in patients with advanced disease, metastasis is already ongoing. In this connection, it has been found that tumor-derived extracellular vesicles (tdEV) play an important role in homeostatic and oncogenic processes and can be informative in relation to the presence, progression, and prognosis of tumors.

In principle, CTC counts are considered more reliable due to a very low background count, but - when CTC counts are low - tdEV may supplement the evaluation.

As a practical example, most patients that are diagnosed with prostate cancer or will develop metastatic disease will initially respond to androgen deprivation, but will eventually progress to castration-resistant prostate cancer (CRPC) and death. In recent years, a plethora of new drugs have been introduced or are being developed for patients that develop mCRPC. A major challenge in evaluating the effectiveness of these new drugs is to adequately assess the response to therapy as the disease of

many patients is restricted to the bone which is not amenable to evaluation by RECIST and bone scans and PSA changes are not reliable before 12-16 weeks of treatment. In multivariate analysis measurement of CTCs has shown to be a superior biomarker for assessing prognosis and disease response in mCRPC. The CTC load is strongly associated with overall survival. To simplify matters in the FDA-cleared CellSearch CTC assay (Menarini Silicon Biosystems), mCRPC patients are divided into those with 5 or more (unfavorable) or less than 5 (favorable) CTC/ 7.5 ml of blood. Response to therapy after 3-6 weeks of treatment was assessed by switching from unfavorable to favorable. Several approaches have been investigated for the best evaluation of treatment response based on changes in CTC. The stumbling block for an accurate assessment of all mCRPC patients is the low frequency of CTCs. In the original prospective CTC study, 38% of patients had less than 5 CTC at all blood draws and no evaluation of the response could be assessed. The observation that CTCs represent only a small portion of the Cytokeratin+, DAPI+, CD45- objects in the CellSearch generated images and that the objects not assigned as CTCs showed similar prognostic value suggested the potential for improving the proportion of patients in which a response evaluation could be made. The open-source image analysis program ACCEPT was used to identify and classify Cytokeratin+, DAPI+, CD45- objects, and within these objects, a population was defined as tumor-derived Extracellular Vesicles (tdEV). These tdEV appeared in a 10-fold higher frequency as compared to CTC and showed a similar relation with overall survival. Moreover, elevated

tdEV in the mCRPC patients with 5 or fewer CTC had a 4.7-fold higher risk to die as compared to those with no elevated tdEV.

Up to now, CTC counts and tdEV counts have been
5 studied separately and individually correlated to disease and patient status.

In addition, the plasma from the collected blood may be used to detect other circulating markers, including small tdEVs, circulating tumor DNA, circulating tumor RNA,
10 and circulating proteins, all of which may have some relation to the metastatic activity of a tumor.

The need is therefore felt to find ways to combine information from CTCs and information on tdEVs to support clinicians in therapy decisions, particularly by assessing
15 the presence and the development of tumors in patients. Such a combination can be useful not only in oncology but also in other fields of medicine. For example, the detection of circulating endothelial cells (CEC) and fragments thereof (hereinafter also indicated as
20 endothelium-derived extracellular vesicles, edEV) may be indicative of cardiovascular diseases.

Summary of the Invention

It is therefore an object of the present invention to provide an improved method to support clinicians in therapy
25 decisions.

This object is achieved by the method as defined in claim 1.

Brief Description of the Drawings

Fig. 1 shows a flowchart of a method according to an
30 embodiment of the present invention.

Fig. 2 shows a graph depicting the combination of CTC counts and tdEV counts in a BTL parameter.

Fig. 3 shows a block diagram of a specific embodiment according to the invention.

5 Fig. 4 shows a block diagram of a specific embodiment according to the invention.

Fig. 5 shows a block diagram of a specific embodiment according to the invention.

Fig. 6 shows graphs, obtained respectively with three
10 different BTL equations, of the hazard ratio for overall survival evaluated using BTL as a continuous variable.

Fig. 7 shows graphs of the continuous hazard ratio for an optimal form of different BTL equations and the hazard ratios (HR) for operator CTC.

15 Fig. 8 shows graphs for the fraction and mean intensity of HER2 marker expression for CTCs only and for the Tumor Marker Expression biomarker (TME, which combines CTCs and tdEVs) in 98 breast cancer patient samples. The top left panel shows the fraction of CTCs that are positive
20 (> 25 above control staining) or weakly positive (> 1 above control staining). Samples are sorted by the fraction positive and no result is available if there are 2 or fewer CTCs. The top right panel shows the TME parameter determined using CTC and tdEV. Samples are sorted by the
25 fraction positive and no result is available if there are 2 or fewer CTCs and/or tdEVs combined. The bottom panels show the mean intensity for the same samples.

Detailed Description of the Invention

The method according to the invention is for
30 diagnosing or assessing the progression of a disorder in an

individual or assessing the responsiveness/response to a treatment in an individual (patient).

In detail, the following description will be focused on the application of this method to circulating tumor cells (CTC) and circulating cell fragments (hereinafter also indicated as tumor-derived extracellular vesicles, tdEV), and other prognostic biomarkers in a sample of organic fluid of a patient. CTCs and tdEV are indicative of metastasizing tumors, in particular carcinoma or melanoma. However, the present method may be applied also to detect other kinds of cells of interest.

The expression "cells of interest" is intended to indicate cells whose presence and number in an organic fluid sample of a patient may be indicative of the presence of a disorder in the patient. In a preferred embodiment, cells of interest are rare cells, whose density in the organic fluid of the patient is low. The ranges of the density for different kinds of cells are listed in the following.

For example, the method according to the invention may also be applied to the detection of circulating endothelial cells (CEC) and circulating cell fragments (hereinafter also indicated as endothelium-derived extracellular vesicles, edEV) indicative of cardiovascular disease, infectious disease, or cancer. Density of CECs in blood range from 0 to 200 / mL whole blood, in particular 0 to 50 / mL whole blood, more in particular around 5 / ml whole blood.

The method may also be applied to the detection of circulating multiple myeloma Cells (CMMC) and circulating

cell fragments (hereinafter also indicated as multiple myeloma-derived extracellular vesicles, mmEV). The presence of CMMC and their number in the blood of a patient may be indicative of their presence in the bone marrow at the time of diagnosis. Furthermore, their number may be monitored to evaluate myeloma, as well as the efficacy of different therapies for this tumor. Density of CMMCs in blood range from 0 to 20000 / mL whole blood, in particular 0 to 2000 / mL whole blood. Typically, medians are about 1000 before therapy, drop to about 5 on remission in response to effective therapy, and increase back up to about 100 on relapse.

In some diseases, the count of more than one type of cell of interest and cellular fragment of interest is particularly informative, such as in the case of CTCs, CECs, and tdEV, edEV for colorectal cancer. There may be additional circulating markers that can be derived from the same sample which have independent prognostic value, including circulating tumor DNA (ctDNA), various types of RNA and protein markers.

Other kinds of rare cells to which the method could be applicable, are fetal cells, respiratory virus cells, and cells collected from cerebrospinal fluid taps.

The expression "cellular fragments of interest" is intended to indicate phospholipid membrane-enclosed structures, in particular cellular particles including e.g. exosomes, microvesicles, apoptotic bodies, whose presence and number in an organic fluid sample of a patient may be indicative of the presence of a disorder in the patient.

The expression "components of interest" is intended to

indicate collectively and generally one or more of "cells of interest", "cellular fragments of interest" and optionally also circulating biomarkers of interest derived from the pathological condition (disorder), e.g. circulating tumor DNA, circulating RNA or circulating proteins.

The expression "responsiveness" is intended to indicate the eligibility for an individual to a specific treatment. The expression "response" is intended to mean the actual effect of the treatment on the individual.

The method according to the present invention comprises the following steps.

First, a number of cells of interest and a number of cellular fragments of interest is counted in a sample of organic fluid of the individual. Optionally, also a number of clusters of cells of interest can be counted. For example, occasionally CTCs in a sample of whole blood are not found isolated but instead grouped in clusters. The count of these clusters and the addition of this information to that relating to the number of cells of interest and the number of cellular fragments of interest can be of additional help in evaluating disease or the progression of disease. Optionally, also the blood plasma may be analyzed for the presence of circulating tumor DNA (ctDNA), disease specific RNAs, disease specific proteins.

As a next step, a circulating disease load (CDL) parameter is determined as a function of the number of cells of interest and the number of cellular fragments of interest, and optionally the number of clusters of cells of interest, the concentration of ctDNA, RNAs, and/or proteins

of interest.

Subsequently, the circulating disease load (CDL) parameter is correlated with the presence or progression of the disorder or response/responsiveness to the treatment in
5 the individual.

The CDL parameter in the patient is a function of the number of cells of interest and the number of cellular fragments of interest, and optionally of clusters of cells of interest, with respect to the background numbers of
10 cells of interest, cellular fragments of interest, and optionally clusters of cells of interest, in a normal individual.

Figure 1 shows a flowchart of the method according to an embodiment of the invention.

15 At a step S01, a sample of organic fluid of a patient, in particular a blood sample, is provided.

Then, step S02, the sample of organic fluid is processed to enable selective imaging of the sample and detection of the presence of cells of interest and cellular
20 fragments of interest (and, optionally, of clusters) in the sample.

In detail, at step S02, the sample of organic fluid is enriched for the components of interest COI (e.g. CTC and tdEV) and labeled.

25 For example, for the detection of CTCs and tdEVs, the blood sample may be subject to immunomagnetic enrichment targeting epithelial cell adhesion molecule (EpCAM).

The enriched sample of organic fluid is labeled with at least one marker specific for the rare cell and
30 fragments thereof, e.g. CTC and tdEV, and/or optionally

with at least one marker specific for cells and/or fragments thereof other than the rare cell and/or the fragment thereof to be detected.

The markers are identifiable by selective imaging.

5 According to a preferred embodiment, the markers are fluorescent markers that can be imaged by fluorescence imaging. For example, for the detection of CTCs and fragments thereof, the enriched sample may be stained with one or more fluorescent markers, in particular DAPI, CD45-
10 APC, and CK-PE.

However, processing of the sample may be different, according to the specific application, to allow detection of the presence or absence of the component of interest, for example using scatter markers or temporal behavior in
15 response to a stimulus, or any other known method.

At a step S03, images of the labeled sample are captured, in a per se known way. In detail, in this embodiment, the images are fluorescent images.

For example, the marked sample may be arranged within
20 a cartridge, and digital images of the surface for each of the fluorophores may be recorded.

According to a preferred embodiment, one or more of steps S02 and S03, in particular both steps S02 and S03, may be performed by the CellSearch System®. The step S03
25 could be performed by other imaging systems such as the DEPArray™.

Then, at a step S04, the captured images are processed by a control unit to determine the presence of cells of interest and cellular fragments of interest in said images
30 and, therefore, in the sample of organic fluid of the

patient. The control unit is configured to detect and enumerate the components of interest in the received images.

In particular, the control unit may be configured to
5 detect and enumerate the cells of interest and cellular fragments of interest by using one or more machine learning algorithms that are trained for identifying if a marker-stained object in an image is a cell of interest or a cellular fragment of interest.

10 In the embodiment illustrated in Figure 1, the method further comprises a step S05 at which the control unit provides a plurality of counters, one for each component of interest COI that has been detected and enumerated at step S04. For example, the counters can be a CTC counter CTC-CNT
15 and a tdEV counter tdEV-CNT.

For example, the various counters may be provided to a user, for example through a visual signal on a graphic user interface.

At step S06, the rare counters are combined to
20 establish a value of the circulating disease load CDL and, as a function of the CDL value, assessing (step S07) a diagnosis of the patient. Steps S06 and S07 are described in detail later with reference to Figure 3.

Optionally, according to the embodiment of Figure 1,
25 after the detection and enumeration of the components of interest at step S04, the method may further comprise:

- computing (step S08) the marker expression of each component of interest that is detected at step S04. The marker expression for each COI may be computed in a per se
30 known way. For example by computing the average marker

expression for CTC and tdEV separately, or by determining the number of CTC and tdEV that are negative, weakly positive, and strongly positive. The thresholds for transitioning from negative to weak to strong are marker
5 and disease-dependent. For example, Her2 expression in breast cancer is negative when at the same level as control cells, weak when up to 20 above the control cells, and strong when above 20; and

- computing (step S09) a tumor marker expression (TME)
10 value that is a function of all the single marker expressions computed for each COI and the counters for CTC and tdEV. For example, weighting the average marker values for CTC and tdEV together in a way that depends on their relative frequency, or weighting the proportion of negative
15 CTC and tdEV, the proportion of weakly stained CTC and tdEV, and the proportion of strongly positive CTC and tdEV in a way that depends on their relative frequency.

Further details about steps S08 and S09 are provided in detail hereinafter with reference to the embodiment of
20 Figure 4.

The TME may be useful to assess whether a patient is eligible for targeted therapy.

According to an embodiment, the method of the invention may comprise additional steps for the analysis of
25 ctDNA, RNAs and/or proteins. For example, at a step S50, the blood plasma may be separated from the cell fraction in the fluid obtained at step S01. However, another tube of blood may be collected in addition to the one drawn for CTC analysis.

30 At step S51, ctDNA, RNA, and/or proteins are each

analyzed using assays optimized per target. For example, a plasma sample may be centrifuged at a high g-force (20,000 x g) to remove any cellular material, and next generation sequencing may be applied to the supernatant of this process to identify circulating DNA with mutations typical for tumors. Then, the extracted concentration of circulating proteins, DNA and/or RNA may be used at step S06 in combinations with the cell counters for providing the CDL.

10 The method according to the present invention comprises:

 - marking the sample with at least one marker specific for the cell of interest and the cellular fragment of interest, and/or optionally at least one marker specific for cells and/or cellular fragments other than the cell of interest and/or the cellular fragment of interest, the marker/s being identifiable by selective imaging,

15 - acquiring at least one image of the marked sample, wherein the image comprises at least one marker-stained object to be classified;

 - classifying the marker-stained object; and
 - determining whether the marker-stained object is a cell of interest or a cellular fragment of interest based on a result of the classification of the marker-stained object.

25 According to the method, counting a number of cells of interest and/or a number of cellular fragments of interest and/or a number of clusters of cells of interest in the sample comprises:

30 increasing a cell of interest counter (CTC-CNT) in

response to each marker-stained object being determined to be a cell of interest, and/or

increasing a cellular fragment of interest counter (tdEV-CNT) in response to each marker-stained object determined to be a cellular fragment of interest, and/or

optionally, increasing a cell of interest cluster counter (CTCcl-CNT) in response to each marker-stained object determined to be a cell of interest cluster.

The classification steps may be performed by running one or more machine learning algorithms based on classification and trained to identify if a marker-stained object in an image comprises a cell of interest or a cellular fragment of interest.

Preferably, the CDL parameter is a function of the difference in the value of the CTC counter CTC-CNT with respect to their relative background level in a normal individual.

In a preferred embodiment, cells of interest are CTCs, fragments are tdEVs and clusters are CTC clusters. In this case, these elements can be marked by cytokeratin and CD45 antibodies and DAPI stain. CTCs, and clusters will be positive for cytokeratin and DAPI and negative for CD45. tdEVs will be positive for cytokeratin and negative for CD45.

Cells of interest, fragments, and clusters may e.g. be counted by using the Cellsearch system (Menarini Silicon Biosystems), which detects, enriches, and enumerates circulating tumor cells or other rare cell types from whole blood in a standardized and reproducible workflow. The enumeration of cells and fragments of interest could also

be performed using other imaging systems such as the DEPArray™ (Menarini Silicon Biosystems).

The value of the circulating disease load parameter CDL may be a linear combination of the values of the CTC counter CTC-CNT and the tdEV counter tdEV-CNT.

The circulating disease load parameter CDL is a function of the difference in the value of the CTC counter CTC-CNT with respect to their relative background level in healthy patients as follows:

10 $CDL = f(CTC - CTC_{BG}, tdEV - tdEV_{BG}),$ wherein

CTC is the number of cells of interest in the individual,

CTC_{BG} is the number of cells of interest in the normal individual,

15 $tdEV$ is the number of cellular fragments of interest in the individual,

$tdEV_{BG}$ is the number of cellular fragments of interest in the normal individual.

Optionally,

20 $CDL = f(CTC - CTC_{BG}, tdEV - tdEV_{BG}, CTC_{clust} - CTC_{clust,BG}),$ wherein

CTC_{clust} is the number of clusters of cells of interest in the individual,

25 $CTC_{clust,BG}$ is the number of clusters of cells of interest in the normal individual.

In a preferred embodiment, the circulating disease load (CDL) parameter ranges from a minimum value, e.g. 0, to a maximum value, e.g. 1, as a function of the CTC counter CTC-CNT and/or the value of the tdEV counter tdEV-CNT, wherein the risk of disease monotonically increases

with the value of the CDL.

Figure 2 shows a graph that exemplifies this possible computation of the CDL, considering the combination of the count of CTCs and the count of tdEV. The CDL parameter is thus renamed "blood tumor load" (BTL) and ranges between a value of 0 (low risk of disease) to a value of 1 (high risk of disease).

The low concentration of CTC limits the reliability of CTC-based diagnostics, and therapy selection nor effect determination is possible in the group of patients who have no detected CTC before therapy. Metastatic patients almost certainly have CTC, but the concentration may be too low to detect a CTC in a single tube of blood. However, the majority of CTC will fail to extravasate and thus may disintegrate at the metastatic sites or in circulation, leading to tdEV. Augmentation of CTC-based diagnostics with tdEV can expand the number of patients for whom a diagnosis is possible, but providing two indicators of the same process means results will be more difficult to interpret. The aim of BTL is to integrate CTC and tdEV (and possibly others) into a single measure that is above background for a larger proportion of patients.

BTL may be applied e.g. for **application a)**, i.e. to assess the risk or probability that a patient is metastasizing, for **application b)** to aid in selection of targeted therapies, and **application c)** to monitor the impact of treatment on the metastatic activity.

The table below summarizes the clinical questions, the current standard approach, and the potential means by which CTC and tdEV could improve diagnosis.

Question	Primary guide	CTC / alternative	tdEV	BTL
Cancer diagnosis	Biopsy, imaging	Presence of any indicates high risk of malignancy and metastases		Probability that patient is metastasizing (has detectable BTL)
Need for treatment	Staging, marker expression	Presence of any indicates high risk of metastases		Probability that patient is metastasizing Marker expression
Treatment selection	Marker expression, treatment conventions	Current biopsy of the most active tumor components		Marker expression
Treatment efficacy	Change in lesion size (imaging)	Elimination of circulating tumor derived material		Significant change in blood tumor load

Referring to the applications mentioned above, to provide a probability that a patient is above the background CDL (application a), Fig 3), the background BTL or the background of CTC and tdEV need to be modeled. This means expressing the background BTL as a random variable, or CTC and tdEV as random variables.

In detail, according to the embodiment of Figure 3, the plurality of counters determined at step S05 of Figure 1 are gathered (Steps S10_1,...,S10_N), for example by a processing unit. The counters are each indicative of the number of a respective COI.

Then, at step S11, a CDL value is determined as a function of the gathered counters. The CDL value may be computed as per any of the equations reported below. In this case, the CDL value may be referred to as a circulating disease load CDL.

Then, step S12, the probability that the so-obtained CDL value was found in a normal or benign patient is computed from reference values for benign and or normal donors using an applicable statistical distribution, for example, a joint Poisson distribution, or a normal distribution. The complement of this probability is taken as the probability that the patient has circulating tumor-derived material, and thus has a metastatic tumor. For example, a probability above 0.8 would warrant additional diagnostics, such as more extensive imaging, tissue biopsies, or similar methods.

At step S13, a signal indicative of the computed probability is provided at output. For example, the signal may be provided to a user through a graphical user interface or any other known interface.

To determine marker expression (application b), Figure 4, a tumor marker expression (TME) parameter can be used in addition to the CDL parameter.

In this particular embodiment, the method according to the invention further comprises the following steps:

- assessing expression of at least one biomarker which is a target for therapy of the disorder in the cells of interest and/or in the cellular fragment of interest;
- determining a tumor marker expression (TME) parameter as a function of the assessed biomarker expression;
- correlating the TME parameter with the responsiveness to treatment in the individual.

In detail, according to the embodiment of Figure 4, the plurality of marker expressions of step S08 of Figure 1

are retrieved (step S14₁,...,S_{14N}).

Then, step S15, a tumor marker expression is determined as a function of all the gathered marker expressions for the COIs. The tumor marker expressions may
5 be computed with the equations described hereinbelow for the TME.

At step S16, a signal indicative of the tumor marker expression is provided at output. For example, the signal may be provided to a user through a graphical user
10 interface or any other known interface.

To determine treatment response (application c), Figure 5, response, stable disease, disease progression) the prior CTC and tdEV counts need to be modeled as random variables and the probability that the posterior counts
15 were drawn from the same distribution need to be computed.

In detail, according to the embodiment of Figure 5, samples of organic fluid from an individual are processed (e.g., steps S01-S04 of Figure 1) at different times. For example, a first sample is processed (step S17) at a time
20 T1 and a second sample is processed (step S20) at a time T2 larger than T1, to assess the progression of the patient.

In response to the processing of both the first and the second samples, the COI counters (e.g. counters for CTC and tdEV, and optionally CTC clusters) are provided (steps
25 S18₁,...,S18_N for the first sample and steps S21₁,...,S21_N for the second sample).

At step S19, a circulating disease load CDL[1] for the first sample is determined as a function of the respective COI counters, as discussed for step S11 of Figure 3.

30 At step S22, a circulating disease load CDL[2] for the

second sample is determined as a function of the respective COI counters, as discussed for step S11 of Figure 3.

Then, step S23, a first probability P_{reduce} is computed, reflecting the probability that the second measurement was
5 not drawn from the same distribution, but from a distribution that was shifted down, i.e. the probability that the true posterior CDL[2] is reduced relative to the prior CDL[1]. For example, assuming the CDL follows a joint Poisson distribution, and using the prior CDL as parameters
10 for this distribution the probability of finding the posterior CDL or any possible lower value for CDL may be computed.

Then, step S24, the first probability P_{reduce} is compared with a threshold, for example 0.8. The threshold
15 may be chosen depending on the specific application.

If the first probability is higher than the threshold, then a (partial) response is provided at the output of the patient assessment evaluation method (step S25). For example, a (partial) response may be provided to a user
20 through a graphical user interface or any other known interface. The partial response output is an indication that the CDL is reducing, and may be interpreted as an effect of therapy, thus supporting the maintenance of such therapy when appropriate.

25 If the first probability P_{reduce} is not higher than the threshold, then a second probability P_{growth} is computed (step S26), reflecting the probability that the second measurement was not drawn from the same distribution, but from a distribution that was shifted up, i.e. the
30 probability that the true posterior CDL2 is increased

relative to the prior CDL1. For example, assuming the CDL follows a joint Poisson distribution, and using the prior CDL as parameters for this distribution the probability of finding the posterior CDL or any possible higher value for
5 CDL may be computed.

The second probability P_{growth} is compared with a threshold (step S27), for example 0.8. The threshold may be chosen depending on the specific application.

If the second probability P_{growth} is higher than the
10 threshold, then a progression is provided at the output of the patient assessment evaluation method (step S28). The progression output indicates that the disease of the patient has worsened between time T1, at which the first sample has been collected, and time T2, at which the second
15 sample has been collected. The progression may be interpreted as a sign that the therapy is not yet yielding any effect, and depending on the therapy, may be interpreted as supporting a modification to the current therapy.

20 If the second probability is not higher than the threshold, then a stable disease is provided at the output of the patient assessment evaluation method (step S28). The stable disease may be a signal of a known type that is provided to a user through a graphical user interface or
25 any other known interface. The stable disease indicates that the disease of the patient has not substantially changed between time T1, at which the first sample has been collected, and time T2, at which the second sample has been collected. Depending on the goals of the current treatment,
30 this may be interpreted as a therapy success, or failure,

to be interpreted by the treating oncologist.

All the above applications rest on the most general concept that BTL is a measure for the metastatic load of a tumor, which should be preferably reduced, or alternatively
5 maintained stable as an effect of therapy. BTL is a function of the enumeration and other features belonging to CTCs, tdEVs, and CTC clusters found in the blood sample, with respect to their relative background levels in normal/benign donors (BG).

10
$$BTL = f(CTC, CTC_{BG}, tdEV, tdEV_{BG}, CTC\ cluster, CTC\ cluster_{BG})$$

BTL has many applications. In the following, some of them will be described, without intending to limit the invention in any way.

15 A first application of the BTL concept is to estimate the marker expression of tumor antigens, which are possible targets for therapy. Some examples of targets are listed in the table below.

Cancer	Target
Breast	Her2 (+)
	ER (+) or PR (+)
	ER (+) or PR (+) plus Her2 (-), sufficiently
	Ki-67+
	Her2 (-), ER (-) and PR (-)
Colorectal	Her2 (+)
	EGFR (+) no RAS-gm, no RAF-gm
Lung	EGFR (+)
	Her2 (+)
	ROS1-gm
	KRAS G12C-gm

	BRAF V600E-gm
	MET exon 14 skipping-gm
	RET fusion-gm
Prostate	PARP and low cell repair
	PSMA
Myeloma	CD38, CD47, CD138, SLAMF7, GPRC5D, FcRH5 and BCMA

Table above is according to the American Society of Clinical Oncology (ASCO, August 2023), wherein (+): cancer has higher than normal expression of target, (-): normal expression, ER: endogen receptor, PR: progesterone receptor, EGFR: epidermal growth factor receptor, PARP: poly-ADP ribose polymerase, genetic mutations indicated by -gm after name of gene or description of aberrancy. In Multiple Myeloma, CD38, CD47, CD138, SLAMF7, GPRC5D, FcRH5 and BCMA are highly expressed by malignant plasma cells.

10 Tumor antigen expression can be measured on CTC and tdEV. However, false positive identifications are most likely negative for the antigen and would thus artificially lower the determined expression. Because of the more favorable false positive rate of CTC compared to tdEV, and
15 because some of the aforementioned treatment targets can be expressed at very low levels, the detection of a treatment target on a CTC should be considered more reliable than the detection on a tdEV. Nevertheless, expression may be detected on both CTC and tdEV, and combined to arrive at a
20 joint value for the fraction of circulating tumor-derived material that expresses the marker of interest. The form of the equation needed to combine the CTC and tdEV expressions

into a marker expression is analogous to the BTL equation. As with BTL the diagnostic information obtained from CTC is combined with information from tdEV whenever the information from CTC alone is not sufficiently informative.

5 This marker expression is a gauge for the susceptibility of a tumor to a specific therapy. The accuracy of the tumor marker expression is a function of the number of events evaluated and can be estimated using a stochastic noise model. This stochastic noise model can also be used to
10 estimate the blood volume that needs to be evaluated for some desired accuracy.

A second application is the use of BTL to determine the sample volume needed to obtain sufficient CTC for molecular characterization. The molecular characterization
15 of CTC from which one can assess the molecular profile of the cancer and the heterogeneity within the cancer is strongly associated with the number of CTC from which a molecular profile can be obtained. Based on morphologic features and nuclear staining one can provide a likelihood
20 of the number of CTC from which a molecular profile can be obtained. Using the success rate of the single cell isolation procedure one can estimate the number of CTC from which a molecular profile is obtained and one can determine whether this is sufficient for the desired molecular
25 profile. For example, from one molecular profile obtained from a CTC one can determine the aberrancy of the cell and whether or not a treatment target is present but this is clearly not sufficient to assess tumor heterogeneity or determine whether or not a patient is a candidate for a
30 certain therapy. In contrast, when 10 molecular CTC

profiles are obtained with all similar profiles the reliability of the assessment is rather high, but when they are all aberrant and different 10 may not be sufficient to obtain a clear picture of the tumor. Because tdEV are correlated to CTC, one can estimate how much blood will need to be investigated to obtain a sufficient number of CTC for molecular characterization. Depending on the blood volume needed, this may be achieved with regular blood draws or with a diagnostic leukapheresis (DLA). Such extrapolations require an estimate of the true CTC concentration with a confidence interval. The relationship to estimate a true CTC concentration from the CTC and tdEV concentration when CTC counts are low (0, 1, 2, etc.) can be derived from the BTL. For molecular characterization cells need to be isolated and the nucleic acids amplified, the better the cell looks the higher the chance that isolation and characterization will be successful. Such features can be extracted from the images and morphologic features derived such that a likelihood of success can be provided. The distribution of very nice looking to very bad looking cells can differ between cancers and treatments. Gathered knowledge of this distribution can be used to add the equitation and an estimate can be provided how much blood is needed for successful molecular characterization.

Another application is the blood myeloma load (BML) based on the identification and enumeration of CMMC and mmEV.

In healthy individuals, epithelial cells do not circulate in the blood. The presence of epithelial cells in blood indicates a pathological condition and, in most

instances, these epithelial cells originate from a tumor (carcinoma) that is shedding tumor cells in the blood which can lead to extravasation and distant metastasis. In contrast, plasma cells are present in blood although in small numbers which makes the detection of malignant plasma cells (CMMC) more difficult. Differences in antigen expression between normal plasma cells and CMMC such as lack of expression of CD27 and CD19 and monoclonality of the CMMC (lambda or kappa) can be used to help discriminate between the normal and malignant plasma cells. Morphological differences can also help to distinguish both. The latter can be utilized using the approach described for CTC and tdEV. After the classification of CMMC by experts and training of deep learning convolutional networks (DLCN) on the identification of CMMC, a second round of classification can be applied by presenting the DLCN objects detected in normal individuals or patients with infectious diseases in which a higher number of normal plasma cells are present and objects detected in CMMC. In addition, differences between the number of EVs derived from normal plasma cells and myeloma cells (mmEV) can aid in increasing the sensitivity and specificity of CMMC detection. Moreover, similar to the BTL a BML can be derived from the CMMC and mmEV count.

Yet another application is the blood endothelial load (BEL) based on the identification and enumeration of CEC and edEV. Endothelial cells and endothelial-derived Extracellular Vesicles can be detected in the blood of healthy individuals and are frequently detected in higher numbers in patients with cardiovascular disease (heart

attack), infectious disease, and cancer. Endothelial cells line the complete vascular system and can be released into circulation as CEC and edEV from all locations. The origin and cause of CEC and edEV release can be quite different in cancer, cardiovascular disease, infectious disease, and healthy donors. The morphology of CEC is very heterogeneous yet a classification that pinpoints the origin of the CEC and the cause of their release has not been made yet. Although a DLCN can be trained on objects classified as CEC and edEV by experts it will not identify their origin. A second round of classification in which CEC and edEV are identified in the first round of DLCN in healthy donors, patients with cardiovascular disease, infectious disease, and cancer can now yield a classification in which the origin of CEC and edEV are identified. As in the other examples, the numbers obtained can be reduced into one Blood Endothelial Load from which the severity of cancer, cardiovascular disease, and or infection can be deduced.

Examples

Example 1 - BTL equations

Several equations were tested. Three of them are explained below.

The main considerations at the basis of the equations are the following.

1) While CTC and tdEV are related to the same pathology, CTC are more important than tdEV.

2) CTC and tdEV are correlated, but at low CTC concentrations, tdEV are comparatively more accurate, whereas at high CTC concentrations, tdEV are comparatively

less accurate.

3) Estimation of a confidence interval on the CTC concentration is fundamental to any extrapolations with regard to the needed sample volume for required molecular analyses. BTL is a relation that allows the use of tdEV for the estimation of such a confidence interval.

A simpler form of BTL is a linear combination of CTC and tdEV counts as shown in equation 1. Here, both counts were log-transformed to reduce the impact of large outliers, and a log of 0 is set to -1. Additionally, the coefficient "a" balances the contribution of tdEV relative to CTC.

$$BTL_{linear} = \log_{10} CTC + a \log_{10} tdEV \quad (eq\ 1)$$

A more advanced form of the BTL equation builds upon the observation that CTC have a high correlation to tdEV, so a high tdEV count means that there is a higher probability that the CTC count was underestimated. This is expressed in equation 2, where the sigmoid function in brackets can be simplified if desired.

$$BTL_{sigmoid} = \log_{10} CTC + 3 a \left(\frac{2}{1 + e^{-\log_{10} tdEV}} - 1 \right) \quad (eq\ 2)$$

Another advanced form of this equation explicitly tapers the impact of the tdEV count when the CTC count is higher.

$$BTL_{tapered} = \log_{10} CTC + a \log_{10} \frac{tdEV}{CTC+1} \quad (eq\ 3)$$

Another advanced form of the BTL equation makes use of the log-logistic function optimized in a previous study,

with the shape and shift parameters for CTC and tdEV obtained separately from fitting the distributions as described in [Coumans, F.A., et al., Challenges in the enumeration and phenotyping of CTC. Clin Cancer Res, 2012. 18(20): p. 5711-8.].

$$BTL_{log-logistic} = \frac{llg(CTC) + llg(tdEV)}{2} \quad (eq\ 4)$$

$$\text{where } llg(x) = \left(\frac{1}{1 + e^{-(shape \ln(x+1) - shift)}} - \alpha \right) / (1 - \alpha)$$

$$\text{with } \alpha = 1 / (1 + e^{shift}).$$

For all of these equations, the CTC / tdEV count can be the absolute count, or the absolute count minus some background count level.

As can be seen, the CDL parameter may be a function of the number of cells of interest and the number of cellular fragments of interest including a transform of the number of cells of interest, and/or a transform of the number of cellular fragments of interest. This transform can be any non-linear function suitable for right-skewed distributions, including square root, cube root, logarithm, or a power transform, or a combination thereof.

Other variations on equations 1-3 include, but are not limited to:

$$BTL_{tapered_full} = \log_{10} CTC + a \log_{10} \frac{tdEV}{CTC + CTC_{cluster} + 1} + b \log_{10} CTC_{cluster}$$

$$BTL_{aggressive\ taper} = \log_{10} CTC + \frac{a}{(CTC + 1)^2} \log_{10} tdEV$$

$$BTL_{mild\ taper} = \log_{10} CTC + \frac{a}{CTC + 1} \log_{10} tdEV$$

$$BTL_{\text{lin cube root}} = \sqrt[3]{CTC + 1} + a \sqrt[3]{tdEV + 1}$$

$$BTL_{\text{complete}} = \log_{10} CTC + a \log_{10} \frac{tdEV}{CTC + 1} + b \text{ctDNA}_{\text{mutations}} + c \text{exRNA}_{\text{PCA3}} + d \text{PSA}$$

As can be noted, some of the above equations take
 5 into account also other circulating biomarkers correlated
 to the pathological condition, such as mutations in the
 circulating tumor DNA (ctDNA_{mutations}), circulating RNA
 (exRNA_{PCA3}) and proteins (PSA).

Many more variations on equations 1-3 are possible,
 10 but equations 1-3 will be applied here to illustrate the
 potential of BTL.

Any of these functions can be scaled and centered to
 achieve a range that is easiest to interpret for a
 clinician, e.g. from 0 to 1, or from 0 to 100.

15 Example 2 - Evaluation of BTL equations on cancer studies

BTL was applied to the study archives from studies in
 metastatic prostate (IMMC38), metastatic colorectal (CAIRO-
 2), and metastatic and non-metastatic breast cancer (IMMC01
 20 and IMMC26 respectively). No background was subtracted from
 the tdEV count in the present evaluation. Values of "a"
 were evaluated in the range of 0.1 to 10, representing an
 underweight of tdEV up to an overweight of tdEV in BTL. The
 hazard ratio for overall survival was evaluated using BTL
 25 as a continuous variable, but scaling the hazard ratio to
 the interquartile range to account for differences in
 scale. The results are shown in Figure 6, relative to the
 hazard ratio of operator/manual CTC.

The following should be noted.

- For all equations, there exists a range of values of the coefficient "a" for which the prognostic value of BTL is higher than the prognostic value of operator CTC (OP-
5 CTC).
- When the tdEV contribution is overweight, BTL is less prognostic. This was expected from the assumption that CTC are more important than tdEV.
- Studies with overall lower CTC concentration (non-
10 metastatic breast and metastatic colorectal) have more benefit from BTL.

Figure 7 shows the continuous hazard ratio for an optimal form of equations 1-3 and the hazard ratios (HR) for operator CTC. The vertical marker shows the HR, and the
15 horizontal bars indicate the 95% confidence interval (CI) on the HR. It should be noted that the 95% CI is affected by the study data (individual patient survivals and censoring) and the properties of BTL. It is reasonable to expect that the study data dominate the confidence
20 intervals.

HR is the change in risk due to an increase of the predictor variable by 1. Because of this, an equation $BTL = 0.5 * CTC$ would have a higher HR even though a simple scale change does not change the prognostic value of the CTC
25 count. To guard against such misinterpretations, the HR shown here is the continuous hazard ratio, scaled to the interquartile range for metastatic studies, and the 10-90 percentile for non-metastatic studies. With this scaling, the scale change equation mentioned above has the same HR
30 as the unscaled version.

The major impact of BTL is to expand the coverage of patients that have low CTC counts. When the hazard ratio is determined on dichotomized data (i.e. split on a value near the median), BTL is expected to have slightly lower hazard ratios because the noise on BTL will be higher than the noise on CTC alone.

The above results demonstrate the potential of the CDL parameter applied to tumors.

Example 3 - TME equations

The tumor marker expression (TME) may indicate a Tumor Marker Expression Fraction, TMEF, wherein the Tumor Marker Expression Fraction TMEF is calculated as:

$$TMEF = \frac{MEF_{RC}^{+} + a MEF_{FR}^{+}}{1 + a}$$

wherein MEF_{RC}^{+} is the fraction of cells of interest (RC) with positive marker expression, MEF_{FR}^{+} is the fraction of cellular fragments of interest (FR) with positive marker expression, and a is a coefficient that balances the contribution of cellular fragments of interest relative to cells of interest.

For example, with reference to the Her2+ biomarker, the Tumor Marker Expression Fraction TMEF may be:

$$Frac.Her2^{+} = \frac{Frac.Her2_{CTC}^{+} + a Frac.Her2_{tdEV}^{+}}{1 + a}$$

$$Frac.Her2^{+} = \frac{Frac.Her2_{CTC}^{+} + a Frac.Her2_{tdEV}^{+} + b ctDNA_{TOP2A}}{1 + a + b}$$

With $a = \frac{25}{(CTC+1)^2}$, Frac Her2+ the fraction of CTC / tdEV with an expression above a threshold value applicable for the Her2 marker expression, and $ctDNA_{TOP2A}$, the copy number

change in TOP2A as determined on circulating tumor DNA.

The tumor marker expression (TME) may indicate, in addition or in alternative to the Tumor Marker Expression Fraction, a Tumor Marker Expression Level, TMEL. The Tumor
5 Marker Expression Level may be calculated as:

$$TMEL = \frac{\frac{\sum_{j=1}^N MEL_{RCj}}{N_{RC}} + a \frac{\sum_{k=1}^N MEL_{FRk}}{N_{FR}}}{1 + a}$$

wherein MEL_{RC} is the marker expression intensity level
10 on the cells of interest (RC), MEL_{FR} is the marker expression intensity level on the cellular fragments of interest (FR), N_{RC} is the number of cells of interest, N_{FR} is the number of cellular fragments of interest, and a is a coefficient that balances the contribution of cellular
15 fragments of interest relative to cells of interest. The expression of the coefficient a is only an example and may be different from the illustrated value.

In other words, the TMEL may be considered as a weighted mean expression of a biomarker.

20 For example, with reference to the Her2+ biomarker, the Tumor Marker Expression Level TMEL may be:

$$Level\ Her2^{+} = \frac{\frac{\sum_{j=1}^N CTC_j I_{Her2j}}{N_{CTC}} + a \frac{\sum_{k=1}^N tdEV_k I_{Her2k}}{N_{tdEV}}}{1 + a} \text{ with } a = \frac{25}{(N_{CTC} + 1)^2}.$$

25 Combining information from multiple sources can fill information deficiencies. Tumor marker expression is increasingly used to guide therapy. However, in most cases

the only available tissue material available to determine expression was taken at the time of diagnosis, sometimes more than 10 years ago. The tumor has evolved since, and no longer expresses the same markers as before. A liquid
5 biopsy in the form of circulating material can provide a current assessment of marker expression. Circulating tumor cells are particularly interesting because they allow determination of proteins, DNA and RNA. However, this determination is obviously impeded if only a few CTC are
10 found in a single tube of blood. As with the CDL, combining information from multiple sources into a single parameter can fill the gaps where needed. Here we will show this for the expression of HER2 on CTC and tdEV. Other targets, and other circulating markers are possible. Figure 8 shows the
15 fraction and mean intensity of HER2 marker expression for CTCs only and for the Tumor Marker Expression (TME) parameter in 98 breast cancer patient samples.

Patients with a majority of CTC positive for HER2 may be susceptible to Trastuzumab, and patients with a majority
20 of CTC at least weakly positive may be susceptible to other HER2 targeting therapies. The left panels in figure 8 shows that, if assessed only on CTCs, the HER2 marker expression (both fraction and mean intensity) is not available in 40 out of 98 patient samples, because there are less than 3
25 CTCs (i.e. not enough CTCs to have a reliable measurement). On the other hand, if HER2 expression measurements on CTCs and tdEVs are combined as a single TME parameter, HER2 marker expression cannot be provided in only 11 out of 98 samples, where there are less than 3 CTCs and/or tdEVs
30 combined, as shown by the right panels in figure 8.

The method of the invention may also comprise a sorting step, which allows to isolate the cells of the organic fluid sample that have been identified as cells of interest (e.g., rare cells, in particular CTCs or fetal
5 cells) or as cellular fragments of interest (e.g., circulating cellular fragments, in particular tdEV) for performing downstream processing, including molecular analysis of the cells of interest (e.g. whole genome amplification, low-pass sequencing, next-generation
10 sequencing, Copy Number Variation, FISH, RT-PCR, qPCR, dPCR, STR analysis, Detection of Mutations, Gene Expression Profiling, etc.) or for culture and proliferation of the cells of interest. The sorting step may be performed by the DEPArray™ or by other known image-based cell sorters.

15

CLAIMS

1. An in-vitro method for diagnosing or assessing the progression of a disorder in an individual or assessing the
5 responsiveness/response to treatment in an individual, comprising:

- marking a sample of organic fluid of the individual with at least one marker specific for a cell of interest and a cellular fragment of interest, the marker/s being
10 identifiable by selective imaging,

- acquiring at least one image of the marked sample, wherein the image comprises at least one marker-stained object to be classified;

- classifying the marker-stained object; and
15 - determining whether the marker-stained object is a cell of interest or a cellular fragment of interest based on a result of the classification of the marker-stained object,

- counting a number of cells of interest and a number
20 of cellular fragments of interest, wherein the cell of interest is a circulating tumor cell (CTC) and the cellular fragment of interest is a tumor-derived extracellular vesicle (tdEV), or the cell of interest is a circulating endothelial cell (CEC) and the cellular fragment of
25 interest is an endothelial-derived extracellular vesicle (edEV), or the cell of interest is a circulating multiple myeloma cell (CMMC) and the cellular fragment of interest is a multiple myeloma extracellular vesicle (mmEV),

wherein counting the number of cells of interest and
30 the number of cellular fragments of interest comprises:

- increasing a cell of interest counter (CTC-CNT) in response to each marker-stained object being determined to be a cell of interest, and/or

- increasing a cellular fragment of interest counter (tdEV-CNT) in response to each marker-stained object determined to be a cellular fragment of interest;

- determining a circulating disease load (CDL) parameter that is a function of the difference of the value of the cell of interest counter (CTC-CNT) and/or the cellular fragment of interest counter (tdEV-CNT) with respect to their relative background level in a normal individual, wherein the function is

$$CDL = f(CTC - CTC_{BG}, tdEV - tdEV_{BG})$$

wherein

CTC is the number of cells of interest in the individual,

CTC_{BG} is the number of cells of interest in the normal individual,

tdEV is the number of cellular fragments of interest in the individual,

$tdEV_{BG}$ is the number of cellular fragments of interest in the normal individual; and

- correlating the circulating disease load (CDL) parameter with the presence or progression of the disorder or responsiveness/response to the treatment in the individual.

2. The method according to claim 1, wherein:

- the step of counting a number of cells of interest and a number of cellular fragments of interest also comprises counting a number of clusters of cells of

interest, wherein a cell of interest cluster counter (CTCcl-CNT) is increased in response to each marker-stained object determined to be a cell of interest cluster; and

- the circulating disease load (CDL) parameter is also a function of the number of clusters of cells of interest.

3. The method according to claim 1 or 2, wherein:

- the step of counting a number of cells of interest and a number of cellular fragments of interest also comprises counting at least one circulating biomarker correlated to the disorder; and

- the circulating disease load (CDL) parameter is also a function of the at least one circulating biomarker correlated to the disorder.

15

4. The method according to any of claims 1 to 3, wherein the step of marking the sample also comprises marking the sample with at least one marker specific for cells and/or cellular fragments other than the cell of interest and/or the cellular fragment of interest.

20

5. The method according to any of the preceding claims, wherein the circulating disease load (CDL) parameter ranges from a minimum value, e.g. 0, to a maximum value, e.g. 1, the circulating disease load (CDL) parameter being a monotonic function of the number of cells of interest, e.g. CTC, and of the number of cellular fragments of interest, e.g. tdEV, and, optionally, of the number of clusters of cells of interest.

30

6. The method according to any of the preceding claims,

wherein the CDL parameter depends on a transform of the number of cells of interest and/or a transform of the number of cellular fragments of interest and, optionally, a transform of the number of clusters of cells of interest, wherein the transform is for example a root function, a logarithmic function, a power function or a combination thereof.

10

7. The method according to any of claims 1 to 6, wherein

$$CDL = \log_{10} N_{RC} + a \log_{10} N_{FR}$$

15 wherein N_{RC} is the number of cells of interest, N_{FR} is the number of cellular fragments of interest, and a is a coefficient that balances the contribution of rare cell fragments relative to cells of interest.

20 8. The method according to any of claims 1 to 6, wherein the CDL parameter depends on a sigmoid function of the number of rare cell fragments.

25 9. The method according to any of claims 1-6, wherein

$$CDL = \log_{10} N_{RC} + 3a \left(\frac{2}{1 + e^{-\log_{10} N_{FR}}} - 1 \right)$$

wherein N_{RC} is the number of cells of interest, N_{FR} is the number of cellular fragments of interest, and a is a

coefficient that balances the contribution of cellular fragments of interest relative to cells of interest.

10. The method according to any of claims 1-6, wherein

$$CDL = \log_{10} N_{RC} + a \log_{10} \frac{N_{FR}}{N_{RC} + 1}$$

wherein N_{RC} is the number of cells of interest, N_{FR} is the number of cellular fragments of interest, and a is a coefficient that balances the contribution of cellular fragments of interest relative to cells of interest.

11. The method according to any of claims 1-6, wherein

$$CDL = \frac{llg(N_{RC}) + llg(N_{FR})}{2}$$

wherein $llg(x) = \left(\frac{1}{1 + e^{-(shape \ln(x+1) - shift)}} - \alpha \right) / (1 - \alpha)$

with $\alpha = 1/(1 + e^{shift})$

wherein N_{RC} is the number of cells of interest, N_{FR} is the number of cellular fragments of interest, and llg is the log-logistic function with *shape* and *shift* parameters for CTC and tdEV obtained separately from fitting the corresponding distributions.

12. The method according to any of claims 1 to 11, wherein the CDL parameter depends on the number of cellular fragments of interest scaled by the number of cells of interest.

13. The method of any of the preceding claims, wherein the disorder is a tumor.

14. The method of any of the preceding claims, wherein

5 - the cells of interest are circulating tumor cells (CTCs) and the fragments are tumor-derived extracellular vesicles (tdEVs), the disorder being carcinoma or melanoma; or

- the cells of interest are circulating multiple myeloma cells (CMMCs) and the fragments are multiple myeloma

10 extracellular vesicles (mmEVs), the disorder being myeloma; or

- the cells of interest are circulating endothelial cells (CECs) and the fragments are endothelial-derived extracellular vesicles (edEV), the disorder being

15 cardiovascular disease.

15. The method according to any of claims 1 to 13, wherein the cells of interest are CTC and CEC and the fragments are tdEV and edEV, the disorder being colorectal

20 cancer.

16. The method according to claim 1, further comprising the steps of:

- assessing expression of at least one biomarker,

25 which is a target for therapy of the disorder, in the cells of interest and/or in the cellular fragment of interest;

- determining a tumor marker expression (TME) parameter as a function of the assessed biomarker expression;

30 - correlating the TME parameter with the

responsiveness to treatment in the individual.

17. The method according to claim 16, wherein the biomarker is selected from the group consisting of Her2,
5 PSMA, MUC1, EGFR, ER, PR, KI67, PDL1, RAS, RAF, ROS1, KRAS, BRAF, MET, RET and PARP.

18. The method according to claim 16 or 17, wherein the tumor marker expression (TME) indicates a Tumor Marker
10 Expression Fraction, TMEF, the Tumor Marker Expression Fraction being:

$$TMEF = \frac{MEF_{RC}^{+} + a MEF_{FR}^{+}}{1 + a}$$

wherein, MEF_{RC}^{+} is the fraction of cells of interest
15 (RC) with positive marker expression, MEF_{FR}^{+} is the fraction of cellular fragments of interest (FR) with positive marker expression, and a is a coefficient that balances the contribution of cellular fragments of interest relative to cells of interest.

20

19. The method according to any of claims 15-17, wherein the tumor marker expression (TME) indicates a Tumor Marker Expression Level, TMEL, the Tumor Marker Expression
25 Level being:

$$TMEL = \frac{\frac{\sum_{j=1}^N MEL_{RCj}}{N_{RC}} + a \frac{\sum_{k=1}^N MEL_{FRk}}{N_{FR}}}{1 + a}$$

wherein MEL_{RC} is the marker expression intensity level

on the cells of interest (RC), MEL_{FR} is the marker expression intensity level on the cellular fragments of interest (FR), N_{RC} is the number of cells of interest, N_{FR} is the number of cellular fragments of interest, and a is a
5 coefficient that balances the contribution of cellular fragments of interest relative to cells of interest.

20. The method according to any of claims 3 to 19, wherein the circulating biomarker correlated to the
10 disorder is at least one circulating protein, an RNA, a DNA, or combinations thereof.

21. The method according to any of the preceding claims, further comprising, by an image-based cell sorter,
15 sorting at least one of the cells that has been determined to be a cell of interest or a cellular fragment of interest.

22. The method according to the preceding claim,
20 wherein the step of sorting is for downstream molecular analysis or for culture and proliferation of the sorted cells.