

NOVEL PROCESS

Field

- 5 The present invention relates to process for the isolation of circulating tumour cell (CTC) clusters and/or circulating tumour cell-white blood cell (CTC-WBC) clusters comprising *inter alia* microfluidic separation of an apheresis sample.

Background

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Cancer is a leading cause of death worldwide, accounting for about 10 million deaths in 2020 (Ferlay et al. 2020). In particular, it is uncontrolled metastatic spread of cancer in patients which leads to death in over 90% of cases. Accordingly, there is a significant unmet clinical need for therapies with particular efficacy in cases of metastatic cancer. There is substantial
15 interest in precision oncology which aims to address the aforementioned need via identification of disease-tailored biomarkers which may in turn be targeted by highly specific, highly effective and minimally toxic treatments, in particular immune-stimulating treatments, such as cancer vaccines and adoptive transfer of immune cells.

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Precision oncology techniques are currently hampered by the insufficient information that can be retrieved using current diagnostic techniques, both in relation to characteristics of tumour cells themselves and in relation to the specific tumour-reactive immune cells in each specific patient. Furthermore, the interaction between tumour cells and tumour-reactive remain cells during cancer dissemination and metastatic spread remains largely uncharacterised (Lambert
25 et al. 2017).

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Circulating tumour cells (CTCs) are tumour-derived cells which are found in the bloodstream and which function as the precursors of metastasis in a number of cancer types, for example breast and lung carcinomas (Aceto et al. 2014; Pantel and Speicher, 2016). Although rare in the circulation relative to single CTCs, CTC clusters (i.e. clusters of single CTCs) have a 23- to 50-fold increased metastatic potential (Aceto et al. 2014). In some cases, CTCs are found in the bloodstream in association with non-malignant cells, and in particular in association with white blood cells (WBCs). Such CTC-WBC clusters have differential expression of specific genes relative to single CTCs alone which support cell cycle progression and consequently
35 enhance the metastatic potential of CTCs (Szczerba et al. 2019).

Utilising CTCs for analysis in liquid biopsies is therefore associated with unique advantages in the clinical context, and it is particularly desirable to ensure isolation and subsequent processing of CTC clusters and/or CTC-WBC clusters since single CTCs within such clusters have high intrinsic metastatic potential. Obtaining a detailed understanding of these metastatic precursors will delimit precision oncology techniques and facilitate development of therapeutics for highly metastatic cancer treatment. For example, a substantial number of CTCs from CTC clusters and/or CTC-WBC clusters would be required to allow sequencing and genotyping techniques to cover the entire sub-clonal heterogeneity of a systemic cancer within a single patient. Pooling such data could then develop a library of CTC markers for use in diagnosis. Alternatively, said data could produce a library of putative novel drug targets, for particular use against CTCs with a high intrinsic metastatic potential.

CTCs from CTC clusters and/or CTC-WBC clusters, can be harvested from small samples of peripheral blood from cancer patients, since the collection procedure is well-tolerated and such blood is easily accessible via the venepuncture process of a peripheral blood vessel. Harvesting CTCs from CTC clusters and/or CTC-WBC clusters in peripheral blood reduces sampling bias relative to organ biopsy techniques since it may provide a representation of tumour cells from all body sites with access to the circulation, including capture of aggressive cancer cells with high metastatic potential.

However, realising the aforementioned advantages of utilising CTCs from CTC clusters and/or CTC-WBC clusters for analysis in liquid biopsies requires a substantial number of CTCs from CTC clusters and/or CTC-WBC clusters to be isolated from a patient with metastatic cancer, so as to provide thorough coverage of the sub-clonal, heterogeneity of a systemic cancer. Indeed, this will enable identification of a large number of metastasis-relevant mutations and tumour characteristics, e.g. tumour-associated antigens and disease-relevant biomarkers, which may serve as a starting point for precision oncology techniques.

Despite the recent implementation of sensitive techniques and protocols for the purification and profiling of individual isolated CTCs, actually isolating such a large number of CTCs from peripheral blood samples is very unlikely due to the rarity of said CTCs in any small peripheral blood sample. This problem is only exacerbated in the context of isolating CTC clusters and/or CTC-WBC clusters, which are desirably isolated due to their enhanced metastatic potential, since said clusters are yet rarer still. Ultra-low harvesting yields are therefore a significant limitation to the potential use of CTCs from CTC clusters and CTC-WBC clusters in precision oncology.

There is therefore a significant need for the development of techniques which enable the isolation of very high numbers of CTC clusters and/or CTC-WBC clusters from patients with metastatic cancer.

5 **Summary of the Invention**

The present invention provides, *inter alia*, a process for the isolation of circulating tumour cell (CTC) clusters and/or circulating tumour cell-white blood cell (CTC-WBC) clusters comprising:

- (i) collecting an apheresis sample from a patient;
 - (ii) debulking the apheresis sample of excess white blood cells;
 - 10 (iii) separating CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample using microfluidic separation; and
 - (iv) isolating separated CTC clusters and/or CTC-WBC clusters;
- wherein the debulking step comprises a flow-based process where flow is driven by gravity and/or minimal applied artificial pressure; wherein the microfluidic separation is performed
- 15 using a microfluidics device capable of separating CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample.

Brief Description of the Figures

- 20 Figure 1 shows, in Panel A, a fluorescence micrograph of a heterotypic cluster i.e. a circulating tumour cell-white blood cell (CTC-WBC) cluster isolated from a patient via the process of the present invention, comprising larger CTCs, labelled with anti-EPCAM, anti-HER2, and anti-EGFR antibodies, and smaller white blood cells (WBCs), labelled with anti-CD45 antibodies. Figure 1 also shows, in Panel B, a graphical illustration of said heterotypic cluster i.e. a
- 25 CTC-WBC cluster comprising CTCs (striped area) and WBCs (dotted area) (see Examples 1 and 2).

- Figure 2 shows, in Panel A, a fluorescence micrograph of homotypic clusters i.e. circulating tumour cell (CTC) clusters isolated from a patient via the process of the present invention, comprising CTCs labelled with anti-EPCAM, anti-HER2, and anti-EGFR antibodies. Figure 2
- 30 also shows, in Panel B, a graphical illustration of said homotypic cluster i.e. CTC cluster comprising CTCs (striped areas) (see Examples 1 and 2).

- Figure 3 shows the quantification of individual circulating tumour cells (CTCs), CTC clusters, and CTC-WBC clusters isolated from a patient via the process of the present invention. Quantification of individual CTCs, CTC clusters, and CTC-WBC clusters isolated from said
- 35 patient is expressed as both an absolute number (i.e. count) of said components, and as a

percentage of the total number of individual CTCs, CTC clusters, and CTC-WBC clusters isolated from said patient (N=8280) (see Example 1 and 2).

Figure 4 shows a FACS contour plot which describes the distribution of the cell population, including any circulating tumour cells (CTCs) and CTC clusters (see boxed areas), in a leukapheresis sample isolated from a patient both pre- (left-hand panel) and post- (right-hand panel) the novel debulking process developed by the present inventors.

Figure 5 shows a FACS contour plot which describes the distribution of the cell population, including any circulating tumour cells (CTCs) and CTC clusters (see boxed area), in a leukapheresis sample isolated from a patient, and debulked using the novel debulking process developed by the present inventors (lower-left panel). Figure 5 further shows a FACS contour plot which characterises the light scattering properties of the CTC and the CTC cluster populations, via a gated sub-analysis which distributes cells according to SSC-a (side light scatter area) and FSC-a (forward light scatter area), resulting in size-related discrimination of putative CTC clusters (see demarcated area of upper-right panel).

Figure 6 shows brightfield micrographs which demonstrate the different effect of performing the process of debulking the apheresis sample of excess white blood cells, both under gravity and using pressure-forced separation, on homotypic clusters i.e. circulating tumour cell (CTC) clusters of metastatic breast cancer cells (see Examples 1 and 4).

Figure 7 shows, brightfield micrographs and fluorescence micrographs, wherein circulating tumour cells (CTCs) were enriched through a microfluidic device and immuno-stained with anti-EPCAM, anti-HER2, and anti-EGFR antibodies, and white blood cells (WBCs) were immuno-stained with anti-CD45 antibodies, which demonstrate the suitability of each of a peripheral blood sample, an apheresis sample, and a debulked apheresis sample for microfluidic separation, and which demonstrate the resulting CTC, CTC cluster and CTC-WBC cluster yield from said microfluidic separation (see Examples 1 and 5).

Detailed Description of the Invention

The process of the present invention is a process for the isolation of circulating tumour cell (CTC) clusters and/or circulating tumour cell-white blood cell (CTC-WBC) clusters.

As used herein, the term circulating tumour cell (CTC) relates to a cell that originates from a tumour (i.e. a cancerous tumour) and subsequently enters the bloodstream, typically prior to

seeding metastasis. A circulating tumour cell (CTC) may originate from an initial, primary, tumour, or a secondary tumour which is itself a result of an established metastasis.

As used herein, the term CTC cluster relates to a group of two or more associated, i.e. aggregated, CTCs. Typically, a CTC cluster will comprise between about 2 and about 100 CTCs, such as between about 2 and about 50 CTCs.

As used herein, the term CTC-WBC (white blood cells) clusters relates to an association of at least one CTC and at least one white blood cell (WBC). In particular, a CTC-WBC cluster will comprise two or more, for example four or more, six or more, eight or more, or ten or more CTCs and one or more, for example two or more, three or more, four or more, or five or more WBCs. Typically, a CTC-WBC cluster will comprise between about 2 and about 100 CTCs, such as between about 2 and about 50 CTCs, and between about 1 and about 10 WBCs, such as between about 1 and about 5 WBCs.

Suitably, a white blood cell (WBC), or leukocyte, found in a CTC-WBC clusters as defined herein may be a cell of either the myeloid (e.g. basophils, eosinophils, monocytes, neutrophils) or lymphoid (lymphocytes) lineage. In particular, a WBC found in a CTC-WBC cluster as defined herein is a cell of the myeloid lineage. For example, a WBC found in a CTC-WBC cluster as defined herein is a neutrophil.

The process of the present invention first comprises step (i), of collecting an apheresis sample from a patient. Use of apheresis sample appears to lead to a higher yield of CTC and CTC-WBC clusters than using peripheral blood (see Figure 7).

As used herein, the process of apheresis relates to an extracorporeal technique in which the blood of a patient is passed out of the body and through an apparatus which separates one particular component of the blood from the remaining components, retaining said particular component of the blood in the apparatus and returning the remaining components to the body of the patient. The skilled person would understand that the process of apheresis could utilise any apparatus or be modified in any way so as to capture any particular component of the blood, or more efficiently capture any particular component of the blood.

Examples of apheresis include plasmapheresis, for separating plasma from the remaining components of the blood, erythrocytapheresis, for separating red blood cells from the remaining components of the blood, and leukapheresis, for separating white blood cells

(WBCs), or other mononucleated cells, such as stem cells, from the remaining components of the blood.

5 In one embodiment of the present invention, the apheresis sample of step (i) is a sample obtained under conditions of leukapheresis, i.e. it is a leukapheresis sample, i.e. it is the component retained as a result of the process of leukapheresis. Suitable conditions of leukapheresis facilitate the separation of white blood cells (WBCs), or other mononucleated cells, such as stem cells, from the remaining components of the blood on the basis of their physical characteristics, such as their size, shape and density. In particular, suitable conditions
10 of leukapheresis facilitate the separation of white blood cells (WBCs), or other mononucleated cells, such as stem cells, from the remaining components of the blood on the basis of their density. Accordingly, the conditions of leukapheresis, or the process of leukapheresis, in addition to facilitating the retention of WBCs, facilitate the retention of mononucleated cells that possess a density similar to WBCs, in particular CTCs and/or CTC clusters and/or CTC-
15 WBC clusters, in the leukapheresis sample.

Suitably, the conditions of leukapheresis comprise conditions of density gradient centrifugation, i.e. the leukapheresis sample may be the component retained as a result of the process of density gradient centrifugation, i.e. the process of leukapheresis may be a process
20 of density gradient centrifugation.

For example, the conditions of leukapheresis may comprise conditions of rate-zonal centrifugation or isopycnic centrifugation, i.e. the leukapheresis sample may be the component retained as a result of the process of rate-zonal centrifugation or isopycnic separation, i.e. the
25 process of leukapheresis may be a process of rate-zonal centrifugation or isopycnic centrifugation. In particular, the conditions of leukapheresis may comprise conditions of polymeric density separation, i.e. the leukapheresis sample may be the component retained as a result of the process of polymeric density separation, i.e. the process of leukapheresis may be a process of polymeric density separation.

30 Alternatively, the conditions of leukapheresis may comprise differential centrifugation, i.e. the leukapheresis sample may be the component retained as a result of the process of differential centrifugation, i.e. the process of leukapheresis may be a process of differential centrifugation.

35 Alternatively, suitable conditions of leukapheresis facilitate the separation of white blood cells (WBCs), or other mononucleated cells, such as stem cells, from the remaining components of the blood on the basis of their size. For example, the conditions of leukapheresis may

comprise conditions of filtration, i.e. the leukapheresis sample may be the component retained as a result of the process of filtration, i.e. the process of leukapheresis may be a filtration process.

5 Suitably, the apheresis sample of step (i) has a volume of between about 10 and about 300 mL, for example between about 25 and about 250 mL, for example between about 50 and about 250 mL, for example between about 50 and about 200 mL, for example between about 50 and about 150 mL, for example about 100 mL.

10 Suitably, the apheresis sample of step (i) is collected over a period of between about 30 and about 240 minutes, for example between about 60 and about 180 minutes, for example between about 90 and about 180 minutes, for example about 120 minutes.

Suitably, the apheresis sample of step (i) is collected using an apparatus suitable for
15 apheresis. For example, the apheresis sample of step (i) is collected using a Spectra Optia® apparatus, COBE® Spectra apparatus or similar.

The process of the present invention further comprises step (ii) of debulking the apheresis sample of excess white blood cells. Use of a debulking step appears to render the apheresis
20 sample suitable for microfluidic separation and ultimately lead to a higher yield of CTC and CTC-WBC clusters than a corresponding process without said debulking step (see Figure 7).

As used herein, the term debulking (e.g. of an apheresis sample) refers to the removal of specific, typically unwanted, components (e.g. excess white blood cells, or other
25 mononucleated cells, in particular excess white blood cells) from a starting material (e.g. an apheresis sample) to both concentrate the remaining desired material (e.g. CTC clusters and/or CTC-WBC clusters) and avoid or at least reduce the downstream processing of said specific unwanted components.

30 As used herein, the term excess in relation to white blood cells (WBCs) refers to white blood cells which are not of relevance to the present process of isolating CTC clusters and/or CTC-WBC clusters, in particular since they are not a component of said CTC clusters and/or CTC-WBC clusters. Typically, excess white blood cells are individual, i.e. single; not clustered, white blood cells, e.g. basophils, eosinophils, monocytes, neutrophils, or lymphocytes. The term
35 excess may further refer to small groups of associated white blood cells, i.e. clusters of WBCs.

It should be noted that as used herein, the term excess in relation to white blood cells does not refer to white blood cells which are associated with CTCs within CTC-WBC clusters. However, the skilled person would understand that step (ii), of debulking the apheresis sample of excess white blood cells, of the present process may nevertheless cause the debulking of
5 some CTC-WBC clusters, in particular small CTC-WBC clusters in which a white blood cell is exposed on the outer surface of the cluster, due to the methods used.

In some embodiments, the apheresis sample of step (i) may contain red blood cells, and in particular may contain visible red blood cells. Therefore, in one embodiment the process of
10 the present invention suitably comprises a further step (i)(a), between the steps (i) and (ii), of debulking the apheresis sample of red blood cells. This step (i)(a) is suitably included when the apheresis sample of step (i) contains red blood cells. More suitably, the step (i)(a) is included when the apheresis sample of step (i) contains visible red blood cells.

15 In one embodiment, the step (i)(a) of debulking the apheresis sample of red blood cells comprises lysing red blood cells. For example, debulking the apheresis sample of red blood cells may suitably comprise incubating the apheresis sample of step (i) with a chemical capable of lysing red blood cells, such as ammonium chloride.

20 In one embodiment, the debulking step of (ii) comprises a process of capture of excess white blood cells. For example, in one embodiment, the debulking step (ii) comprises the capture of excess white blood cells using a capture device.

In one embodiment, the capture device comprises an immobilised affinity label which is
25 capable of binding to one or more antigens on the surface of said excess white blood cells.

In another embodiment, the debulking step (ii) comprises the step of treating the apheresis sample with a bifunctional molecule having a first functional component which is capable of binding to an antigen on the surface of said excess white blood cells and a second functional
30 component which is capable of binding the capture device.

For example, the first functional component is an affinity label which is capable of binding to an antigen on the surface of said excess white blood cells and the second functional component is an affinity label which is capable of binding to an antigen immobilised on the
35 capture device. Alternatively, the first functional component is an affinity label which is capable of binding to an antigen on the surface of said excess white blood cells and the second functional component is an antigen which is capable of binding an affinity label immobilised

on the capture device. Alternatively, the first functional component is an affinity label which is capable of binding to an antigen on the surface of said excess white blood cells and the second functional component is a magnetic component (such as a magnetic bead) which is capable of binding a ferrous component of the capture device.

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The first and second functional components may be joined by a linker (e.g. a peptide linker). In a first embodiment, the affinity label is an antibody protein. As used herein, the term "antibody protein" refers to any antibody, antibody fragment, or fusion protein comprising one or more antibody fragments, or a derivative of any of the aforementioned which is capable of
10 binding to an antigen, preferably immunospecifically. The term antibody protein includes monoclonal antibodies, which can be, for example, mammalian (e.g. murine or camelid) or avian, chimeric, for example, human/mouse or human/primate chimeras, humanized antibodies or fully human antibodies. The term antibody protein includes 4-chain antibodies which comprise 2 light and 2 heavy chains and heavy chain only antibodies (e.g. from
15 camelids) which comprise 2 heavy chains. The term antibody protein further embraces an immunoglobulin, such as IgG, including IgG1, IgG2, IgG3 or IgG4, IgM, IgA, such as IgA1 or IgA2, IgD, IgE or IgY. The term antibody protein also includes single chain antibodies, antibody fragments such as Fc, Fab, and ScFv fragments and the like, and fragments of heavy chain only antibodies such as VHHs.

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In an alternative embodiment, the affinity label is a small molecule capable of binding to an antigen on the surface of excess white blood cells. As used herein, the term "small molecule" defines a molecule with a molecular weight of about 1200 g/mol or less, for example about 1000 g/mol or less, such as about 900 g/mol or less such as about 800 g/mol or less. Typically,
25 a small molecule has a molecular weight of about 100 g/mol or more, for example about 200 g/mol or more, such as about 300 g/mol or more. Therefore, a small molecule may have a molecular weight of about 100-1200 g/mol, for example about 200-1000 g/mol, for example about 300-800 g/mol.

30 In an embodiment, the capture device is a chromatographic device, such as a device comprising a chromatographic column. A molecule such as an antigen or affinity label which is immobilised on the capture device may be bound to the stationary phase of the chromatographic device e.g. chromatography column. When the capture device has a ferrous component for binding to a second functional component which is a magnetic component, the
35 stationary phase of the chromatographic device e.g. chromatography column may comprise or consist of said ferrous component.

Other examples of a capture device include a centrifugation device, a filtration device or a microfluidics device.

5 In one preferred embodiment, the debulking step of (ii) comprises a process of magnetic capture of excess white blood cells. In one embodiment, the debulking step (ii) comprises the magnetic capture of excess white blood cells magnetically labelled via one or more antigens on the surface of said excess white blood cells as a result of a prior step of treating the apheresis sample with affinity labelled magnetic particles, wherein the affinity labelled magnetic particles are capable of binding to one or more antigens on the surface of excess
10 white blood cells.

The affinity label of the affinity labelled magnetic particles is a molecule capable of binding to an antigen on the surface of excess white blood cells. The affinity labelled magnetic particles may, individually or collectively, comprise more than one different affinity label each of which
15 binds a different antigen on the surface of excess white blood cells.

In a preferred embodiment, a molecule capable of binding to an antigen on the surface of excess white blood cells, for example the affinity label of the affinity labelled magnetic particles, is an antibody protein capable of binding to an antigen on the surface of excess
20 white blood cells.

In one embodiment, when the debulking step of (ii) comprises a process of magnetic capture of excess white blood cells, the magnetic particles of the affinity labelled magnetic particles are magnetic beads. In one embodiment, the magnetic particles of the affinity labelled
25 magnetic particles comprise ferrous components, and optionally further comprise derivatives covalently bound to the particles. In one embodiment, the magnetic particles of the affinity labelled magnetic particles do not comprise agarose.

Suitably, the magnetic particles of the affinity labelled magnetic particles have a diameter
30 between about 10 and about 100 nm, for example between about 25 and about 75 nm, for example about 50 nm.

Suitably, when the debulking step of (ii) comprises a process of capture, in particular magnetic capture, of excess white blood cells the one or more antigens on the surface of excess white
35 blood cells are selected from the group consisting of CD45, CD31, CD16, and CD11b. More suitably, the one or more antigens on the surface of excess white blood cells are selected from the group consisting of CD45, CD31, and CD11b.

In one embodiment, when the debulking step of (ii) comprises a process of capture, in particular magnetic capture, of excess white blood cells, excess white blood cells are bound by a molecule, and in particular are magnetically labelled, via one antigen on the surface of said excess white blood cells. Suitably, the one antigen on the surface of said excess white blood cells is selected from the group consisting of CD45, CD31, CD16, and CD11b, and in particular is selected from the group consisting of CD45, CD31, and CD11b. For example, the one antigen on the surface of said excess white blood cells is CD45.

10 In a preferred embodiment, when the debulking step of (ii) comprises a process of capture, in particular magnetic capture, of excess white blood cells, excess white blood cells are bound by a molecule, and in particular are magnetically labelled, via two or more, for example two or three, antigens on the surface of said excess white blood cells. Suitably the two or more antigens on the surface of said excess white blood cells are selected from the group consisting of CD45, CD31, CD16, and CD11b, and in particular are selected from the group consisting of CD45, CD31, and CD11b. For example, the two or more antigens on the surface of said excess white blood cells are CD45 and one or more further antigens selected from the group consisting of CD31 and CD11b. In a particularly preferred embodiment, when the debulking step of (ii) comprises a process of capture, in particular magnetic capture, of excess white blood cells, excess white blood cells are bound by a molecule, and in particular are magnetically labelled via two antigens on the surface of said excess white blood cells. For example, the two antigens on the surface of said excess white blood cells are selected from the group consisting of CD45, CD31, and CD11b. For example, the two antigens on the surface of said excess white blood cells are CD45 and a further antigen selected from the group consisting of CD31 and CD11b.

In one embodiment, the debulking step of (ii) comprises a flow-based process. For example, in one embodiment, the debulking step of (ii) comprises a process of capture of excess white blood cells, wherein the capture process comprises a flow-based process. In particular, in one embodiment, the debulking step of (ii) comprises a process of magnetic capture of excess white blood cells, wherein the magnetic capture process comprises a flow-based process.

Suitably, said flow-based process is performed under minimal pressure and in particular does not involve the application of artificial pressure to drive flow in the flow-based process. As used here in applied "artificial pressure" means a pressure applied to the system by artificial means, such as by a mechanical device such as a pump. Pumps include vacuum pumps, peristaltic

pumps, compressor pumps and the like. Applied artificial pressure is to be distinguished from pressure resulting from the natural force of gravity.

Thus, suitably, the flow-based process is not accelerated (or decelerated) by the application of artificial pressure to the ongoing flow-based process under gravity, such as by pumping, compressing or expanding the flow vessel, applying pressure to the input sample via a syringe plunge etc.

Therefore, in a preferred embodiment, the debulking step of (ii) comprises a flow-based process where flow is driven by gravity and/or minimal applied artificial pressure. For example, in one embodiment, the debulking step of (ii) comprises a process of capture of excess white blood cells, wherein the capture process comprises a flow-based process where flow is driven by gravity. In particular, in one embodiment, the debulking step of (ii) comprises a process of magnetic capture of excess white blood cells, wherein the magnetic capture process comprises a flow-based process where flow is driven by gravity. That is, in a preferred embodiment, the debulking step of (ii) comprises a flow-based process which does not involve the application of any significant (or any) artificial pressure to the ongoing flow-based process. For example, the debulking step of (ii) comprises a process of capture of excess white blood cells, wherein the capture process comprises a flow-based process which does not involve the application of artificial pressure to the ongoing flow-based process. In particular, in a preferred embodiment, the debulking step of (ii) comprises a process of magnetic capture of excess white blood cells, wherein the magnetic capture process comprises a flow-based process which does not involve the application of artificial pressure to the ongoing flow-based process.

Thus, the flow-based process may be performed under minimal applied artificial pressure, such as an applied artificial pressure of not more than 15 psi, for example between 2 and 12 psi, for example between 5 and 12 psi, such as about 10 psi. Minimal applied artificial pressure may be applied via a pump such as a vacuum pump. The flow-based process may be performed using minimal applied artificial pressure without gravity or with gravity. However suitably the flow-based process is driven by gravity without application of artificial pressure i.e. no artificial pressure is applied beyond that imposed by natural gravity force.

In one embodiment, the flow-based process of the debulking step of (ii) is a chromatographic process. In particular, the flow-based process of the debulking step of (ii) is a chromatographic process wherein the stationary phase in the chromatography is capable of preventing the elution of excess white blood cells. For example, in one embodiment, the flow-based process

of the debulking step of (ii), which comprises a process of capture of excess white blood cells, is a chromatographic process, in particular wherein the stationary phase in the chromatography binds i.e. captures, and thus prevents elution of, excess white blood cells. In particular, in a preferred embodiment, the flow-based process of the debulking step of (ii),
5 which comprises a process of magnetic capture of excess white blood cells, is a chromatographic process, in particular wherein the stationary phase in the chromatography binds i.e. captures (i.e. magnetically captures), and thus prevents elution of, magnetically labelled excess white blood cells. For example, the stationary phase in the chromatography is capable of binding to magnetically labelled excess white blood cells i.e. capturing and thus
10 preventing elution of said bound magnetically labelled excess white blood cells by the flow of the flow-based process, wherein said flow is driven by gravity and/or minimal applied artificial pressure. The gravity-driven flow (with minimal and preferably no applied artificial pressure), appears to be beneficial to the process because it helps preserve, i.e. preserve the structure of, the CTC and/or CTC-WBC clusters and avoids that they be broken down to individual cells
15 (see Figure 6). Suitably, when the debulking step of (ii) comprises a process of magnetic capture of excess white blood cells, the stationary phase comprises ferrous components which bind to magnetically labelled excess white blood cells. Suitably, binding between the ferrous components of the stationary phase and magnetically labelled excess white blood cells prevents elution by the flow of the flow-based process, in particular wherein said flow is driven
20 by gravity and/or minimal applied artificial pressure.

In one embodiment, the flow-based process of the debulking step of (ii) is a chromatographic process, wherein the mobile phase in the chromatography is a liquid. Suitably, the mobile phase is a liquid comprising phosphate-buffered saline and/or bovine serum albumin and/or
25 ethylenediaminetetraacetic acid (EDTA). Suitably, the mobile phase is a liquid further comprising a kinase inhibitor, for example a Rho kinase inhibitor.

In one embodiment, the flow-based process of the debulking step of (ii) is a chromatographic process that operates under saturating conditions.
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In one embodiment, the chromatographic process is a column-based chromatographic process. Suitably, in such a column-based chromatographic process, the apheresis sample is passed through a column by a flow-based process where flow is driven by gravity and/or minimal applied artificial pressure. In one embodiment, when the chromatographic process is
35 a column-based chromatographic process, the column is loaded at maximal capacity such that the column-based chromatographic process operates under saturating conditions.

In one embodiment, the debulking step of (ii) is optimised for the debulking of excess white blood cells. In particular, in one embodiment, the debulking step of (ii) is optimised for the debulking of individual white blood cells. For example, the debulking step of (ii), which comprises a process of capture of excess white blood cells, is optimised for the capture of excess white blood cells. For example, the debulking step of (ii), which comprises a process of capture of excess white blood cells, is optimised for the capture of individual white blood cells. In particular, in one embodiment, the debulking step of (ii), which comprises a process of magnetic capture of excess white blood cells, is optimised for the magnetic capture of excess white blood cells. In particular, in one embodiment, the debulking step of (ii), which comprises a process of magnetic capture of excess white blood cells, is optimised for the magnetic capture of individual white blood cells.

In one embodiment, the flow-based process of the debulking step of (ii) is optimised for the debulking of excess white blood cells, in particular individual white blood cells. For example, the flow-based process of the debulking step of (ii), which comprises a process of capture of excess white blood cells, is optimised for the capture of excess white blood cells, in particular individual white blood cells. In particular, the flow-based process of the debulking step of (ii), which comprises a process of magnetic capture of excess white blood cells, is optimised for the magnetic capture of excess white blood cells, in particular individual white blood cells. In one embodiment, the flow-based process of the debulking step of (ii) is a chromatographic process which is optimised for the debulking of excess white blood cells, in particular individual white blood cells. For example, the flow-based process of the debulking step of (ii), which comprises a process of capture of excess white blood cells, is a chromatographic process which is optimised for the capture of excess white blood cells, in particular individual white blood cells. In particular, the flow-based process of the debulking step of (ii), which comprises a process of magnetic capture of excess white blood cells, is a chromatographic process which is optimised for the magnetic capture of excess white blood cells, in particular individual white blood cells. In a particular embodiment, the flow-based process of the debulking step of (ii) is a column-based chromatographic process which is optimised for the debulking of excess white blood cells, in particular individual white blood cells. For example, the flow-based process of the debulking step of (ii), which comprises a process of capture of excess white blood cells, is a column-based chromatographic process which is optimised for the capture of excess white blood cells, in particular individual white blood cells. In particular, the flow-based process of the debulking step of (ii), which comprises a process of magnetic capture of excess white blood cells, is a column-based chromatographic process which is optimised for the magnetic capture of excess white blood cells, in particular individual white blood cells. A column-based chromatographic process is suitable for the debulking of excess white blood cells, in particular

individual white blood cells, because the column comprises a stationary phase e.g. matrix beads, which provide a particularly suitable surface for binding excess i.e. individual white blood cells. The column-based chromatographic process may be optimised for the debulking of excess white blood cells, in particular individual white blood cells, by loading the column at
5 maximal capacity such that the column-based chromatographic process operates under saturating conditions.

Suitably, the debulking step of (ii) takes no longer than about 150 minutes, for example no longer than about 120 minutes. For example, the debulking step of (ii) may take between about
10 30 and about 150 minutes, for example between about 60 and 150 minutes, for example between about 90 and 150 minutes, for example about 120 minutes.

Most suitably, the debulking step of (ii) is entirely distinct, i.e. separate, from step (iii) of separating CTC clusters and/or CTC-WBC clusters from other components of the debulked
15 apheresis sample using microfluidic separation (see Example 1 below). For example, when step (iii) is performed using a microfluidics device, the debulking step of (ii) is not performed using a microfluidics device, and instead uses an entirely distinct, i.e. separate, apparatus. Most preferably, the debulking step of (ii) is not performed using a microfluidics device. Indeed, as described in Examples 1 and 5 below, the debulking step (ii) has an important role in
20 rendering an apheresis sample suitable for use in a microfluidics device and so suitable for step (iii) of separating CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample using microfluidic separation.

The process of the present invention further comprises step (iii) of separating CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample using
25 microfluidic separation. Suitably, the microfluidic separation of (iii) is performed using a microfluidics device capable of separating CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample.

As noted above, the microfluidic separation of step (iii) is for separating CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample. As used herein, other components of the debulked apheresis sample, i.e. components which are not CTC clusters or CTC-WBC clusters, may include both cellular and non-cellular components. For example, other cellular components of the debulked apheresis sample may include, but are
35 not limited to, red blood cells, excess, i.e. individual, white blood cells (WBCs), and single circulating tumour cells (CTCs). For example, other non-cellular components of the debulked apheresis sample may include, but are not limited to, free antibodies, blood proteins, plasma,

- and platelets. It will be understood by the skilled person that although the process of apheresis is for the separation of one particular component of the blood from the remaining components, the process may be inefficient such that the apheresis sample nevertheless contains a small amount of the remaining components. Similarly, the steps (i)(a) and (ii) may fail to entirely
- 5 debulk the apheresis sample of red blood cells and excess white blood cells respectively. Accordingly, the skilled person would therefore understand that the microfluidic separation of step (iii) is useful for separating CTC clusters and/or CTC-WBC clusters from other components, e.g. cellular and non-cellular, of the debulked apheresis sample
- 10 In one embodiment, the microfluidic separation of (iii) is performed using a microfluidics device which comprises a cell separation cassette. As used herein, a cell separation cassette refers to a disposable unit which may be introduced into, or removed from, a microfluidics device, and which comprise the microfluidics structure within which microfluidic separation occurs. For example, the cell separation cassette comprises the microfluidics structure which is
- 15 responsible for separating CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample.

The skilled person would understand that microfluidic separation, for example the microfluidic separation of (iii), is suitably performed by passing the sample comprising the components to be separated, for example the debulked apheresis sample comprising CTC clusters and/or CTC-WBC clusters and other components, in a small volume of a liquid, through a cell separation cassette and therefore through a microfluidics device. Suitably, in one

20 embodiment, the liquid comprises phosphate-buffered saline and/or bovine serum albumin and/or ethylenediaminetetraacetic acid (EDTA). Suitably, the liquid further comprises a kinase inhibitor, for example a Rho kinase inhibitor.

25

In one embodiment, the cell separation cassette comprises a stepped cassette configuration (Miller et al. 2018). As used herein, a stepped cassette configuration refers to a configuration whereby, in the forward direction through the cell separation cassette, and therefore the

30 microfluidics device, there is continuous series of steps of increasing height from the bottom of the cell separation cassette to the top of the cell separation cassette. The region above the continuous series of the steps is the flow channel, i.e. the region through which the sample, such as the debulked apheresis sample flows. As the continuous series of steps increases in height, the distance between the top of the step and the top of the cell separation cassette

35 becomes progressively smaller in the forward direction through the microfluidics device. The distance between the top of the step and the top of the cell separation cassette is known as the critical gap. Accordingly, in the forward direction through the microfluidics device, the flow

channel is defined by a progressively smaller critical gap. Therefore, in one embodiment the cell separation cassette comprises a stepped cassette configuration wherein the steps define flow channels of different critical gap wherein the critical gap becomes progressively smaller in the forward direction through the microfluidics device. The stepped cassette configuration facilitates the separation of CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample by their failure to pass through the critical gap at a step in the cell separation cassette. The skilled person would understand that CTC clusters, and similarly CTC-WBC clusters, have a degree of variability in their physical characteristics, in particular in their size, shape, and deformability. Moreover, CTC clusters and CTC-WBC clusters may vary in their physical characteristics, in particular in their size, shape, and deformability. Therefore, in one embodiment, CTC clusters and/or CTC-WBC clusters are suitably separated from other components of the debulked apheresis sample by their failure to pass through the critical gap at a single step in the cell separation cassette. In an alternative embodiment, CTC clusters and/or CTC-WBC clusters are suitably separated from other components of the debulked apheresis sample by their failure to pass through the critical gap at two or more steps in the cell separation cassette.

It should be noted that the cell separation cassette may comprise alternative microfluidics structures/configurations which allow particular isolation of CTC and/or CTC-WBC clusters. For example, the cell separation cassette may comprise a serpentine channel configuration (e.g. the MyCTC chip system).

Alternative microfluidics structures/configurations allow particular isolation of single i.e. individual CTCs. Examples of such structures/configurations include filtration-based configurations (e.g. the ISET™ system) or inertial focusing-based configurations (e.g. the iCTC™ chip system) or the FDA-approved, CellSearch™ system, which is configured as a ferrofluidic magnet device that exclusively captures EPCAM-labelled CTCs.

In one embodiment, the cell separation cassette comprises a unidirectional configuration (Miller et al. 2018). That is, the configuration that facilitates the separation of CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample exists in only one direction, typically the forward direction, through the cell separation cassette, and therefore the microfluidics device. In an alternative embodiment, the cell separation cassette comprises a bidirectional configuration. That is, the configuration that facilitates the separation of CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample exists in two directions through the cell separation cassette. In an alternative embodiment, the cell separation cassette comprises a multidirectional configuration. That is,

the configuration that facilitates the separation of CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample exists in two or more, for example three, four, five, or more, directions through the cell separation cassette.

- 5 In one particular embodiment, the cell separation cassette comprises a stepped cassette configuration in a unidirectional configuration. In an alternative embodiment, the cell separation cassette comprises a stepped cassette configuration in a multidirectional configuration, for example in a bidirectional configuration.
- 10 In one embodiment, the cell separation cassette comprises a looped cassette (Miller et al. 2018). That is, the configuration that facilitates the separation of CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample is arranged in a loop throughout the cell separation cassette such that there is a number of parallel stretches of the configuration arranged along the length of the cell separation cassette. In an alternative
- 15 embodiment, the cell separation cassette comprises a linear cassette. That is, the configuration that facilitates the separation of CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample is arranged in a line across the width of the cell separation cassette.
- 20 In one particular embodiment, the cell separation cassette comprises a looped cassette comprising a stepped cassette configuration. In one particular embodiment, the cell separation cassette comprises a looped cassette comprising a stepped cassette configuration in a unidirectional configuration. In an alternative embodiment, the cell separation cassette comprises a linear cassette comprising a stepped cassette configuration, for example wherein
- 25 the stepped cassette configuration is in a unidirectional configuration.

- In one embodiment, the microfluidics device of step (iii), which is for use in microfluidic separation is selected from the group consisting of the Parsortix® PR1 device, the Parsortix® PC1 device, and the MyCTC device (Schwab et al. 2022). In a preferred embodiment, the
- 30 microfluidics device of step (iii), which is for use in microfluidic separation is a Parsortix® PR1 device or Parsortix® PC1 device.

- In one embodiment, the microfluidic separation of step (iii) is performed such that CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample on the
- 35 basis of their physical characteristics. Suitably, the physical characteristics are selected from size, shape and deformability, or a combination thereof. More suitably, the physical characteristics are selected from size and deformability, or a combination thereof.

In one embodiment, the microfluidic separation of step (iii) is performed using a microfluidics device capable of separating CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample on the basis of their physical characteristics. Suitably, the physical characteristics are selected from size, shape and deformability, or a combination thereof. More suitably, the physical characteristics are selected from size and deformability, or a combination thereof.

Suitably, the microfluidic separation of step (iii) is performed under low, and preferably extremely low artificial pressure, and in particular is performed under a pressure which is less than or equal to, in particular less than, the pressure which CTC clusters and/or CTC-WBC clusters would be subject to by shearing forces in the human circulation. For example, the microfluidic separation of step (iii) is performed under a pressure of 4 psi or less, for example 3 psi or less, for example 2 psi or less, for example 1 psi or less.

The process of the present invention further comprises step (iv) of isolating separated CTC clusters and/or CTC-WBC clusters.

In one embodiment, the step (iv) of isolating separated CTC clusters and/or CTC-WBC clusters comprises removing the cell separation cassette from the microfluidics device and washing the cell separation cassette with a liquid.

In one particular embodiment, the step (iv) of isolating separated CTC clusters and/or CTC-WBC clusters comprises eluting separated CTC clusters and/or CTC-WBC clusters from the microfluidics device by passing a liquid in the reverse direction through the cell separation cassette, and therefore the microfluidics device.

In an alternative embodiment, the step (iv) of isolating separated CTC clusters and/or CTC-WBC clusters comprises removing the cell separation cassette from the microfluidics device and isolating separated CTC clusters and/or CTC-WBC clusters from the cell separation cassette via direct physical extraction. Such direct physical extraction typically comprises exposing separated CTC clusters and/or CTC-WBC clusters in the cell separation cassette, for example by dismantling the cell separation cassette, and directly collecting separated CTC clusters and/or CTC-WBC clusters from the cell separation cassette, for example using a serological pipette or micropipette.

The process of the present invention may optionally further comprise step (v), which follows step (iv) of sorting single i.e. individual CTCs derived from isolated CTC clusters and/or CTC-WBC clusters, by a process of cytometric fluorescence-activated cell sorting (FACS). That is, CTC clusters and/or CTC-WBC clusters isolated in step (iv) may be dispersed into the single
5 i.e. individual CTCs which form said cluster and subsequently the single i.e. individual CTCs are sorted by a process of cytometric fluorescence-activated cell sorting (FACS). Alternatively, step (v) may comprise sorting CTC clusters and/or CTC-WBC clusters, the structural integrity of which is preserved, i.e. CTC clusters and/or CTC-WBC clusters are not dispersed into the single i.e. individual CTCs which form the CTC clusters and/or CTC-WBC clusters prior to, or
10 during, sorting.

In one embodiment, the FACS of step (v) comprises labelling of single i.e. individual CTCs via one or more antigens on the surface of said single i.e. individual CTCs and subsequently positively selecting said single i.e. individual CTCs on the basis of the labelling of the one or
15 more antigens. Suitably, the one or more antigens on the surface of said single i.e. individual CTCs comprises epithelial cell adhesion molecule (EPCAM). Suitably, the label(s) used in the labelling of the one or more antigens on the surface of single i.e. individual CTCs are one or more antibody proteins.

In one embodiment, the FACS of step (v) comprises labelling of white blood cells (WBCs) via one or more antigens on the surface of said WBCs and subsequently excluding said WBCs on the basis of the labelling of the one or more antigens. Suitably, the one or more antigens on the surface of said WBCs are selected from the group consisting of CD45, CD31, and CD11b. Suitably, the label(s) used in the labelling of the one or more antigens on the surface
25 of WBCs are one or more antibody proteins.

In one embodiment, the FACS of step (v) comprises the exclusion of cells with inappropriate physical characteristics, i.e. physical characteristics which are not observed for single i.e. individual CTCs. Suitably, physical characteristics include size, shape and deformability. For
30 example, the FACS may comprise the exclusion of cell debris and/or cell multiples, in particular cell doublets.

In a particular embodiment, the FACS of step (v) comprises (a) the labelling of single i.e. individual CTCs via one or more antigens on the surface and subsequent selection of said
35 single i.e. individual CTCs on the basis of the labelling; and/or (b) the labelling of WBCs via one or more antigens on the surface and subsequent exclusion of said WBCs on the basis of the labelling; and/or (c) comprises the exclusion of cells with inappropriate physical

characteristics, i.e. physical characteristics which are not observed for single i.e. individual CTCs. The steps (a), (b), and (c) may occur simultaneously or consecutively.

5 The process of the present invention may optionally further comprise step (vi), which follows step (iv), or more preferably follows optional step (v), of sequencing isolated, and optionally sorted, single i.e. individual CTCs derived from isolated CTC clusters and/or CTC-WBC clusters.

10 For example, sequencing isolated, and optionally sorted, single i.e. individual CTCs derived from isolated CTC clusters and/or CTC-WBC clusters may be performed in order to identify specific mutations present in individual CTCs and/or to identify mutations commonly present in CTCs. Furthermore, sequencing isolated, and optionally sorted, single i.e. individual CTCs derived from isolated CTC clusters and/or CTC-WBC clusters may be performed in order to inform how CTC clusters and/or CTC-WBC clusters, or secondary tumours derived from said
15 CTC clusters and/or CTC-WBC clusters, will respond to specific treatments. In addition, sequencing isolated, and optionally sorted, single i.e. individual CTCs derived from isolated CTC clusters and/or CTC-WBC clusters may be performed in order to identify new cancer or metastasis markers which may be utilised in diagnostics, and/or identify new cancer- or metastasis-specific molecules which may be a novel drug target.

20 In one embodiment, the process of the present invention is a process for the isolation of circulating tumour cell (CTC) clusters, such that the isolate resulting from step (iv) comprises CTC clusters. For example, in one embodiment the process of the present invention is a process for the isolation of circulating tumour cell (CTC) clusters but not circulating tumour
25 cell-white blood cell (CTC-WBC) clusters, such that the isolate resulting from step (iv) comprises CTC clusters but is substantially free of, for example is free of, CTC-WBC clusters.

30 In one embodiment, the process of the present invention is a process for the isolation of circulating tumour cell-white blood cell (CTC-WBC) clusters, such that the isolate resulting from step (iv) comprises CTC-WBC clusters. For example, in one embodiment the process of the present invention is a process for the isolation of circulating tumour cell-white blood cell (CTC-WBC) clusters but not circulating tumour cell (CTC) clusters, such that the isolate resulting from step (iv) comprises CTC-WBC clusters but is substantially free of, for example
35 is free of, CTC clusters.

As used herein, the term “substantially free of” refers to the fact that the isolate resulting from step (iv) comprises a *de minimis* amount of CTC clusters or CTC-WBC clusters.

In one embodiment, the process of the present invention, that is a process for the isolation of circulating tumour cell (CTC) clusters and/or circulating tumour cell-white blood cell (CTC-WBC) clusters, is such that CTC clusters and/or CTC-WBC clusters are preserved. In particular, the process for isolation is such that the structural integrity of CTC clusters and/or CTC-WBC clusters is preserved, i.e. CTC clusters and/or CTC-WBC clusters are not disrupted into the single i.e individual CTCs which form the CTC clusters and/or CTC-WBC clusters.

Suitably, the process of the present invention is such that the structural integrity of CTC clusters and/or CTC-WBC clusters is preserved since the debulking step of (ii) comprises a flow-based process performed under minimal pressure and the microfluidic separation of (ii) is performed under lower pressure and in particular is performed under a pressure which is less than or equal to, in particular less than, the pressure which CTC clusters and/or CTC-WBC clusters would be subject to in the human circulation. That is, preventing the exposure of CTC clusters and/or CTC-WBC clusters to a high pressure, in particular a pressure greater than the pressure which CTC clusters and/or CTC-WBC clusters would be subject to in the human circulation, allows for the structural integrity of CTC clusters and/or CTC-WBC clusters to be preserved.

In particular, the process of the present invention is such that the structural integrity of CTC clusters and/or CTC-WBC clusters is preserved since the debulking step of (ii) comprises a flow-based process where flow is driven by gravity and/or minimal applied artificial pressure i.e. does not involve the application of any significant artificial pressure (such as exceeding 15 psi) to the ongoing flow-based process, and the microfluidic separation of (iii) is performed under low or extremely low pressure and in particular is performed under a pressure which is less than or equal to, in particular less than, the pressure which CTC clusters and/or CTC-WBC clusters would be subject to by shearing forces in the human circulation.

In one embodiment, the process of the present invention is a process for the isolation of circulating tumour cell (CTC) clusters and/or circulating tumour cell-white blood cell (CTC-WBC) clusters from a patient with cancer. In particular, the process of the present invention is a process for the isolation of circulating tumour cell (CTC) clusters and/or circulating tumour cell-white blood cell (CTC-WBC) clusters derived from a tumour in a patient with a cancer.

Therefore, in one embodiment, the apheresis sample of step (i) is an apheresis sample from a patient that has cancer, or from a patient that is suspected of having cancer. For example, the apheresis sample of step (i) is a sample obtained under conditions of leukapheresis from

a patient that has cancer, or from a patient that is suspected of having cancer. In one embodiment, is an apheresis sample from a patient that has been diagnosed with cancer. For example, the apheresis sample of step (i) is an apheresis sample, in particular a sample obtained under conditions of leukapheresis, from a patient that has been diagnosed with cancer.

In one embodiment, the cancer is a solid tumour cancer. For example, in one embodiment, the cancer is an epithelial tumour i.e. carcinoma, for example an adenocarcinoma, basal cell carcinoma, or squamous cell carcinoma. In an alternative embodiment, the cancer is a lymphoma. In a further alternative embodiment, the cancer is a melanoma. In an additional alternative embodiment, the cancer is a sarcoma, for example fibrosarcoma, Kaposi's sarcoma, osteosarcoma, or rhabdomyosarcoma. In an alternative embodiment, the cancer is a blastoma.

In one embodiment, the cancer is bladder cancer, brain cancer, breast cancer, cervical cancer, colon cancer, gallbladder cancer, liver cancer, oesophageal cancer, ovarian cancer, pancreatic cancer, prostate cancer, rectal cancer, renal cancer, skin cancer e.g. basal cell carcinoma, stomach cancer, thyroid cancer, or uterine cancer. In a particular embodiment, the cancer is breast cancer, colon cancer, lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, or skin cancer e.g. basal cell carcinoma.

In one embodiment, the cancer is a metastatic cancer. For example, in one embodiment, the cancer is a metastatic carcinoma. In an alternative embodiment, the cancer is a metastatic lymphoma. In a further alternative embodiment, the cancer is a metastatic melanoma. In an additional alternative embodiment, the cancer is a metastatic sarcoma. In an alternative embodiment, the cancer is a metastatic blastoma.

In one embodiment, the cancer is a metastatic cancer. For example, the cancer is metastatic bladder cancer, metastatic brain cancer, metastatic breast cancer, metastatic cervical cancer, metastatic colon cancer, metastatic gallbladder cancer, metastatic liver cancer, metastatic oesophageal cancer, metastatic ovarian cancer, metastatic pancreatic cancer, metastatic prostate cancer, metastatic rectal cancer, metastatic renal cancer, metastatic skin cancer, metastatic stomach cancer, metastatic thyroid cancer, or metastatic uterine cancer. In a particular embodiment, the cancer is metastatic breast cancer, metastatic colon cancer, metastatic lung cancer, metastatic ovarian cancer, metastatic pancreatic cancer, metastatic prostate cancer, or metastatic skin cancer.

Suitably, the efficiency and sensitivity of the process of the present invention allows for the isolation of circulating tumour cell (CTC) clusters and/or circulating tumour cell-white blood cell (CTC-WBC) clusters from a patient, irrespective of any previous CTC cluster and/or CTC-WBC cluster isolation from the same patient. Therefore in one embodiment, the process of the present invention is a process for the isolation of circulating tumour cell (CTC) clusters and/or circulating tumour cell-white blood cell (CTC-WBC) clusters from a patient who has previously received apheresis and/or who has previously had CTC clusters and/or CTC-WBC clusters isolated from them. In an alternative embodiment, the process of the present invention is a process for the isolation of circulating tumour cell (CTC) clusters and/or circulating tumour cell-white blood cell (CTC-WBC) clusters from a patient who has not previously received apheresis and/or who has not previously had CTC clusters and/or CTC-WBC clusters isolated from them

The Invention may be further defined by the following clauses:

1. A process for the isolation of circulating tumour cell (CTC) clusters and/or circulating tumour cell-white blood cell (CTC-WBC) clusters comprising:
 - (i) collecting an apheresis sample from a patient;
 - (ii) debulking the apheresis sample of excess white blood cells;
 - (iii) separating CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample using microfluidic separation; and
 - (iv) isolating separated CTC clusters and/or CTC-WBC clusters;

wherein the debulking step comprises a flow-based process where flow is driven by gravity and/or minimal applied artificial pressure; wherein the microfluidic separation is performed using a microfluidics device capable of separating CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample.

2. The process according to clause 1, wherein the apheresis sample of (i) is a sample obtained under conditions of leukapheresis.

3. The process according to clause 1 or clause 2, wherein the apheresis sample of (i) has a volume of between about 50 and about 250 mL, for example between about 50 and about 200 mL, for example between about 50 and about 150 mL, for example about 100 mL.

4. The process according to any one of clauses 1 to 3, wherein the debulking step of (ii) comprises a process of capture of excess white blood cells, wherein the capture process

comprises a flow-based process where flow is driven by gravity without application of artificial pressure.

5. The process according to any one of clauses 1 to 4, wherein the wherein the debulking
5 step of (ii) comprises a process of magnetic capture of excess white blood cells.

6. The process according to clause 5, wherein the debulking step of (ii) comprises the
magnetic capture of excess white blood cells magnetically labelled via one or more antigens
on the surface of said excess white blood cells as a result of a prior step of treating the
10 apheresis sample with affinity labelled magnetic particles, wherein the affinity label of the
affinity labelled magnetic particles is capable of binding to one or more antigens on the surface
of excess white blood cells.

7. The process according to clause 6, wherein the affinity label of the affinity labelled
15 magnetic particles is an antibody protein capable of binding to one or more antigens on the
surface of excess white blood cells.

8. The process according to clause 6 or clause 7, wherein the one or more antigens on
the surface of said excess white blood cells is selected from the group consisting of CD45,
20 CD31 and CD11b.

9. The process according to any one of clauses 1 to 8, wherein the flow-based process
of the debulking step of (ii) is a chromatographic process wherein the stationary phase in the
chromatography is capable of preventing the elution of excess white blood cells.
25

10. The process according to clause 9, wherein the flow-based process of the debulking
step of (ii), which comprises a process of capture of excess white blood cells, is a
chromatographic process wherein the stationary phase in the chromatography is capable of
preventing the elution of captured excess white blood cells.
30

11. The process according to clause 10, wherein the flow-based process of the debulking
step of (ii), which comprises a process of magnetic capture of excess white blood cells, is a
chromatographic process wherein the stationary phase in the chromatography is capable of
preventing the elution of magnetically labelled excess white blood cells.
35

12. The process according to any one of clauses 9 to 11, wherein the chromatographic process is a column-based chromatographic process and the apheresis sample is passed through a column by a flow-based process where flow is driven by gravity.
- 5 13. The process according to clause 12, wherein the column is loaded at maximal capacity such that the column-based chromatographic process operates under saturating conditions.
14. The process according to any one of clauses 9 to 13, wherein the stationary phase comprises ferrous components which bind to magnetically labelled excess white blood cells.
- 10 15. The process according to any one of clauses 9 to 14, wherein the debulking step of (ii) is optimised for the debulking of excess white blood cells.
16. The process according to clause 15, wherein the debulking step of (ii) is optimised for the capture of excess white blood cells.
- 15 17. The process according to clause 16, wherein the debulking step of (ii) is optimised for the magnetic capture of excess white blood cells.
18. The process according to clause 15, wherein the debulking step of (ii) is optimised for the debulking of individual white blood cells.
- 20 19. The process according to clause 18, wherein the debulking step of (ii) is optimised for the capture of individual white blood cells.
- 25 20. The process according to clause 19, wherein the debulking step of (ii) is optimised for the magnetic capture of individual white blood cells.
21. The process according to any one of clauses 1 to 20, wherein the debulking step of (ii) takes no longer than about 150 minutes, for example no longer than about 120 minutes.
- 30 22. The process according to any one of clauses 1 to 21, wherein the microfluidics device of (iii) comprises a cell separation cassette.
23. The process according to clause 22, wherein the cell separation cassette comprises a stepped cassette configuration wherein the steps define flow channels of different critical gap wherein the critical gap becomes progressively smaller in the forward direction through the

microfluidics device such that CTC clusters and/or CTC-WBC clusters are separated from other components of the debulked apheresis sample by their failure to pass through the critical gap at a step in the cell separation cassette .

5 24. The process according to clause 23, wherein the stepped cassette configuration is unidirectional.

25. The process according to any one of clauses 1 to 24, wherein the microfluidics device of (iii) is a Parsortix® PR1 device or Parsortix® PC1 device.

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26. The process according to any one of clauses 1 to 25, wherein the microfluidic separation of step (iii) is performed using a microfluidics device capable of separating CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample on the basis of their physical characteristics.

15

27. The process according to clause 26, wherein the physical characteristics are selected from size, shape and deformability, or a combination thereof.

28. The process according to any one of clauses 1 to 27, wherein the step (iv) of isolating
20 separated CTC clusters and/or CTC-WBC clusters step comprises eluting separated CTC clusters and/or CTC-WBC clusters from the microfluidics device by passing a liquid in the reverse direction through the microfluidics device.

29. The process according to any one of clauses 1 to 28, wherein the process further
25 comprises the step (v), which follows step (iv), of sorting individual circulating tumour cells (CTCs) derived from isolated circulating tumour cell (CTC) clusters and/or circulating tumour cell-white blood cell (CTC-WBC) clusters, by a process of cytometric fluorescence-activated cell sorting (FACS).

30 30. The process according to any one of clauses 1 to 29, wherein the process is a process for the isolation of circulating tumour cell (CTC) clusters, such that the isolate resulting from step (iv) comprises CTC clusters.

31. The process according to any one of clauses 1 to 29, wherein the process is a process
35 for the isolation of circulating tumour cell-white blood cell (CTC-WBC) clusters, such that the isolate resulting from step (iv) comprises CTC-WBC clusters.

32. The process according to any one of clauses 1 to 31, wherein the apheresis sample is an apheresis sample from a patient that has a cancer.
33. The process according to clause 32, wherein the cancer is a solid tumour cancer.
- 5 34. The process according to clause 33, wherein the cancer is an epithelial tumour i.e. carcinoma.
- 10 35. The process according to any one of clauses 32 to 34, wherein the cancer is breast cancer, colon cancer, lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, or skin cancer e.g. basal cell carcinoma.
- 15 36. The process according to any one of clauses 32 to 35, wherein the cancer is a metastatic cancer, for example metastatic breast cancer, metastatic colon cancer, metastatic lung cancer, metastatic ovarian cancer, metastatic pancreatic cancer, metastatic prostate cancer, or metastatic skin cancer.
37. The process according to clause 32 or clause 33, wherein the cancer is a lymphoma.
- 20 38. The process according to clause 37, wherein the cancer is a metastatic lymphoma.
39. The process according to clause 32 or clause 33, wherein the cancer is a melanoma.
- 25 40. The process according to clause 39, wherein the cancer is a metastatic melanoma.
41. The process according to any one of clauses 1 to 31, wherein the process is a process for the isolation of circulating tumour cell (CTC) clusters and/or circulating tumour cell-white blood cell (CTC-WBC) clusters derived from a tumour in a patient with a cancer.
- 30 The invention also provides a process for the isolation of circulating tumour cell (CTC) clusters and/or circulating tumour cell-white blood cell (CTC-WBC) clusters comprising:
- (a) debulking of excess white blood cells an apheresis sample collected from a patient;
 - (b) separating CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample using microfluidic separation; and
 - 35 (c) isolating separated CTC clusters and/or CTC-WBC clusters;

wherein the debulking step comprises a flow-based process where flow is driven by gravity and/or minimal applied artificial pressure; wherein the microfluidic separation is performed using a microfluidics device capable of separating CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample.

5

The merits of the process invention may include the following:

- (i) CTC clusters and/or CTC-WBC clusters may be isolated with high efficiency and thus good yield.
- (ii) The clusters isolated are representative of the clusters within the entire blood volume of the subject.
- (iii) The process provides a sensitivity suitable for liquid biopsy methods useful in the diagnosis or treatment of cancer.

10

Examples

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Example 1: An Exemplary Protocol for the Isolation of circulating tumour cell (CTC) clusters and/or circulating tumour cell-white blood cell (CTC-WBC) clusters from a patient

20 Step 1: Recruit a patient upon signed informed consent and appropriate permission from ethical review boards.

Step 2: Subject the patient to a 120 minute leukapheresis session using a Spectra Optia® device, wherein the collection volume of apheresis product is set to 100 mL in a sterile and hermetically sealed Leukopak® collection bag.

25

Step 3: If necessary, store, for a maximum of 30 minutes, the apheresis product (in the Leukopak® bag) in a closed ice box during transportation to a Biosafety Level 2 Laboratory.

30 Step 4: Spray the Leukopak® bag with 70% (v/v) ethanol and wipe the surface of the bag. Move the bag into an active laminar flow cabinet.

Step 5: Using sterile scissors, cut the Leukopak® bag open under active laminar flow. Proceed to prepare the apheresis sample (see steps 6 to 17) in a period of maximum 45 minutes.

35

Step 6: Using sterile serological pipettes, harvest and transfer the apheresis product to pre-chilled (4°C) sterile Falcon tubes, filling them to their maximum (50 mL) capacity.

Step 7: Wash, twice, the empty Leukopak® bag with 100 mL of pre-chilled (4°C) sterile phosphate-buffered saline (PBS; pH 7.4), and harvest and transfer the wash PBS to pre-chilled (4°C) sterile Falcon tubes, filling them to their maximum (50 mL) capacity.

5

Step 8: Combine the harvested apheresis product with the wash PBS volumes and centrifugate in a pre-chilled (4°C) Eppendorf centrifuge at 300g for 10 minutes.

Step 9: Remove the supernatant volume using sterile serological pipettes and centrifugate said supernatant in a pre-chilled (4°C) Eppendorf centrifuge at 300g for 10 minutes. Repeat this step another time.

10

Step 10: Resuspend each of the pellets in 5 mL of a ten-fold dilution of Miltenyi Red Blood Cell Lysis Solution (in water). Incubate the suspension for 3 minutes.

15

Step 11: Bring the final volume of each of the pellet resuspensions to 50 mL by adding pre-chilled (4°C) sterile PBS.

Step 12: Centrifugate each of the pellet resuspensions in a pre-chilled (4°C) Eppendorf centrifuge at 300g for 10 minutes.

20

Step 13: If a pellet resulting from Step 12 contains visible red blood cells proceed to (optional) step 14 for this pellet. If a pellet resulting from Step 12 is a uniformly white pellet with no visible red blood cells, proceed to step 17 for this pellet.

25

(Optional) Step 14: Resuspend each relevant pellet in 5 mL of Gibco ACK Lysis Buffer. Incubate the suspension on ice for 3 minutes.

(Optional) Step 15: Centrifugate each of the relevant pellet resuspensions in a pre-chilled (4°C) Eppendorf centrifuge at 300g for 10 minutes.

30

(Optional) Step 16: If a pellet resulting from (optional) step 15 contains visible red blood cells, repeat (optional) steps 14 and 15 for this pellet. If a pellet resulting from (optional) step 15 is a uniformly white pellet with no visible red blood cells, proceed to step 17 for this pellet.

35

Step 17: Collect each of the pellets in one pool and resuspend the collected pellets in 50 mL of pre-chilled (4°C) Miltenyi AutoMACS Rinsing Solution supplemented with Miltenyi Bovine

Serum Albumin Stock Solution (to a final concentration of 0.5% v/v) and supplemented with Merck Millipore Y-27632 Rho kinase inhibitor (to a final concentration of 10 μ mol/L. This solution (less the pellets) is hereby referred to as YPEB Buffer. Unless stated otherwise, YPEB Buffer is always used as pre-chilled (4°C) and sterile.

5

Step 18: Perform an automated cell count on the resuspended pellets using an Invitrogen Countess II device. The resuspended pellets are subjected to a ten-fold pre-dilution in YPEB buffer followed by a two-fold dilution in Invitrogen Trypan Blue Stain (0.4%). Micropipettes are used to prepare the dilutions in technical triplicates and the Invitrogen Countess II device is used in automated cell counting mode according to the manufacturer's instructions. The cell counting process should take a period of maximum 15 minutes, and the resuspended pellets should be maintained on ice throughout this process.

Step 19: Subject the resuspended pellets to white blood cell debulking via magnetic capture (see steps 19 to 30). The debulking process, i.e. magnetic capture, should take a maximum of 150 minutes .

Step 20: Calculate the required number of Miltenyi MultiMACS machine runs, and therefore the number of Miltenyi MultiMACS columns and/or machines required to process the entire resuspended pellet suspension, in view of the cell count of the resuspended pellets (i.e. apheresis product). Note that each MultiMACS machine run should take about 18 minutes and that each column should process a minimum of about 1×10^8 labelled cells and a maximum of about 2×10^8 labelled cells.

Step 21: Reduce the resuspended pellets back to pellets by centrifugating in a pre-chilled (4°C) Eppendorf centrifuge at 300g for 10 minutes.

Step 22: Resuspend the resultant pellet in YPEB Buffer at a ratio of 1 mL YPEB Buffer per 10^8 cells, supplemented with 200 μ L per 10^8 cells of pre-chilled (4°C) Miltenyi CD45 MicroBeads (human) and 200 μ L per 10^8 cells of pre-chilled (4°C) Miltenyi CD31 MicroBeads human and mouse.

Step 23: Incubate the suspension at 4°C for 30 minutes.

Step 24: Bring the final volume of the suspension to 50 mL by adding pre-chilled (4°C) YPEB Buffer.

Step 25: Centrifugate the suspension in a pre-chilled (4°C) Eppendorf centrifuge at 300g for 10 minutes.

5 Step 26: Resuspend the resultant pellet in YPEB Buffer at a ratio of 1 mL YPEB Buffer per 10⁸ cells.

Step 27: Run the Miltenyi MultiMACS device on the "DEplete" program, using a Miltenyi Multi-24 Column Block previously equilibrated with 200 µL per well YPEB Buffer.

10 Step 28: Apply, to the equilibrated Miltenyi Multi-24 Column Block, a volume of the suspension such that a minimum of 1 x 10⁸ cells and a maximum of 2 x 10⁸ cells are administered per column. Subsequently allow the cells to flow through the columns by gravity and wash each column with 2 mL of YPEB Buffer. Collect the flow-through volume and the wash volume together in a disposable sterile Nalgene Robotic Reservoir deep-well plate.

15 Step 29: Repeat the steps 27 and 28 as many times as is required to process the entire suspension, which contains the entire cell count from the apheresis process.

20 Step 30: Collect all the flow-through and wash volumes and centrifugate in a pre-chilled (4°C) Eppendorf centrifuge at 300g for 10 minutes. Resuspend the resultant pellet in 50 mL pre-chilled (4°C) YPEB Buffer.

Step 31: Repeat step 18 above on the suspension, to obtain the post-debulking cell count.

25 Step 32: Subject the entire suspension to microfluidic separation, for the isolation of circulating tumour cell (CTC) clusters and/or circulating tumour cell-white blood cell (CTC-WBC) clusters, using the Parsortix® PR1 microfluidics device. The microfluidic separation process should take a maximum of 90 minutes.

30 Step 33: Centrifugate the entire suspension in a pre-chilled (4°C) Eppendorf centrifuge at 300g for 10 minutes. Resuspend the resultant pellet in 2 mL YPEB buffer. Transfer the suspension to a pre-chilled (4°C) sterile Falcon tube for loading into the Parsortix® PR1 microfluidics device.

35 Step 34: Prepare a single-use Parsortix® GED3D6.5 cell separation cassette according to the manufacturer's instructions.

Step 35: Insert the prepared Parsortix® GED3D6.5 cell separation cassette into the Parsortix® PR1 microfluidics device and run the priming program PX2_PF.

- 5 Step 36: Insert the pre-chilled (4°C) sterile Falcon tube containing the suspension into the sample loading tubing line of the Parsortix® PR1 device, as soon as the priming program PX2_PF is complete, which should take a maximum of about 15 minutes.

- 10 Step 37: Run the cell separation program PX2_S502 until it is complete, which should take somewhere between 30 and 45 minutes. During the program, the Falcon tube containing the suspension should be regularly agitated to ensure intake of the entire suspension.

- 15 Step 38: At this stage, CTC and/or CTC-WBC clusters should have been separated from other components of the debulked apheresis sample and should be isolated within the Parsortix® GED3D6.5 cell separation cassette. To verify isolation of CTC and/or CTC-WBC clusters, prepare an antibody solution of 470 µL PBS containing 1% (v/v) Bovine Serum Albumin, 10 µL CST anti-human EPCAM AlexaFluor488, 10 µL GeneTex anti-human EGFR FITC, 10 µL Biolegend anti-human CD340 (erbb2/HER2) AlexaFluor488, which is loaded into a 15 mL Corning tube.

- 20 Step 39: Run the in-cassette staining program STAIN_B1 after loading the antibody solution-containing 15 mL Corning tube into Line 1 and 1 mL of PBS containing 1% (v/v) Bovine Serum Albumin into Line 2 of the Parsortix® PR1 device. This staining process should take a maximum of about 15 minutes.

- 25 Step 40: Unload the stained Parsortix® GED3D6.5 cell separation cassette from the Parsortix® PR1 device and proceed to count fluorescently labelled CTC and/or CTC-WBC clusters under a fluorescent microscope lamp. This process should take between about 30 and about 45 minutes.

- 30 Step 41: Once counting of fluorescently labelled CTC and/or CTC-WBC clusters is complete, load the stained Parsortix® GED3D6.5 cell separation cassette back into the Parsortix® PR1 device and run the cell recovery program PX2_Hv3 to collect the isolated CTC and/or CTC-WBC clusters.

- 35 **Example 2: Isolation of circulating tumour cell (CTC) clusters and circulating tumour cell-white blood cell (CTC-WBC) clusters from a patient with cancer**

A patient with metastatic breast cancer was identified and provided their consent regarding undergoing the process described in Example 1. Permissions from the appropriate ethical review boards were also acquired. The patient was subjected to the process described in Example 1 in order to isolate circulating tumour cell (CTC) clusters and circulating tumour cell-
5 white blood cell (CTC-WBC) clusters from said patient.

Following isolation of CTC and/or CTC-WBC clusters from the patient, said clusters were stained (i.e. fluorescently labelled) and subsequently counted in order to assess the efficiency of the isolation process. The results of this process are illustrated in Figures 1 to 3. Figures 1
10 and 2 respectively depict a fluorescently labelled heterotypic cluster, i.e. a CTC-WBC cluster comprising CTCs and white blood cells, and a fluorescently labelled homotypic clusters, i.e. a CTC clusters comprising CTCs only. CTCs are stained with anti-EPCAM, anti-EGFR and anti-HER2 antibodies. White blood cells are stained with anti-CD45 antibodies. Figure 3 depicts the number of individual CTCs, CTC clusters (i.e. homotypic clusters), and CTC-WBC clusters
15 (i.e. heterotypic clusters) isolated via the process described in Example 1, which is a process of the present invention. Specifically, in this representative patient with cancer, the process resulted in the isolation of a total of 8280 CTCs or clusters thereof, of which 5256 were individual CTCs, 605 were CTC clusters (i.e. homotypic clusters), and 2419 were CTC-WBC clusters (i.e. heterotypic clusters).

20 In comparison, in an alternative study, of 70 patients with invasive breast cancer, only 34 patients were found to have detectable CTCs (or clusters thereof) following microfluidic separation of a 7.5 mL peripheral blood sample (Szczerba et al. 2019). The mean number of CTCs (or clusters thereof) detected in said patients was 22 (Szczerba et al. 2019). The
25 majority of the CTCs or clusters thereof were single i.e. individual CTCs (88.0%), with relatively smaller fractions of CTC clusters (8.6%) and CTC-WBC clusters (3.4%) (Szczerba et al. 2019).

Therefore, the process of the present invention is a sensitive process which reliably isolates CTCs, and in particular CTC and/or CTC-WBC clusters from patients with cancer. Moreover,
30 the process of the present invention allows for the isolation of a much higher yield of CTCs from patients with cancer, in particular metastatic cancer. In addition, the process of the present invention enriches i.e. proportionally increases, the number of CTC clusters and CTC-WBC clusters present in the isolate, relative to existing processes using peripheral blood samples, which is advantageous since it is these clusters which comprises CTCs with the
35 highest intrinsic metastatic potential.

Example 3: Determining the ability of the process of debulking the apheresis sample of excess white blood cells to enrich the sample with circulating tumour cells (CTCs) and clusters thereof

5 The ability of the process of debulking the apheresis sample of excess white blood cells, as described between steps 19 and 31 of Example 1 above, to enrich the sample with circulating tumour cells (CTCs) and clusters thereof was assessed. The initial apheresis sample, pre-debulking, and the debulked apheresis sample, were each subjected to cytometric fluorescence-activated cell sorting (FACS) analysis in order to characterise the cell
10 populations within each sample. The sample was obtained from a patient with metastatic breast cancer. The results are shown in Figure 4.

These results demonstrate that the novel debulking process developed by the present inventors enriches a debulked apheresis sample, i.e. proportionally increases the number of
15 relative to the sample pre-debulking, with circulating tumour cells (CTCs) and presumably clusters thereof, in particular CTC clusters and CTC-white blood cell (CTC-WBC) clusters. Simultaneously, the debulked apheresis sample comprises a proportionally lower number of WBCs relative to the sample pre-debulking. The debulked apheresis sample is therefore a more suitable input sample for microfluidic separation.

20 In view of the above, a debulked apheresis sample obtained by the process of the present invention was further characterised via FACS to investigate the ability of the process of debulking the apheresis sample of excess white blood cells, as described between steps 19 and 31 of Example 1 above, to specifically enrich the sample with CTC and/or CTC-WBC
25 clusters. The presence of said CTC and/or CTC-WBC clusters was identified via a sub-gated analysis of the post-debulking apheresis sample, which distributes cells according to SSC-a (side light scatter area) and FSC-a (forward light scatter area), resulting in size-related distribution. The sample was obtained from a second patient with metastatic breast cancer. This patient is the same patient referred to in Examples 1, 4 and 5. The results are shown in
30 Figure 5.

These results demonstrate that the novel debulking process developed by the present inventors, in addition to enriching the debulked apheresis sample with circulating tumour cells (CTCs), was able to specifically enrich an apheresis sample with CTC clusters and/or CTC-
35 WBC clusters, the structural integrity of which was maintained during the novel debulking process. The debulked apheresis sample is therefore a particular suitable input for microfluidic

separation since it comprise a proportionally large number of CTC clusters and/or CTC-WBC clusters, which are considered to have particularly enhanced metastatic potential.

Example 4: Determining the impact of pressure on the yield of circulating tumour cell (CTC) clusters and circulating tumour cell-white blood cell (CTC-WBC) clusters following the process of debulking the apheresis sample of excess white blood cells

The impact of pressure on the yield of circulating tumour cell (CTC) clusters and circulating tumour cell-white blood cell (CTC-WBC) clusters following the process of debulking the apheresis sample of excess white blood cells, as described between steps 19 and 31 of Example 1 above, was investigated. Samples containing homotypic clusters i.e. CTC clusters, of metastatic breast cancer cells to debulking under natural gravity (no artificial pressure in addition to that imposed by gravity force) and under artificial pressure were analysed.

Specifically an apheresis sample from a patient with metastatic breast cancer was treated with Miltenyi CD45 MicroBeads (human) (see step 22 of Example 1 above). Prior to debulking the treated apheresis sample was viewed using brightfield microscopy to visualise the frequency of homotypic clusters i.e. CTC clusters, of metastatic breast cancer cells. Half of said treated apheresis sample was run through the Miltenyi MultiMACS device on the "DEplete" program, which is driven by gravity only (see steps 23 to 28 of Example 1 above, elution time ca 1 minute), and half was run through the Miltenyi MultiMACS device on the "POS_SEL" program, which comprises vacuum-pressure elution (pressure up to 20 psi, elution time < 1 second). Post debulking i.e. magnetic capture via chromatographic separation, the sample was again viewed using brightfield microscopy and the number of homotypic clusters i.e. CTC clusters, of metastatic breast cancer cells which are intact, partially intact or shattered i.e. entirely disrupted were counted. Figure 6 depicts the treated apheresis sample pre- and post-debulking, wherein the debulking is driven by either gravity only, or under pressure. In particular, Figure 6 illustrates that homotypic clusters i.e. CTC clusters, of metastatic breast cancer cells remain largely intact during debulking driven by gravity only, but that said homotypic clusters are typically disrupted during debulking comprising pressure-forced separation. Table 1 below quantifies the above observations of Figure 6:

Table 1: The proportion of homotypic clusters i.e. CTC clusters, of metastatic breast cancer cells which are intact, partially intact or shattered following debulking i.e. via magnetic capture driven by either gravity only, or under pressure-forced separation.

	Proportion (%) of homotypic clusters i.e. CTC clusters	
	Flow driven by gravity only	Flow driven under pressure
Intact	74	5
Partially Intact	21	44
Shattered (i.e. entirely disrupted)	5	51

Therefore, homotypic clusters i.e. CTC clusters which include single CTCs with high intrinsic metastatic potential, are commonly disrupted, i.e. shattered into individual CTCs, when pressure is applied during debulking. The resultant sample will therefore contain very few CTC clusters, such that the CTC cluster yield from subsequent microfluidic separation techniques will be low and limiting to further use. Therefore, debulking, for example via magnetic capture, should be performed under gravity i.e. without application of pressure such that CTC clusters, or indeed CTC-white blood cell (CTC-WBC) clusters, remain intact and available for subsequent isolation.

10

Example 5: Comparing the efficiency of the process of Example 1 to alternative processes for isolating circulating tumour cell (CTC) clusters and/or circulating tumour cell-white blood cell (CTC-WBC) clusters

15 In order to demonstrate the merits of the process of the present invention, and the importance each step in recognising said merits, the present inventors have performed the microfluidic separation process described in Example 1 above (see steps 32 to 41) on (i) a peripheral blood sample, (ii) an apheresis sample which has not been debulked (i.e. see steps 1 to 18 and 32 to 41 of Example 1), and (iii) an apheresis sample which has been debulked (i.e. see
20 steps 1 to 41 of Example 1), each of which was derived from a patient with metastatic breast cancer.

The cell separation cassettes which were loaded with the respective samples were visualised using both brightfield microscopy and fluorescence microscopy techniques. Regarding the
25 latter, CTCs were immune-stained with anti-EPCAM, anti-HER2, and anti-EGFR antibodies, and WBCs were immune-stained with anti-CD45 antibodies. The results of this analysis are presented in Figure 7.

Specifically, the peripheral blood sample was suitable for microfluidic separation but included
30 a very small number of CTCs and WBCs, and no visible CTC clusters or CTC-WBC clusters.

The apheresis sample contained a very large number of WBCs which made the sample unsuitable for microfluidic separation as the cell separation cassette became saturated and blocked. The debulked apheresis sample was suitable for microfluidic separation and the cell separation cassette isolated a large number of CTCs, CTC clusters, and CTC-WBC clusters.

- 5 Therefore, microfluidic separation of a peripheral blood sample or apheresis sample alone fails to isolate significant number of CTCs, and in particular CTC clusters and/or CTC-WBC clusters. However, the novel process developed by the present inventors, comprising apheresis, a novel debulking process, and microfluidic separation, facilitates isolation of very high numbers of CTCs, and in particular CTC clusters and/or CTC-WBC clusters, from
- 10 patients.

Miscellaneous

Unless specified otherwise, physical parameters such as pressures are determined at 20 °C.

- 15 The unit psi (pounds per square inch) is equivalent to 6985 Pa.

All references referred to in this application, including patent and patent applications, are incorporated herein by reference to the fullest extent possible.

- 20 Throughout the specification and the claims which follow, unless the context requires otherwise, the word 'comprise', and variations such as 'comprises' and 'comprising', will be understood to imply the inclusion of a stated integer, step, group of integers or group of steps but not to the exclusion of any other integer, step, group of integers or group of steps.

- 25 The application of which this description and claims forms part may be used as a basis for priority in respect of any subsequent application. The claims of such subsequent application may be directed to any feature or combination of features described herein. They may take the form of product, composition, process, or use claims and may include, by way of example and without limitation, the following claims.

- 30 As used herein when referring to the composition of the invention, the term "consists of" means that no further components are included in the composition other than those listed. As used herein when referring to the composition of the invention, the term "consists essentially of" means that further components can be present but said components do not materially affect the essential characteristics of the composition, and are typically present in a *de minimis*
- 35 amount.

The invention embraces all combinations of preferred and more preferred groups and suitable and more suitable groups and embodiments of groups recited above.

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Claims

1. A process for the isolation of circulating tumour cell (CTC) clusters and/or circulating tumour cell-white blood cell (CTC-WBC) clusters comprising:
 - 5 (i) collecting an apheresis sample from a patient;
 - (ii) debulking the apheresis sample of excess white blood cells;
 - (iii) separating CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample using microfluidic separation; and
 - (iv) isolating separated CTC clusters and/or CTC-WBC clusters;
- 10 wherein the debulking step comprises a flow-based process where flow is driven by gravity and/or minimal applied artificial pressure; wherein the microfluidic separation is performed using a microfluidics device capable of separating CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample.
- 15 2. The process according to claim 1, wherein the apheresis sample of (i) is a sample obtained under conditions of leukapheresis.
3. The process according to claim 1 or claim 2, wherein the debulking step of (ii) comprises a process of capture of excess white blood cells, wherein the capture process
 - 20 comprises a flow-based process where flow is driven by gravity and/or minimal applied artificial pressure.
4. The process according to claim 3, wherein the debulking step of (ii) comprises the magnetic capture of excess white blood cells magnetically labelled via one or more antigens
 - 25 on the surface of said excess white blood cells as a result of a prior step of treating the apheresis sample with affinity labelled magnetic particles, wherein the affinity label of the affinity labelled magnetic particles is capable of binding to one or more antigens on the surface of excess white blood cells.
- 30 5. The process according to claim 6, wherein the affinity label of the affinity labelled magnetic particles is an antibody protein capable of binding to one or more antigens on the surface of excess white blood cells, for example, wherein the one or more antigens on the surface of said excess white blood cells is selected from the group consisting of CD45, CD31 and CD11b.
- 35 6. The process according to any one of claims 1 to 5, wherein the flow-based process of the debulking step of (ii) is a chromatographic process wherein the stationary phase in the

chromatography is capable of preventing the elution of excess white blood cells and, for example, is a process of magnetic capture of excess white blood cells wherein the stationary phase in the chromatography is capable of preventing the elution of magnetically labelled excess white blood cells.

5

7. The process according to claim 6, wherein the chromatographic process is a column-based chromatographic process and the apheresis sample is passed through a column by a flow-based process where flow is driven by gravity without application of artificial pressure.

10 8. The process according to any one of claims 1 to 7, wherein the microfluidics device of (iii) comprises a cell separation cassette.

9. The process according to claim 8, wherein the cell separation cassette comprises a stepped cassette configuration wherein the steps define flow channels of different critical gap wherein the critical gap becomes progressively smaller in the forward direction through the microfluidics device such that CTC clusters and/or CTC-WBC clusters are separated from other components of the debulked apheresis sample by their failure to pass through the critical gap at a step in the cell separation cassette .

20 10. The process according to claim 9, wherein the stepped cassette configuration is unidirectional.

11. The process according to any one of claims 1 to 10, wherein the microfluidic separation of step (iii) is performed using a microfluidics device capable of separating CTC clusters and/or CTC-WBC clusters from other components of the debulked apheresis sample on the basis of their physical characteristics, such as their size, shape and deformability, or a combination thereof.

12. The process according to any one of claims 1 to 11, wherein the step (iv) of isolating separated CTC clusters and/or CTC-WBC clusters step comprises eluting separated CTC clusters and/or CTC-WBC clusters from the microfluidics device by passing a liquid in the reverse direction through the microfluidics device.

13. The process according to any one of claims 1 to 12, wherein the process further comprises the step (v), which follows step (iv), of sorting individual circulating tumour cells (CTCs) derived from isolated circulating tumour cell (CTC) clusters and/or circulating tumour

cell-white blood cell (CTC-WBC) clusters, by a process of cytometric fluorescence-activated cell sorting (FACS).

14. The process according to any one of claims 1 to 13, wherein the apheresis sample is
5 an apheresis sample from a patient that has a cancer.

15. The process according to any one of claims 1 to 14, wherein the process is a process
for the isolation of circulating tumour cell (CTC) clusters and/or circulating tumour cell-white
blood cell (CTC-WBC) clusters derived from a tumour in a patient with a cancer.

10