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Circulating tumor cells: a review of non EpCAM-based approaches for cell enrichment and isolation

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Key words: Circulating Tumor Cells (CTCs), EpCAM, epithelial-mesenchymal transition (EMT), metastatic process, recurrent disease, biomarker

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24 **Abstract**

25

26 **Background:** Circulating tumor cells (CTCs) are biomarkers for non-invasively
27 measuring the evolution of tumor genotypes during treatment and disease progression.
28 Recent technical progress has made it possible to detect and characterize CTCs at the
29 single-cell level in blood.

30 **Content:** Most current methods are based on epithelial cell adhesion molecule
31 (EpCAM) detection, but numerous studies have demonstrated that EpCAM is not a
32 universal marker for CTC detection since it fails to detect both carcinoma cells that
33 undergo epithelial-mesenchymal transition (EMT), and CTCs of mesenchymal origin.
34 Moreover, EpCAM expression has been found in patients with benign diseases. A large
35 proportion of the current studies and reviews about CTCs describe EpCAM based
36 methods, but there are evidences that not all tumor cells can be detected using this
37 marker. Here we describe the most recent EpCAM-independent methods for enriching,
38 isolating and characterizing CTCs, based on physical and biological characteristics, and
39 point out their main advantages and disadvantages.

40 **Summary:** CTCs offer an opportunity to obtain key biological information required for
41 the development of personalized medicine. However there is no universal marker of
42 these cells. To strengthen the clinical utility of CTCs, it is important to improve existing
43 technologies and develop new, non-EpCAM based systems to enrich and isolate CTCs.

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49 Circulating tumor cells (CTCs) are defined as cells that originate in primary tumors,
50 recurrences, or metastases. They circulate freely in peripheral blood, and have antigenic
51 and genetic characteristics specific to the tumor of origin [1]. CTCs are important
52 because the majority of deaths from cancer are linked to the development of
53 disseminated metastases [2]. In the last few years, emerging data have challenged the
54 traditional theory of sequential metastasis development [3] (Supplemental Figure 1).
55 Several studies have pointed out that CTCs can be isolated in patients at relatively early
56 stages of tumor growth [4, 5], even before the primary tumor mass is detected by
57 conventional methods [6]. Furthermore, current high-resolution imaging technology is
58 not sensitive enough to detect micro-metastases or early tumor cell dissemination,
59 which are the key events in tumor progression (Supplemental Figure 1).

60 Because they can be obtained by non-invasive methods, CTCs can be used as
61 therapeutic markers for monitoring treatment effectiveness in real-time, and for
62 detecting recurrent disease. CTCs also have potential for evaluating drug resistance
63 mechanisms, and may have utility in estimating the risk of metastatic relapse and
64 progression. Unlike the characterization of primary tumors, which only provides a static
65 view at the time of diagnosis, analyzing CTCs may improve understanding of the
66 different steps involved in the metastatic cascade, from invasion of tumor cells into the
67 blood stream to the formation of clinically-detectable metastases [7].

68 Although studying circulating tumor cells is a promising approach for better
69 characterizing cancer, there are certain issues inherent to the nature of CTCs that should
70 be considered. CTCs are rare events, which are present at very low concentrations in the
71 blood (i.e. one tumor cell in the background of millions of blood cells) [8]. In addition,
72 only a restricted number of CTCs has the ability to generate metastases [9] and
73 consequently it is necessary to characterize them precisely to be able to distinguish

74 metastatic and non-metastatic CTCs. Numerous methods have been developed to isolate
75 tumor cells, most of which are based on epithelial cell adhesion molecule (EpCAM)
76 detection. Indeed, EpCAM is a conventional marker expressed by cancer cells of
77 epithelial origin and has been then used for carcinoma cell isolation. However, as
78 described below, EpCAM is not expressed by all CTCs and alternative approaches need
79 to be considered. There are multiple recent useful reviews on CTC isolation methods
80 [10-12] but none of them have exclusively focused on non-EpCAM based methods. The
81 aim of this review is to provide an overview of the most recent EpCAM-independent
82 methods for enriching, isolating and characterizing CTCs.

83

84 **EpCAM is not a universal biomarker for isolating CTCs**

85 A great deal of effort and resources has been invested into developing methods for
86 detecting CTCs in peripheral blood. In the last decade, several methods have emerged
87 for detecting and characterizing CTCs. However, these methods and consequently the
88 biological characterization of CTCs are still technically challenging. The first step in the
89 detection of CTCs was the discovery that EpCAM was expressed at variable degrees on
90 epithelial derived carcinomas and related cancers but was absent in the peripheral blood
91 cells [13]. This finding resulted in the investigation and development of different
92 methods for enriching and isolating CTCs based on the EpCAM marker [14, 15] and led
93 to the first and only automated EpCAM-based system (CellSearch[®]) currently approved
94 for clinical use by the United States Food and Drug Administration for the detection of
95 CTCs. CellSearch[®] is thus considered the gold standard for CTC detection methods [16,
96 17]. However, recent evidence has challenged the suitability of this method; EpCAM-
97 positive circulating epithelial cells have been reported in patients with benign colon
98 diseases [18] and are a potential source of false-positive findings. In addition,

99 carcinoma cells can undergo epithelial-mesenchymal transition (EMT) which results in
100 decreased expression of epithelial markers, such as EpCAM and CK [19], and the
101 appearance of mesenchymal markers. The loss of epithelial markers may therefore
102 result in false-negative findings. In this context, the EpCAM marker is not suitable for
103 isolating CTCs from carcinomas that have undergone EMT or those cancers with
104 primary mesenchymal origin. Consequently EpCAM cannot be considered as a
105 universal marker for CTC detection. This highlights the need to develop non-EpCAM
106 based technologies for isolating and detecting CTCs.

107

108 **Enrichment of circulating tumor cells: conventional methods**

109 The major challenge for isolating and characterizing CTCs is their low concentration
110 compared to the other cell types in the peripheral blood. Enrichment approaches take
111 into consideration several parameters: capture efficiency/recovery rate, purity, cell
112 viability, processing speed, blood sample capacity, sample pre-processing requirements,
113 cost of consumables and equipment, repeatability and reliability. The optimal
114 enrichment solution may require a compromise between these performance parameters
115 and the intended downstream application. Current enrichment approaches include a
116 wide range of technologies based on the different properties of CTCs that distinguish
117 them from surrounding normal hematopoietic cells, including biological properties (cell
118 surface protein expression, viability, invasive capacity) and physical properties (size,
119 density, electric charges, deformability) (Figure 1).

120

121 *Methods based on physical properties*

122 Cytological analyses have revealed that CTCs exhibit a greater nuclear to cytoplasm
123 ratio, are larger in size, and have different nuclear morphology compared to normal

124 cells [20]. These cytological alterations result in the differences of their mechanical
125 properties, providing CTCs with several capabilities. The cytoskeletal stiffness of CTCs
126 is dynamically modified. This flexibility may facilitate their invasion to distal sites from
127 the primary tumor, and may confer their resistance to damage from fluid shear stress
128 within the blood vessels during the metastatic process [21]. These modifications in the
129 stiffness alter the conservation of the membrane structure, which in turn affects their
130 surface charge and electrical properties [21]. Various approaches have been used to
131 exploit the differences in physical properties between tumor cells and blood cells as a
132 means of enriching and separating CTCs from blood samples (Figure 1).

133

134 Density gradient centrifugation is a conventional approach for separating blood
135 components based on differences in their sedimentation coefficients. As whole blood is
136 deposited in the liquid gradient and subjected to centrifugation, cells will distribute
137 along the gradient depending on their density (Figure 1, Table 1). Erythrocytes or
138 polymorphonuclear leukocytes migrate to the bottom, while mononuclear leukocytes
139 and CTCs remain at the top as a buffy coat [22]. Percoll, Ficoll-Hypaque® (GE
140 Healthcare Life Sciences, Little Chalfont, United Kingdom) and OncoQuick® (Greiner
141 Bio-One, Kremmünster, Austria) are the most commonly used density gradient media in
142 pre-clinical and clinical research. Ficoll-HyPaque®, formed by the copolymerization of
143 sucrose and epichlorohydrin, is mainly used in biology laboratories to recover
144 peripheral blood mononuclear cells. Despite its long history of use in laboratories, there
145 are some pitfalls associated to this technique, such as the possible loss of tumor cells
146 that migrate either to the plasma fraction or to the bottom of the gradient due to the
147 formation of aggregates [22]. It has been suggested that this cell loss may be due to the
148 cytotoxicity of the density medium [23]. Alternative to Ficoll, there is Percoll density

149 (GE Healthcare Life Sciences) gradient medium made of a colloidal silica particle
150 suspension. The main advantages over Ficoll include reduced toxicity and a wider
151 density gradient range [23]. There are certain discrepancies in the literature regarding
152 the use of Percoll since some studies report a high purity rate [24] while others report
153 low isolation efficiencies compared to Ficoll [25]. A third density system named
154 OncoQuick® is composed of a 50 mL tube with a porous barrier inserted above a
155 separation medium. Cells are separated and pass through the barrier depending on their
156 different buoyancy densities during centrifugation. CTCs, together with the
157 lymphocytes, will remain above the porous barrier, making them easily accessible for
158 subsequent collection. OncoQuick® has mildly higher reported recovery rate compared
159 to Ficoll density gradient, 87% and 84%, respectively [26]. Moreover the mononuclear
160 cell depletion using the OncoQuick® system is significantly higher compared to Ficoll;
161 this facilitates processing higher sample volumes, which is beneficial for CTC
162 characterization [26, 27]. However, during the isolation process, CTCs migrate into the
163 plasma fraction and are frequently lost [29]. Overall major advantages of all the density
164 centrifugation methods are that they are inexpensive and reliable (Table 1). However,
165 the disadvantages include the loss of large CTCs and CTC aggregates that fall to the
166 bottom [29], as well as the fact that leukocytes cannot easily be eliminated, resulting in
167 very low purity. It is therefore necessary to combine centrifugation with another
168 enrichment method.

169

170 Microfiltration enrichment methods process circulating cells through an array of
171 microscale constrictions in order to capture target cells based on their size or a
172 combination of size/deformability. There are multiple different microfiltration devices,
173 some are available on the market and others remain currently prototypes (Figure 2).

174 Membrane microfilters are composed of a semipermeable membrane with a 2D
175 array of micropores. A membrane with a pore size diameter of $8\mu\text{m}$ has been
176 demonstrated to be optimal for CTC retention [30]. The typical configuration used for
177 microfiltration is dead-end filtration (Figure 2A), in which the blood flow is
178 perpendicular to the membrane. The main limitation of this strategy is that the layer of
179 cells retained on the membrane can reduce the efficiency of recovery due to the build-up
180 of filtration resistance [32] (Table 1). To overcome this issue, Zheng et al. created a 3D
181 membrane microfilter consisting of two pored layers (Figure 2B), between which CTCs
182 are retained [32]. In contrast to conventional microfiltration devices, this system reduces
183 the tension stress on the cell plasma membrane and demonstrates a high recovery rate
184 (86% with a theoretically fast throughput of 3.75 mL/min) [32].

185 Another system based on a 2D membrane slot filter (Figure 2C) was proposed
186 by Lu et al., in which the forces exerted on the cells are reduced, reaching viability of
187 90% with a high recovery [33]. The bead-packed filtration device consists of a chamber
188 where uniform beads measuring $45\ \mu\text{m}$ in diameter and non-uniform beads (with
189 diameters ranging from 15 to $100\ \mu\text{m}$) are packed and act as the filtration element
190 (Figure 2D) that retains CTCs and allows red and white blood cells to pass through [33].
191 Studies performed by Lin *et al.* reported a low recovery rate (between 21% and 40%) in
192 contrast to filtration performed using membrane systems [34].

193 There are systems available that make it possible to enrich and isolate CTCs in a
194 single step. For example, ScreenCell[®] technology (ScreenCell, Sarcelles, France), is an
195 innovative single-use and low-cost device. It is based on a filter that isolates and sorts
196 tumor cells by size. There are three different types of device, depending on the
197 downstream analysis: ScreenCell[®] Cyto (molecular techniques that require fixed cells),
198 ScreenCell[®] CC (cell culture) and the ScreenCell[®] MB (RNA or DNA analysis) [35,

199 36]. The main advantages of this system are its low-cost, small format and ease of use.
200 Another platform in development is the parylene-C slot microfilter that measures
201 telomerase activity from captured, viable CTCs. It has 90% recovery rate [36]. The 90%
202 of the cells recovered are viable and yield 200-fold sample enrichment [36]. In contrast
203 to ScreenCell, parylene-C only detects viable CTCs and can be re-used.

204 Filtration allows for rapid CTC enrichment from large volumes of blood in
205 minutes, with minimal processing. Recovery rates are around 90%, but further
206 processing is required for certain downstream applications, as the final purity is
207 typically around 10% or less. The main disadvantages associated with filtration are: the
208 heterogeneity in CTC size, cluster formation, the possibility of membrane clogging,
209 difficulties in the detachment of cells retained in the filter, as well as the background
210 signal on the filters after immunostaining for CTC detection.

211

212 Microfluidics includes several separation methods, which makes it possible to
213 manipulate very small volumes of biological fluids. The past decade has seen many new
214 technologies proposed for biological cell sorting and analysis on microchips. Arrays
215 with pillars of varying geometries have been used to fractionate cells in blood and
216 capture tumor cells [37]. Similarly, crescent-shaped trap arrays with a fixed 5 μm gap
217 width within microfluidic chambers have been used to enrich CTCs from whole blood
218 without pre-processing [38]. Parsortix system (Angle, Guildford, United Kingdom)
219 (Figure 3A) is microfluidic technology that captures CTCs based on their less
220 deformable nature and larger size compared to other blood components. With this
221 system, it has been reported a higher number and purity of isolated CTCs in patient
222 samples than with the Cell Search. Moreover, the processing time of 7.5 mL of whole
223 blood is 2 h in contrast to the 4 h reported for the Cell Search (Cell Search, Jansen

224 Diagnostic, Raritan, NJ, USA). It is worth to remark that with the second version of
225 Parsortix 10 mL can be processed in 2.5 h. The main drawback of this technique is the
226 difficulty of eliminating all leukocytes due to size overlap with CTCs [39].

227 In addition to the previous devices described above, ClearCell[®] FX (Clearbridge
228 Biomedics, Singapore) recovers viable cells in small sample volumes and in a short
229 period of time (e.g. 1 mL of blood in 10 minutes) (Figure 3B) [40]. ClearCell[®] FX does
230 not require pre-processing of the blood; this decreases the possibility of losing cells of
231 interest (Table 1). This system takes advantage of the inertial and centrifugal forces
232 causing the smaller red and white blood cells to flow along the channel's outer wall and
233 the larger CTCs to flow along the inner wall, recovering both fractions in different
234 channels of the system. Unfortunately, CTCs of different sizes may escape through the
235 white/red cell channels, and certain white blood cells can be captured in the CTC
236 fraction.

237 To limit CTC loss to white and red cell channels, CTC-iChip technology (D.A.
238 Harber, Massachusetts General Hospital Cancer Center; M. Toner, Harvard Medical
239 School; Boston, MA) was developed (Figure 3C). CTC-iChip technology combines
240 continuous deterministic lateral displacement for size-based separation of red blood
241 cells/platelets from tumor cells obtained from whole blood, inertial focusing for precise
242 positioning of cells in a microchannel, and microfluidic magnetophoresis for
243 immunomagnetic depletion of white blood cells. This is a fast system (it can process 8
244 mL of blood per hour) allowing the recovery of any viable cancer cell types according
245 available for their characterization. Unfortunately, in the deterministic lateral
246 displacement step, small CTCs are lost, and undesired large cells and aggregates pass on
247 to the next step, due to particle deformability and can limit the use of this device [41].

248 Carefully applied microfluidic approaches are capable of achieving both
249 excellent purity of more than 80% and high recovery rates with little disturbance to the
250 CTCs. However, these advantages come at the expense of lower throughput requiring
251 either reduced sample volumes or prolonged periods of time to process samples (e.g.,
252 several hours to process a full tube of blood).

253

254 Dielectrophoresis (DEP) has been initially described by Pohl as “the translational
255 motion of neutral matter caused by polarization effects in a non-uniform electric field”
256 [42]. To move a particle by dielectrophoresis, the particle needs to be polarizable once an
257 electrical field is applied [43]. This phenomenon has inspired new approaches for the
258 separation of cells based on their electrical properties. Because the DEP force is
259 inversely proportional to the length scale [44], micro-scale chambers named microchips
260 have been developed for isolating rare cell events. These microchips integrate arrays of
261 electrodes to generate a non-uniform alternating current field characteristic of the DEP
262 technology.

263 Interdigitated gold electrodes have been used to separate cancer cells from blood
264 cells [45]. Tumor cells were attracted towards the electric field generated by the
265 electrodes by means of positive DEP, while other cells were flushed away. When the
266 electric field was turned off, the cell initially retained were released and recovered with
267 an approximate rate of 95%. Moon *et al.* created a system with a DEP module
268 integrated into a size-based hydrodynamic step, used as the enrichment stage to remove
269 excess blood cells [46]. The first commercial instrument based on DEP field flow
270 fractionation was the ApoStream™ system (ApoCell Inc., Houston, TX) (Figure 4A).
271 To use this methodology, an initial enrichment step is required. Recovery rate is over
272 70% and the viability more than 97%; however, the purity obtained is less than 1%,

273 although this can be significantly improved with additional enrichment stages at the risk
274 of reduced recovery rate [47]. The DEPArray™ technology (Silicon Biosystems,
275 Bologna, Italy) combines the ability to manipulate individual cells using
276 dielectrophoretic technology with high quality image-based cell selection (Figure
277 4B). The most attractive characteristics of this technology are the single cell
278 resolution, high fidelity recovery, cell viability and, in the most recent version, the
279 possibility of isolating individual cells from paraffin embedded samples [48].

280 Despite the many advantages presented by DEP-based enrichment methods,
281 there are also some limitations, such as low sample volumes that are processed in a
282 non-continuous manner (Table1). Furthermore, the dielectric characteristics of cells
283 can gradually change due to ion leakage; this requires the isolation to be completed
284 within a short period of time after the sample processing starts [49]. In addition, the
285 electric conductivity of the medium used must be low, which is not achievable for all
286 samples studied.

287

288 *Methods based on biological properties*

289 Antibody-based CTC isolation takes advantage of highly specific affinity reactions
290 between capture antibodies and the target antigens present on the cells of interest. CTCs
291 can be captured directly (positive selection) or indirectly (negative selection). Various
292 antigens have been used to detect or isolate CTCs. The most commonly used antibody is
293 EpCAM as it is expressed in all epithelial cells but is absent from blood cells [13, 50].
294 However, the universality of EpCAM may be reduced when carcinoma cells have
295 undergone the EMT process or when detecting tumor cells of mesenchymal origin.

296 Results from our laboratory have revealed the presence of EpCAM-expressing
297 and non-expressing CTCs after the injection of either EpCAM expressing or non-

298 expressing tumoral cells in mouse paratibias (Supplemental Figure 2). Several organ- or
299 tumor-specific markers, such as CEA, EGFR, PSA, HER-2, MUC-1, EphB4, IGF-1R,
300 cadherin-11 and CSV have also been reported for antibody-based isolation of CTCs
301 (Supplemental Table 1).

302 Immunoaffinity-based CTC isolation is based on antibody-conjugated magnetic
303 nanoparticles or microbeads that often bind to a specific surface antigen [51, 52]. After
304 antigen-antibody interaction, the sample is exposed to a non-uniform magnetic field to
305 capture labeled cells. This method can attain high recovery and purity rates, with single-
306 step detection and isolation of CTCs [51, 53]. The performance of the immunomagnetic
307 method depends directly on both the expression and specificity of the target antigen, as
308 well as on the binding quality of the associated antibody, the efficiency of the
309 immunomagnetic labeling process and magnetic particles, and the separation
310 mechanism designed to isolate labeled cells. A “cocktail” of antibodies targeting
311 multiple antigens can also be used to partially overcome the lack of specificity of
312 current tumor markers [51, 54]. Another approach is negative isolation of CTCs by first
313 lysing erythrocytes and using specific markers to magnetically deplete leukocytes.
314 CD45 is the most frequently used marker for leukocyte depletion. The RosetteSep[®]
315 (STEMCELL Tech, Cambridge, United Kingdom), is a CTC negative selection system
316 based on a mixture of antibodies that specifically crosslink red blood cells to each other
317 and to white blood cells, forming cell rosettes consisting of multiple red and white
318 blood cells. Due to the higher density of these clusters, they can effectively be separated
319 from CTCs by a single centrifugation step. Negative selection methods are completely
320 independent with regard to CTC phenotype, so they are not biased by a particular CTC
321 marker. Negative selection also leaves CTCs untouched, which may result in higher
322 viability. To achieve an acceptable degree of CTC purity, this separation method

323 requires a very high specificity to remove all the leukocytes and needs to avoid non-
324 specific CTC binding. The binding between primary antibody and magnetic particles
325 can be a direct (single-step) or indirect (two-step) method. The latter is composed of
326 secondary antibodies that are already bound to magnetic particles and can specifically
327 bind to an epitope on the primary antibody, potentially reaching higher labeling
328 efficiency. This indirect approach shows a 15-fold increase in labeling efficiency
329 compared to direct methods [55].

330 Regarding the use of the magnetic separation procedure to recover labeled cells,
331 there are many different alternatives. In the batch separation approach, the whole
332 labeled sample is subjected to a magnetic field at once, resulting in the migration of
333 labeled cells to the regions of higher magnetic frequency [56]. The EasySep™ system
334 (STEMCELL Tech, Cambridge, United Kingdom), MojoSort™ (Biolegend, San Diego,
335 CA) and Dynabeads® (ThermoFisher, Waltham, Massachusetts) are based on this
336 principle. Variations of these systems have been developed to increase the processed
337 volume. Thus, continuous-flow separation can be used in which the sample is
338 continuously fed through the separation module. This module can have an activated
339 filter to capture and retain the labeled cells, like the commercially available MACS®
340 (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and MagniSort™ (eBioscience,
341 San Diego, CA). Alternatively, the magnetophoresis mode can be used to selectively
342 manipulate the direction of labeled cells within the flow and collect them at designated
343 outputs [57, 58]. Reported recovery rates using these magnetic enrichment systems have
344 shown significant variations (10–90%) [59 60]. This variation can be explained because
345 the magnetic gradient generated by the separation structure can only attract labeled cells
346 within a limited distance. The MagSweeper® system is a proposed (Figure 5A) solution
347 [61] that uses a robotic arm equipped with a magnetic rod that binds labeled cells. This

348 was initially demonstrated for the recovery EpCAM-positive cells but can be adapted
349 for other CTC markers. Recovery rates of 60% using this device have been reported
350 [54].

351 Micro-scale separation devices have been also developed. Isolation efficiency in
352 an immunomagnetic microfluidic chip is mainly governed by an equilibrium between
353 hydrodynamic and magnetic forces acting on the labeled cells [62]. Hoshino *et al.*
354 described immunomagnetic capture system for CTCs, based on a microchannel on top
355 of a stack of permanent magnets (Figure 5B). As the sample flows into the
356 microchannel, the magnetic gradient attracts the labeled cells. Recovery rates around
357 86% have been attained with this system [63].

358 Finally, CTCs can be recovered using adhesion-based methods that exploit the
359 ability of CTCs to bind to a surface whose biochemical and topographical properties
360 have been modified without the need to label the cells. In static adhesion-based assays,
361 the sample is first incubated on the capture surface. Non-adherent, supposedly non-
362 target cells are washed off, leaving the CTCs attached to the surface. On the basis of
363 this approach, cell adhesion matrix (CAM) has been used to detect and isolate the most
364 invasive CTCs from patients with metastatic and local carcinomas of different origins
365 [64, 65]. Microfluidic adhesion-based devices consist of microchannels coated with an
366 antibody against CTCs. Their design determines both the efficiency of the cell binding,
367 and the recovery rate by influencing the flow rate [66, 67]. Among these devices,
368 OnQChip™ (On-Q-ity, Waltham, MA) and the CEE™ chip (Biocept Laboratories, San
369 Diego, CA) are two commercialized microfluidic devices that have incorporated 3D
370 structures (microposts) to increase the effective surface, thus promoting cell adhesion
371 (Figure 5C). The first combines antibody affinity and size selection for the capture of
372 CTCs and the second is based in immunoaffinity. In this field, Hughes *et al.* have

373 developed, for instance, a microfluidic system based on the binding of E-selectin, a
374 molecule present in the endothelium on to which CTCs adhere prior to their
375 extravasation [68]. This approach has attained high flow rates compared to the other
376 adhesion-based methods (4.8 mL/h) and approximately 50% of capture efficiency.
377 Interestingly, this device demonstrated a higher efficient based on the number of CTCs
378 isolated, compared to the CellSearch® system [68].

379

380 **Concluding remarks**

381 We have highlighted the non-EpCAM-based methods for CTC enrichment/isolation.
382 The major advantage of these techniques is that they can enrich for CTCs that do not
383 have EpCAM expression. However, many challenges associated with current
384 methodologies must be faced, such as the need to improve purity and recovery rates,
385 throughput, cell viability after recovery, and enrichment rates.

386 It would be beneficial to identify properties exclusive to CTCs, which may take
387 the form of a single “master” marker or a combination of antibodies able to recognize
388 all the CTCs present in the sample. Moreover, it would be desirable that those
389 properties or markers were able to distinguish between metastatic and non-metastatic
390 CTCs. Unfortunately, current knowledge does not make it possible to clearly identify
391 and classify CTCs. This information is of most importance in clinics, for the prognosis
392 of the disease, treatment decisions or the effectiveness of the treatment applied.

393 Despite the numerous methods for isolating CTCs described in the literature,
394 some are still at the proof of concept stage with evidences only in cultured cells. The
395 main drawback is that cell lines do not reflect effectively CTCs in a natural biological
396 fluid, especially in terms of heterogeneity [69]. It would be interesting to develop new
397 cell lines that exhibit the genomic and transcriptomic heterogeneity of cancer cell lines.

398 Recently, Alix-Panabières *et al.* [70] and Haber *et al.* [71] have reported the isolation of
399 CTCs and their growth in culture for the establishment as a cell line to examine tumor
400 heterogeneity. Another important point is the necessary sample volume required for
401 CTC isolation. In most cases, the inability to process whole blood is due to high cell
402 concentration or the necessity for reducing sample volume due to the device's capacity.
403 A frequently proposed solution is the dilution of samples; however, this is not ideal
404 since dilution reduces the probability of CTC capture and the prolonged enrichment
405 time compromises cell viability. In addition, the biological characteristics of the cells
406 can be altered by the composition of the dilution buffer.

407 When using immunologically-based enrichment methods, the wide range of
408 phenotypes presented by CTCs make it necessary to use specific cocktails for cell
409 surface epithelial and mesenchymal markers, that not cross-react with other blood cells
410 [72]. Yokobori *et al.* described Plastin 3 as a good alternative for avoiding the use of
411 large cocktails of antibodies, because this marker is not downregulated in CTCs during
412 their EMT and is not expressed in blood cells [73]. Although positive selection is very
413 specific and a high purity can be obtained, the presence of some uncharacterized CTCs
414 in each individual blood sample should be taken into consideration. This can be avoided
415 by negative selection, in which the blood sample is depleted of leukocytes using
416 antibodies against CD45 and other leukocyte antigens (not expressed on carcinomas or
417 other solid tumors). However, cytokeratin⁺ and CD45⁺ sub-cell populations have been
418 described and may be related to various artifacts such as cell doublets or non-specific
419 antibody bindings [74] or circulating cancer-associated macrophage-like cells [75]. The
420 role of EMT in tumor cell dissemination stimulates the development of technologies
421 based on the depletion of normal CD45⁺ hematopoietic cells to limit loss of CTCs with

422 high phenotypic plasticity. However, it should be noted that not all CD45⁻ cells in the
423 blood are tumor cells (e.g., circulating endothelial cells) [11].

424 In the last decade, the strong interest of CTCs has accelerated the development
425 of numerous isolation technologies based on EpCAM independent methods.
426 Technologies based on physical approaches (density gradient centrifugation,
427 microfiltration, microfluidics, dielectrophoresis) or biological properties of CTCs (e.g.
428 membranous markers) have been demonstrated. However, further improvements in pre-
429 enrichment steps will improve the capture and characterization of these cells.

430

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698 **FIGURE AND TABLE LEGENDS**

699 **Figure 1. Methods for CTC isolation from whole blood.** 1) Methods based on
700 biological properties: immunoaffinity-based techniques target specific markers to
701 selectively enrich CTCs or leukocyte depletion. 2) Physical properties such as size,
702 deformability, density and electrical properties can also be used to separate CTCs from
703 blood cells.

704

705 **Figure 2. Microfiltration devices for CTC enrichment.** A) Dead end filtration; B) 3D
706 membrane microfilter. The smaller cells can easily traverse the gap while the large cells
707 (e.g., tumor cells) will be trapped. Two types of force are exerted in the trapped cell
708 such that force is caused by hydrodynamic pressure from the top and supporting force
709 from the bottom membrane; C) 2D membrane slot filter design; D) Bead pack based
710 filtration. The microchannel entrance is blocked by packing large sized beads. Different
711 bead sizes were used to implement a blood/plasma separator at the inlet of the
712 microchannel. When whole blood was dropped into the inlet of the microchannel, the
713 structure allowed for the capillary flow of blood through the hetero-packed beads.
714 During this movement of blood, the red blood cells pass through small pores while large
715 cells such as CTCs are blocked from flowing into the channel.

716

717 **Figure 3. Microfluidic devices for isolating CTCs.** A) Parsortix (Angle). The patented
718 microfluidic technology inside a cassette captures CTCs based on their less
719 deformability and larger size compared to other blood components; Left diagram (plan
720 view) and right diagram (cross section to see in details the device). B) ClearCell[®] FX
721 (Clearbridge Biomedics). The inertial and centrifugal forces transport the smaller red

722 and white blood cells along the channel's outer wall and the larger CTCs along the inner
723 wall recovering both fractions in different channels of the system; C) CTCi-chip
724 technology, combines continuous deterministic lateral displacement (DLD) for size-
725 based separation of blood cells, inertial focusing for precise positioning of cells in a
726 microchannel and microfluidic magnetoforesis for immunomagnetic depletion of white
727 blood cells.

728

729 **Figure 4. Dielectrophoretic based approaches.** A) ApoStream™ from ApoCell
730 (adapted from [47]); B) DepArray™ technology from Silicon Biosystems
731 (<http://www.siliconbiosystems.com/depparray-system>).

732

733 **Figure 5. Antibody-based CTC isolation approaches.** A) MagSweeper (figure
734 adapted from [61]). Magnetic beads were coated with an antibody targeting surface
735 markers and mixed into blood samples to bind cancer cells, which are captured with the
736 magnetic rod. After several washings, the cells are extracted using a magnetic source.
737 B) Microchip –based immunomagnetic assay. The sample is pumped in continuously
738 through the microchannel, causing non-captured blood cells to exit the chip, whereas
739 CTCs are retained due to the magnetic force. C) Diagram representation of OnCChip™
740 (On-Q-ity) and CEE™ (Biocept) devices. These cell enrichment technologies exploit
741 the placement of posts and flow rates through mathematical modeling to enhance
742 isolation, and capture CTCs within a microfluidic channel.

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747 **Table 1. Major advantages and disadvantages of CTCs enrichment methods.**

Method	Advantages	Disadvantages
Density gradient centrifugation	Inexpensive Reliable	Loss of large CTCs and cell aggregates Low purity Additional enrichment techniques required
Microfiltration	Rapid processing of large volumes High efficiency	Low purity Membrane clogging Different size of CTCs Difficulties to detach CTCs from the filter
Microfluidics	Excellent purity High capture rates Little cell disturbance	Long-time consuming Sample pre-processing requirement to reduce volume
Dielectrophoresis	Single cell isolation High cell viability High efficiency	Limited volume Low purity in some devices Cell electrical properties can be affected during the procedure Large number of parameters must be controlled simultaneously
Immunoaffinity based methods	High recovery High purity rates High cell viability using negative selection	Lack of cancer specific markers Heterogeneous expression of markers in cells Problems with the antibody affinity or specificity

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Figure 1

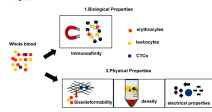


Figure 2

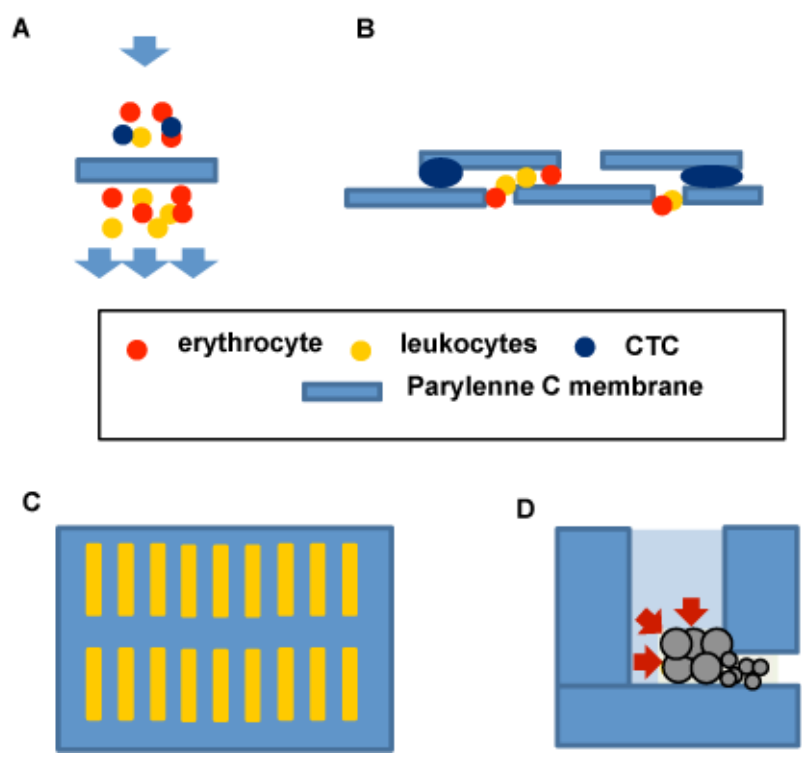
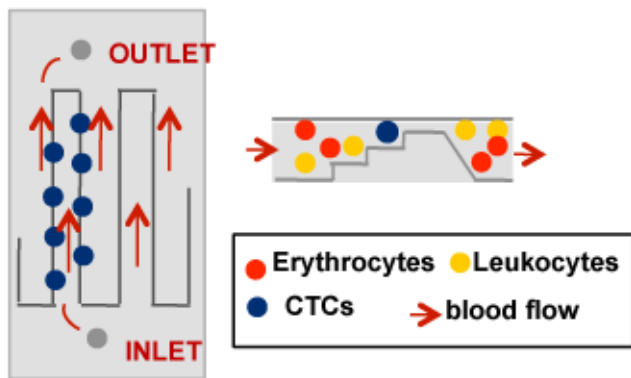
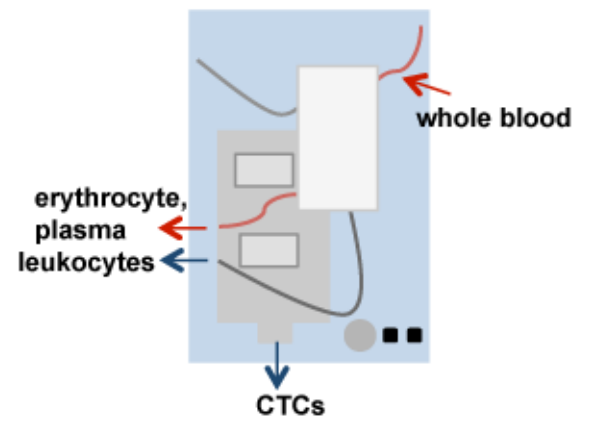


Figure 3

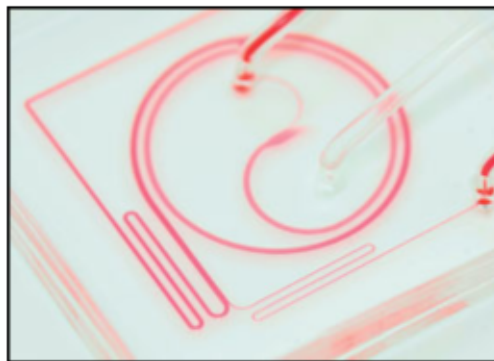
A

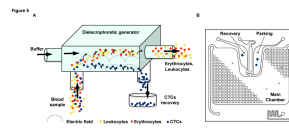


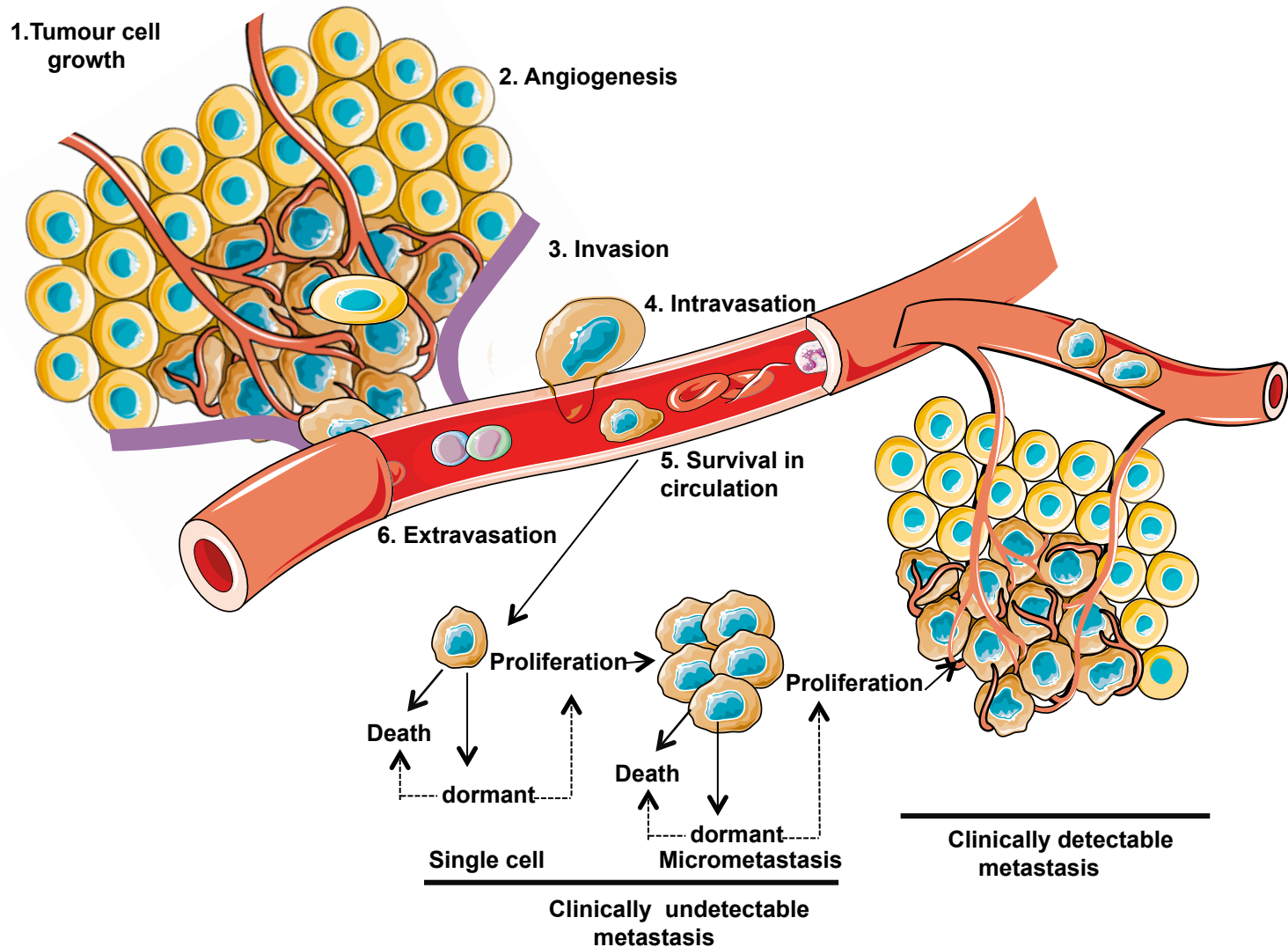
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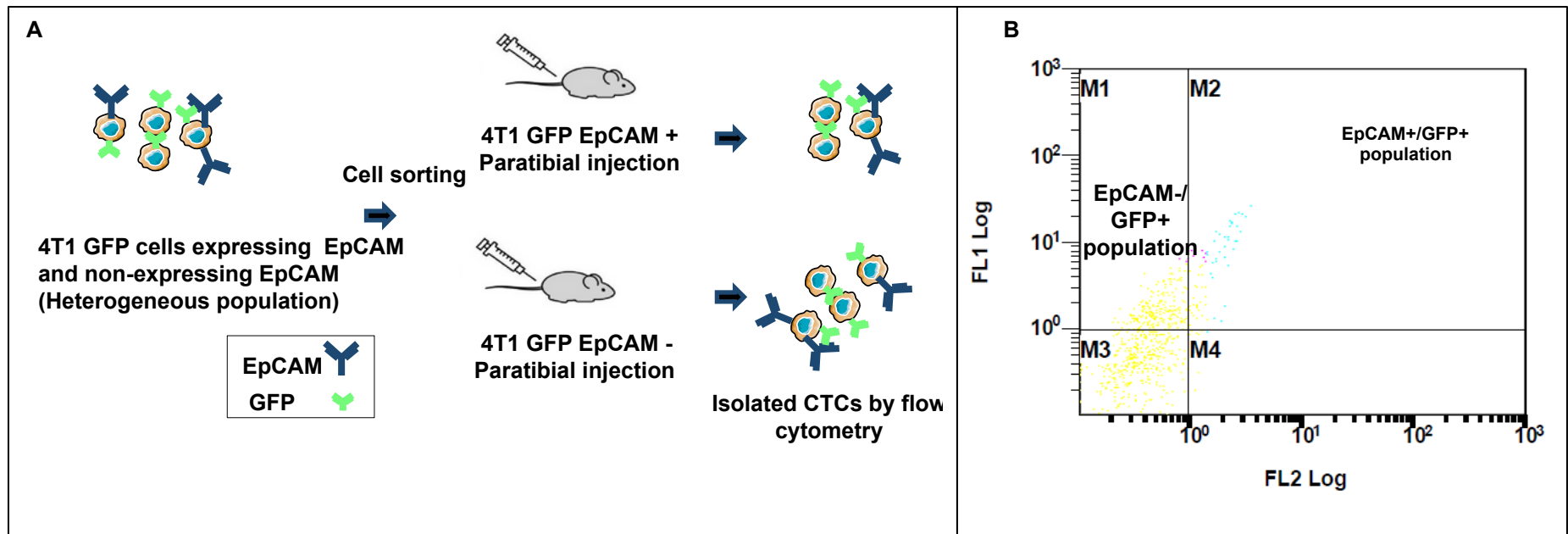
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Supplemental Figure 1. Overview of the key molecular events in metastasis. During the metastatic process, cancer cells proceed through a series of limiting steps to form a secondary tumor. In the initial stages, tumour growth (1) is associated with neoangiogenesis (2). Cancer cells detach from the primary tumor mass, invade adjacent tissues (3) and then enter the lymphatic or circulatory systems (4), which transport them to distant sites (5) from where they extravasate (6) and enter the surrounding microenvironment. At this point, specific factors determine whether the cells will proliferate to form a clinically detectable metastasis or if they are to remain dormant as single cells or micrometastases.



Supplemental Figure 2. Flow-through experiment with paratibial injection of 4T1 cells in Nude mice. A) 4T1 GFP cells were sorted by EpCAM expression. We inoculated 1.5 million of 4T1GFP EpCAM⁺ or EpCAM⁻ in paratibias site of 8 NUDE mice respectively. After 25 days we collected blood from animals and we analyzed using flow-cytometry the presence of CTCs in each mouse as, well as EpCAM expression; B) Cytometry plot showing the coexistence of EpCAM⁺/GFP⁺ and EpCAM⁻/GFP⁺ CTCs in blood after 25 days of the injection.