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PII: S0304-3835(18)30204-0

DOI: 10.1016/j.canlet.2018.03.013

Reference: CAN 13803

To appear in: *Cancer Letters*

Received Date: 24 November 2017

Revised Date: 1 February 2018

Accepted Date: 9 March 2018

Please cite this article as: G. Marsavela, C.A. Aya-Bonilla, M.E. Warkiani, E.S. Gray, M. Ziman, Melanoma circulating tumor cells: Benefits and challenges required for clinical application, *Cancer Letters* (2018), doi: 10.1016/j.canlet.2018.03.013.

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Melanoma Circulating Tumor Cells: Benefits and Challenges Required for Clinical Application

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Keywords: circulating tumor cells; biomarker; melanoma; cancer; liquid biopsy.

The implementation of novel therapeutic interventions has improved the survival rates of melanoma patients with metastatic disease. Nonetheless, only 33% of treated cases exhibit long term responses. Circulating tumor cell (CTC) measurements are currently of clinical value in breast, prostate and colorectal cancers. However, the clinical utility of melanoma CTCs (MelCTCs) is still unclear due to challenges that appear intrinsic to MelCTCs (i.e. rarity, heterogeneity) and a lack of standardization in their isolation, across research laboratories. Here, we review the latest developments, pinpoint the challenges in MelCTC isolation and address their potential role in melanoma management.

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The implementation of novel therapeutic interventions has improved the survival rates of melanoma patients with metastatic disease. Nonetheless, only 33% of treated cases exhibit long term responses. Circulating tumor cell (CTC) measurements are currently of clinical value in breast, prostate and colorectal cancers. However, the clinical utility of melanoma CTCs (MelCTCs) is still unclear due to challenges that appear intrinsic to MelCTCs (i.e. rarity, heterogeneity) and a lack of standardization in their isolation, across research laboratories. Here, we review the latest developments, pinpoint the challenges in MelCTC isolation and address their potential role in melanoma management.

Introduction

Although melanoma is potentially curable when detected in its earliest stages, it can metastasize to other tissues, drastically reducing survival rates [5]. Recent advances in immune- and targeted therapies have improved survival for metastatic cutaneous melanoma [35]. However, immunotherapies are highly toxic and effective in only a proportion of patients [40, 48, 57], and the majority of patients undergoing targeted therapies with MAPK inhibitors rapidly develop drug resistance [47, 49, 54]. In order to overcome these challenges, biomarkers that can guide treatment decisions, monitor response to treatment and identify resistance, are urgently required in the clinical setting.

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33 During the last decade, circulating tumor cells (CTCs) have received widespread
34 attention as prognostic biomarkers [1, 18] (see Table I for the full list of terms). These cells
35 are derived from primary and/or metastatic tumors and can be assessed at any point during
36 disease course. Thus, their presence in peripheral blood can serve as a ‘‘liquid biopsy’’ of
37 solid tumors, particularly when a biopsy cannot be undertaken due to inaccessibility of the
38 tumor, or when multiple metastases are present in a patient. For increased clinical benefit, the
39 analysis of these tumor-derived cells needs to assist with (a) disease prognosis, (b) prediction
40 of clinical outcome to specific treatment, (c) patient-tailored, real time monitoring of
41 response, (d) early detection of treatment resistance or recurrence and progression, and (e)
42 discovery of new therapeutic targets and mechanisms of resistance. Additionally, the
43 molecular characterization of these rare cells harbors significant information about cancer
44 dissemination.

45 The enrichment and detection of CTCs from patients is critically challenging, mainly due
46 to the limited amount of blood sample available and the very low concentration of these cells
47 in peripheral blood. For melanoma, the difficulties are magnified because common CTC
48 markers, such as EpCAM, used in CTC enrichment of epithelial cancers including breast and
49 prostate cancers, are not commonly expressed by MelCTCs, since melanocytes originate
50 from the neural crest and not the epithelium [19]. In addition, MelCTCs are a very
51 heterogeneous population of cells [21, 25, 29], yet current techniques used to enrich
52 melanoma cells from blood do not commonly consider this factor, as their principle for CTC
53 detection relies on the expression of only one or two markers. The isolation of MelCTCs
54 usually follows two common steps: first, CTCs are enriched from the background of millions
55 of blood cells and second, CTCs are detected and characterized in the enriched fraction.

56 Here we detail the progress of MelCTC isolation techniques, from the use of single
57 surface markers to novel methodologies that rely on physical characteristics of MelCTCs. We

58 also describe the clinical significance of current MelCTC studies, addressing the issues that in
59 our informed opinion, hamper the progress of this research field.

60 **Melanoma CTC Enrichment Techniques**

61 Despite MelCTC heterogeneity [25, 29, 53], methods for their capture and enrichment
62 have relied predominantly on the expression of one or two known cell surface markers
63 (Figure 1). For example, immunomagnetic enrichment with magnetic beads coupled to
64 antibodies against known melanoma-specific antigens has been used to enrich CTCs (positive
65 selection). Alternate methods that deplete white blood cells (WBCs) using beads targeting
66 common leukocyte (CD45 or CD34) antigens (negative selection) are also widely used [28,
67 34, 44, 52].

68 The CellSearch™ system (Veridex LCC) involves immunomagnetic capture of CTCs
69 followed by cancer-specific marker staining for CTC detection. This system is the only FDA-
70 approved CTC enumeration platform for breast, prostate and colorectal cancer, where
71 EpCAM is used to capture CTCs followed by immunostaining with cytokeratin [2, 10-12].
72 This adhesion molecule has been described as important in tumor growth, EMT and
73 metastasis [37, 38]; EpCAM is however, expressed exclusively in epithelial-derived
74 neoplasms. A different CellSearch™ kit was therefore developed for MelCTC isolation,
75 which captures CTCs expressing melanoma cell adhesion molecule (MCAM) from whole
76 blood and detects CTCs by immunostaining with MCSP/CSGP4/HMW-MAA (melanoma-
77 associated chondroitin sulphate proteoglycan).

78 Khoja and colleagues [28] used this melanoma specific CellSearch™ kit to detect CTCs
79 in 101 metastatic melanoma patients, and found 0-36 CTCs/7.5 mL of blood prior to
80 treatment, with 40% of patients having at least one CTC. Similarly, Rao and colleagues,
81 found 0 to 8,042 CTCs/7.5 mL of blood in 23% of the patients (n = 44), with greater than 10
82 CTCs detected in only three patients (4%) [44]. Given the low frequency of CTCs detected

83 by targeting only one marker for enrichment, other approaches increased the number of
84 antigens targeted, in order to isolate a larger number of MelCTCs.

85 Freeman and colleagues [21] found that using a combination of MCSP, MCAM, ATP-
86 binding cassette sub-family B member 5 (ABCB5), and cluster of differentiation 271
87 (CD271) targeting antibodies, captured MelCTCs in a significantly higher proportion of
88 metastatic melanoma patients than did the use of MCSP or MCAM alone. This finding
89 demonstrated for the first time the high diversity of MelCTCs and improved the sensitivity of
90 bead-based CTC enrichment compared with experiments that targeted single markers. In this
91 study, patients from all stages showed significantly higher numbers of CTCs than controls
92 (n=15), detecting at least 1 CTC/mL of blood in 73.9% of patients (range: 0-2.5 CTCs/mL of
93 blood). However, despite this multimarker approach aimed at improving the sensitivity of
94 CTC capture, it still yielded low capture efficiency (34%) in spiking experiments [21],
95 suggesting that only a few MelCTcs were being isolated.

96 To improve capture efficiency, the herringbone-chip (HB-chip) technology has been
97 developed. This device uses micro-vortices generated by herringbone-shaped grooves to
98 direct cells toward channel walls coated with a combination of antibodies targeting
99 melanoma-specific antigens. The combination of HB-chip technology with a pool of 12
100 melanoma-specific antibodies for detection, allowed capture of CTCs (on average 8
101 CTCs/2.5 mL) by immunostaining in 32/41 (79%) metastatic melanoma patients at various
102 stages of treatment [36]. These results underscore the need for multiple markers to identify
103 MelCTCs given their remarkable heterogeneity. Nevertheless, these methods are not able to
104 capture all CTCs present within a patient, as those that do not express the antigen of interest
105 are missed.

106 To avoid capture bias, other studies have used negative selection procedures which
107 capture leukocytes with anti-CD45 antibody coated beads followed by WBC depletion using

108 magnetic separation. Systems such as EasySep™ or RosetteSep™ use this approach. Fusi
109 and colleagues, using the EasySep™ method and detecting CTCs using gp100 and MLANA
110 (melanoma antigen recognized by T cells 1) by flow cytometry, found 28/32 (87.5%)
111 metastatic patients had CTCs with a median of 53 CTCs/10 mL of blood [22]. Using
112 RosetteSep™, Girotti and colleagues successfully enriched CTCs and injected them into
113 NSG mice to generate CDX models [23]. While the CTC quantities used to generate the
114 models are unknown, it is likely that relatively large numbers of cells were isolated for
115 successful tumor uptake.

116 Although negative selection is advantageous for removing cells that do not express the
117 most common melanoma markers, the purity of CTCs obtained in the enriched fraction is
118 low, hampering their quantification and downstream analysis. In fact, a spiking experiment
119 comparing the recovery and purity of CD45 depletion with positive enrichment, or a
120 combination of both methods, showed that the greatest recovery was found by using negative
121 selection (58% recovery rate). However, the greatest purity of the CTC fraction was obtained
122 by using the combination method (background reduced from $3x10^7$ to $1.5x10^3$of WBCs) [34].

123 To further improve CTC capture, alternative techniques have been developed recently
124 that exploit the larger cell size of MelCTCs compared to WBCs. Although it has been shown
125 that MelCTCs can have a diverse range of cell sizes [4, 41], most of the CTCs are thought to
126 be larger (10-20 µm) than other blood components, such as RBCs (6-8 µm), leukocytes (7-12
127 µm) or platelets (2-3 µm). Taking advantage of this perceived difference in cell size, the
128 “enrichment by size of epithelial tumor cells” (ISET®) technique was developed [58]. The
129 ISET® system uses polycarbonate filters with 8 µm diameter circular pores for CTC
130 enrichment and detection of cells trapped in filters. De Giorgi and colleagues detected CTCs
131 in 29% and 62.5% of patients with primary invasive and metastatic melanoma respectively
132 using qPCR to detect Tyrosinase transcripts after ISET® filtration; the limit of detection was

133 1 CTC/mL of blood [14]. However, this approach also detected benign circulating nevus cells
134 [15], suggesting the inability of this assay to distinguish between benign nevus cells and
135 melanoma cells. Alternatively, when using the same ISET enrichment technique, with CTCs
136 defined by positive immunohistochemistry expression of S100 and negative expression for
137 CD45 or CD144 (leucocyte and endothelial cell markers, respectively), 51/90 (57%)
138 metastatic melanoma patients had detectable CTCs (1-44 CTCs/mL of blood) [29]. The low
139 percentage of metastatic patients with high-burden disease found with CTCs, shows the
140 unsuitability of this method for detecting all CTCs present in patients. This drove the
141 combination of technologies that rely on physical properties of the MelCTCs with those
142 detecting expression of specific surface markers.

143 The CTC-iChip separates cells based on size using deterministic lateral displacement and
144 inertial focusing followed by negative depletion. Using this chip, CTCs from two metastatic
145 melanoma patients were successfully enriched and detected as positive by staining for the
146 melanoma antigen recognized by T cells 1 (MART-1/MLANA) [41].

147 The OncoQuick® system is another size-based technique that incorporates a filter for
148 CTC separation in conjunction with density-based centrifugation [50]. Spiking experiments
149 showed a ≥60% recovery rate of 4, 20, 100 and 500 spiked SkMel28 cells when assessed by
150 qPCR amplification of cytokeratin 8 (*KRT8*) and 18 (*KRT18*) RNA. The SkMel28 cell line
151 strongly expresses these intermediate filament proteins and *KRT18* expression has been
152 previously identified as an adverse prognostic factor in melanoma [8]. For melanoma CTCs,
153 when transcript levels of *MLANA*, *MIF* (Macrophage Migration Inhibitory Factor), *TYR*, and
154 *MITF* (Melanogenesis Associated Transcription Factor) were assessed by qPCR after
155 OncoQuick® enrichment, results showed that about 1/3 of patients (mostly early-stage)
156 expressed elevated levels of *MIF* and *MLANA* transcripts, in comparison with healthy
157 controls ($p<0.0001$ and $p<0.001$, respectively) [9]. The authors suggest that identification of

158 early-stage patients with CTCs may be used to delineate those that would benefit from a more
159 aggressive therapy at an earlier stage.

160 Previous studies have found circulating tumor microemboli (CTM) or CTC clusters in
161 the blood of melanoma patients, raising the idea that cells enter the bloodstream via collective
162 cell migration, allowing them to survive shear stress and anoikis forces [29, 42]. Recently, the
163 Cluster-Chip was developed to specifically isolate CTC clusters of two or more cells from 4
164 mL of blood, independently of tumor-specific marker expression. This microchip technology
165 relies on the strength of the cluster union and on their behavior when a flow speed is applied
166 through a set of triangular pillars. Captured CTC clusters were identified and detected by
167 immunostaining in 30% (~0.15 CTCs/mL) of 20 tested metastatic melanoma patients.
168 Interestingly, no correlation was found between the number of CTC clusters and the number
169 of single CTCs isolated (n = 19) [53].

170 While these methods have proven the ability to capture CTCs, clinical validation of their
171 prognostic value in large clinical samples is still needed.

172 **Melanoma CTC Detection Methods**

173 While the above techniques have been developed to improve isolation of CTCs,
174 optimization of detection methods is also required. Detection of MelCTCs without a previous
175 enrichment step has been reported by Ruiz and colleagues [51], where the Epic Sciences
176 platform detected MelCTCs from whole blood, using a panel of seven anti-MCSP
177 monoclonal antibodies. MCSP is a cell surface protein involved in melanoma proliferation,
178 spreading and migration of cells and it is overexpressed in more than 90% of melanoma
179 tumor tissue samples [6, 13]. Using this marker for detection, 1-250 CTCs were detected in 8
180 mL of blood (0.5 to 371.5 CTCs/mL of blood) from 22/40 metastatic melanoma patients
181 (55%). Interestingly, this method also enabled whole genome amplification and copy number
182 variation (CNV) analyses of single MelCTCs, which revealed deletions of *CDKN2A* and

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183 *PTEN*, and amplifications of melanoma related genes, *TERT*, *BRAF*, *KRAS* and *MDM2*
184 amongst others [51].

185 Most commonly, once CTCs are enriched by the techniques discussed above they are
186 detected by methods that assess their morphology and/or protein expression using
187 immunocytochemistry (ICC) or flow cytometry. In these techniques a cocktail of antibodies
188 against cell surface or intracellular markers associated with melanocyte biology or melanoma
189 pathogenesis [21, 25, 30, 55] are used to recognize the cells. Alternately, molecular
190 approaches that detect RNA or DNA from enriched MelCTCs, by quantitative real-time PCR
191 (qRT-PCR) [4, 39] or droplet digital PCR (ddPCR) [45], respectively, have been used for
192 CTC detection and characterization. A new and promising method based on the presence of
193 elevated telomerase activity commonly found in melanoma cells, is being trialed for CTC
194 detection [61].

195 Based on our previous identification of heterogeneous MelCTCs [21], we recently
196 developed a flow-cytometry multimarker approach to detect and analyze CTCs for the
197 presence of melanoma-associated markers, such as MCSP and MCAM, in combination with
198 melanoma stem cell markers, such as ABCB5, RANK (receptor activator of NF-κβ) and
199 CD271 [25]. Using this approach we provided for the first time, a detailed insight into the
200 diversity of MelCTCs within each patient, and showed that the prognostic utility of MelCTCs
201 may not rely on the total count of CTCs but on the CTC subpopulations circulating within an
202 individual. This study indicated that a high number of MelCTCs express melanoma-initiating
203 or stem cell markers (ABCB5 and RANK) while only very low numbers of CTCs express
204 melanoma markers MCSP and MCAM [25]. Importantly, the common expression of these
205 melanoma-initiating markers by MelCTCs did not correlate with the expression of these
206 markers in patient-matched tumors, where a low frequency of melanoma tumor cells positive
207 for these markers was observed. This finding provides evidence that most CTCs, at least in

208 melanoma, are derived from rare subpopulations of tumor cells which may have the ability to
209 seed new metastases, and not from the bulk melanoma cells shaping the tumor [25].

210 Aya-Bonilla and colleagues [4] more recently interrogated for the first time, the
211 enrichment of MelCTCs using spiral microfluidic technology [59, 60]. With this device,
212 recovery rates of greater than 55% and a 2.5-3 log depletion of WBCs were observed in
213 spiking experiments using melanoma cell lines with different cell sizes which represents an
214 improvement to depletion rates similar to those obtained by the CTC-iChip [41]. After
215 microfluidic enrichment of blood from 20 metastatic melanoma patients, MelCTCs were
216 identified by flow cytometry, gene expression analysis and immunostaining, in 40%, 54%
217 and 43% of cases, respectively. As found previously [21, 25, 29], MelCTCs showed diversity
218 in their marker population with CTCs analyzed by flow cytometry most commonly
219 expressing *ABCB5* alone or in combination with *RANK*, a marker of treatment resistance.
220 Gene expression analysis of the CTC-enriched fractions also detected transcripts of *PAX3*,
221 alone or in combination with *ABCB5* expression in 6 out of 7 metastatic melanoma patients
222 positive for melanoma transcripts; transcripts of the melanocytic gene, *MLANA*, were
223 detected in the remaining patient. *MLANA*, *PAX3* and *ABCB5* are highly expressed in
224 melanoma tumors and have been described to play an important role in melanoma
225 pathogenesis and resistance [17, 20, 43]. In this study, isolated CTCs were also characterized
226 by multimarker immunostaining for intracellular melanocytic proteins gp100, S100 and
227 MLANA (1-4 CTCs/ 8 mL of blood), which indicated that MelCTCs are also diverse in cell
228 size (range: 13-21 μm) [4]. This study unmistakably confirmed the phenotypic and molecular
229 heterogeneity of MelCTCs.

230 Although great advances have been made in MelCTC isolation (Figure 1; Table II), their
231 quantification remains challenging given the low numbers of CTCs identified even when a
232 variety of multimarker assays are used for their detection. This is presumably due to our

233 limited knowledge of the spectrum of diverse MelCTCs. Studies are needed to investigate
234 MelCTC phenotypes, their role in melanoma biology and prognosis as well as their
235 differential pharmacodynamic responses to treatment [31].

236 **Are CTCs of clinical utility in melanoma?**

237 Studies to date show CTCs are a suitable biomarker of disease status. Furthermore,
238 monitoring the levels of CTCs before and during melanoma treatment has, in limited studies,
239 been shown to be informative with respect to prognosis and therapy response in melanoma
240 [25, 28, 31, 36, 46].

241 Using RT-PCR to detect transcripts in blood, Reid and colleagues showed that in 230
242 patients, the presence of *MLANA* and *ABCB5* transcripts were associated with disease
243 recurrence and the expression of *MCAM* was significantly more common in patients with a
244 poor treatment outcome [46]. Also, the presence of multiple melanoma markers in patient
245 blood significantly correlated with their AJCC stage [32], and the detection of more than one
246 marker at baseline and at any time during treatment administration was a negative prognostic
247 factor for disease-free survival (DFS) and for overall survival (OS) [26].

248 Several studies using immunomagnetic enrichment have also shown that the number of
249 MelCTCs is higher in the blood of patients with advanced disease [21, 36, 62]. Moreover, the
250 number of CTCs was also shown to be associated with treatment failure and shorter median
251 OS when $\geq$ 2 CTCs per 7.5 mL are found during the time that patients are receiving treatment
252 [28, 44]. By contrast, a low CTC count at baseline ($<$ 2 CTCs) or a decrease in CTCs after
253 treatment initiation was associated with response to treatments and longer progression free
254 survival (PFS) rates [31].

255 Recently, using flow cytometry to separate CTC subtypes, we showed that the presence
256 of CTCs was associated with disease stage and PFS [25]. Interestingly, early-stage patients
257 were generally positive for a single marker compared to late-stage patients who had larger

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258 numbers of CTCs expressing a variety of markers. Additionally, patients with higher number
259 of CTCs (>5 $RANK^+$ cells) in 4 mL of blood had significantly lower PFS than those with
260 fewer or no CTCs (<5 $RANK^+$ cells) [25]. Importantly, we demonstrated that prognostic
261 utility might be found not merely by using total CTCs counts but by studying specific
262 subpopulations of CTCs and response to therapy. Patients (n=16) who relapsed after targeted
263 BRAF inhibitor therapy were most likely to exhibit greater numbers of RANK CTC subtypes.
264 Conversely, the presence of CTCs expressing PD-L1 was associated with response to anti-
265 PD1 blockade [27].

266 New experiments with patient-derived xenografts (PDX) are providing new information
267 that can inform treatment decisions for each patient. Particularly where tumors are
268 inaccessible, CTC-derived xenografts (CDX) or *in vitro* growth of CTCs may provide a
269 powerful tool for drug efficacy testing for each patient. Girotti and colleagues [23] have been
270 successful in generating CDXs in 6 out of 21 cases (28.6%) and showed that CDX models
271 established from advanced stage patients could aid in the prediction of patient responses to
272 treatments [23]. While the isolation of only a few CTCs capable of developing xenografts
273 may underestimate the tumor heterogeneity, these are excellent first steps, addressing several
274 of the issues surrounding the clinical benefit of CTC characterization, including the fact that
275 CTCs that develop xenografts are capable of seeding metastases and therefore harbor
276 significant information about the metastatic process. Additionally, further studies
277 characterizing CTC subpopulations prior to or concurrently with injecting them into mice will
278 provide crucial information about CTC phenotypes that are most likely to develop CDXs.

279 Although CTCs quantification shows great potential, the role of MelCTCs in melanoma
280 management is still under investigation. For example, the continued presence or an increase
281 of MelCTCs after therapy initiation may suggest disease progression. Contrarily, a decrease
282 or a continued value of zero MelCTCs might suggest response to treatment. Additionally,

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283 changes in CTC phenotype after treatment initiation may be an early indicator of the
284 emergence of resistance, leading to an early change of therapy. Although these changes are
285 difficult to assess when low numbers of MelCTCs are being isolated, these measurements
286 could offer unique prognostic information. Furthermore, MelCTCs could be tested for
287 molecular evolution of tumors prior to therapy, to identify markers of intrinsic resistance; and
288 during therapy, to identify the development of drug resistance to targeted therapies.

289 There is also a need to evaluate the ability of CTC analysis to inform treatment decision
290 in patients with AJCC stage III and resected stage IV melanomas, as there are now more
291 clinical trials and FDA approved therapeutics for melanoma in stage III and stage IV [3, 35].

292 The lack of standardization and the variety of methodologies used for their isolation has
293 hampered the ability to implement the analysis of MelCTCs into large clinical studies.
294 Therefore, there is an urgent need to standardized protocols for MelCTC enrichment,
295 detection, and quantification across different laboratories.

296 **What additional isolation steps are required to identify the full spectrum of melanoma**
297 **CTC subtypes and their prognostic potential?**

298 Melanoma CTCs are rare and very heterogeneous. The comprehension of their
299 aggressiveness and their application in clinical settings is still limited by the capacity to
300 successfully and routinely isolate viable heterogeneous CTCs from the majority of patients.

301 New platforms that enable effective and repetitive isolation of MelCTCs should be
302 unbiased, which means that CTCs should be enriched and detected without relying on known
303 expression of CTC markers, rather, methods should be based on broader traits of CTCs, such
304 as physical properties (cell size, morphology, rigidity, nuclear/cytoplasm ratio). In addition,
305 isolation methods should be highly efficient in enriching the vast majority of CTCs present in
306 the blood at high purity (i.e. low WBC background). Moreover, methods should allow
307 isolation of viable and intact CTCs for “omic” characterization and, ultimately, the

308 establishment of CTC-derived cell cultures and xenografts (CDX). Furthermore, methods are
309 required that allow high throughput, are low cost and accessible in both research and clinical
310 environments.

311 Integration of genomic and transcriptomic data from bulk tumors [7] together with
312 single-cell RNA-seq of melanoma tumors [56] have reinforced the abundant diversity
313 between and within melanoma tumors. Thus CTCs derived from such tumors would similarly
314 carry heterogeneous features. However, the challenges in isolating all CTCs have
315 significantly flawed the interrogation of the real genomic, transcriptomic and proteomic
316 diversity of MelCTCs.

317 Finding a system able to capture and detect CTCs independently of their cell marker
318 characteristics is urgently needed in melanoma, and a few studies trialing this isolation
319 approach have been reported [4, 14, 29, 41]. Advances remain hampered however by the
320 uncertain biology of these cells, and the lack of optimal technologies along with robust
321 standardization and validation of these technologies.

322 Although challenging, studying the gene expression and mutational landscape of single
323 MelCTCs, their relationship with tumor tissue cells and their connection with treatment
324 response and resistance, will significantly increase the clinical value of this biomarker.
325 Improvements along these lines will dramatically advance CTC use in the clinic. The recent
326 implementation of devices capable of isolating viable and label-free MelCTCs paves the way
327 for studies aimed at dissecting their real heterogeneity and the mechanisms underlying their
328 role in melanoma spreading. Moreover, the isolation and study of MelCTC clusters will
329 provide an insight into their role in melanoma progression and metastasis and in the tumor-
330 immune interactions.

331 **Conclusion**

332 In conclusion, a variety of isolation methods have been developed in order to study the 333 prognostic and predictive applications of CTCs in melanoma. Moving forward, the 334 implementation of optimal isolation techniques allowing phenotypic, genomic and 335 transcriptomic approaches is critical in order to unveil the diversity of MelCTCs and provide 336 new insights into their clinical opportunities (Figure 2). The latest studies suggest that 337 examining CTC subpopulations instead of quantifying CTCs, could significantly impact their 338 clinical utility. However, the variety of subpopulations needs to be identified and clinical 339 trials assessing the biomarker utility of these subpopulations needs to be undertaken to draw 340 meaningful conclusions. Also, critical factors such as the time of blood collection (i.e., 341 different time points), site of collection of blood sample [24], sample handling, transport and 342 storage must not be overlooked and remains to be standardized. Although promising, 343 MelCTC isolation and study still holds technological limitations that ought to be considered 344 by MelCTC specialists worldwide to maximize their potential applications in clinical 345 practice.

346 **Conflict of Interest**

347 The authors declare no conflicts of interest.

348 **Acknowledgements**

349 This work was funded by a National Health and Medical Research (NHMRC) grant 350 APP1046711 and a Western Australia Cancer Council grant, to M.Z and E. G. and, a Western 351 Australia Cancer Council Suzanne Cavanagh Early Career Investigator (2016) and an Edith 352 Cowan University Early Career Research grant (2016) to C.A.B. E.G. is supported by a 353 fellowship from the Cancer Research Trust. A Western Australian Department of Health 354 Research Translation Grant and a Spinnaker grant has also been awarded to M.Z. and E.G. 355 G.M. is supported by an ECU PhD Scholarship.

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570 **Figure Legends**

571 **Figure 1.** The most widely used circulating tumor cell enrichment, detection and molecular
572 characterization technologies for melanoma and other cancers. NGS: next generation
573 sequencing. DEP: dielectrophoresis. ddPCR: droplet digital polymerase chain reaction. FISH:
574 fluorescence *in situ* hybridization. RT-PCR: real time polymerase chain reaction.

575 **Figure 2.** Potential applications and clinical benefits of melanoma circulating tumor cells.

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577 **Table I.** List of terms.

ABCB5	ATP binding cassette subfamily B member 5
AJCC	American Joint Committee on Cancer
BRAF	proto-oncogene B-Raf; v-Raf murine sarcoma viral oncogene homolog B
CD144	VE-Cadherin
CD271	LNGFR (low-affinity nerve growth factor receptor) p75 NTR (neurotrophin receptor)
CD34	cluster of differentiation 34
CD45	cluster of differentiation 45
CDKN2A	cyclin-dependent kinase Inhibitor 2A
CDX	Cancer or CTC derived xenografts
CNV	copy number variation
CTC	circulating tumor cell
CTM	circulating tumor microemboli
ddPCR	droplet digital PCR
EpCAM	epithelial cell adhesion molecule
FDA	US Food and Drug Administration
gp100	glycoprotein 100; melanocyte protein PMEL
HB-chip	herringbone-chip
ICC	immunocytochemistry
ISET	isolation by size of epithelial tumor cells
KRAS	proto-oncogene K-Ras; Kirsten rat sarcoma virus
KRT18	Cytokeratin 18
KRT8	Cytokeratin 8
MAPK	mitogen-activated protein kinase
MCAM	melanoma cell adhesion molecule
MCSP/CSGP4/HMW-MAA	melanoma-associated chondroitin sulphate proteoglycan
MDM2	proto-oncogene MDM2
MelCTC	melanoma circulating tumor cells
MIF	macrophage migration inhibitory factor
MITF	microphthalmia-associated transcription factor
MLANA/MART-1	melanoma antigen recognized by T cells 1
NSG	NOD scid gamma mice
PAX3	paired box gene 3
PD-1	programmed cell death protein 1
PD-L1	programmed Death-ligand 1
PDX	patient derived xenografs
PFS	progression free survival
PTEN	phosphatase and tensin homolog
RANK	receptor activator of NF-κβ
RBCs	red blood cells
TERT	telomerase reverse transcriptase
WBCs	white blood cells

578

Table II. Assessment of MelCTC enrichment and detection techniques.

MelCTC Enrichment	Platform	Advantages	Disadvantages	References
Positive Immunomagnetic Enrichment	CellSearch	(CellSearch FDA-approved method.) High specificity.	Relies on prior knowledge of target cell surface markers.	[28, 44]
	HB-chip			[36]
	Magnetic beads			[21]
Negative Immunomagnetic Enrichment	EasySep	Retrieves heterogeneous and viable MelCTCs.	Relies on no marker expression by CTCs and high expression by leukocytes. High WBC background.	[22]
	RosetteSep			[23]
	CTC-iChip		Low specificity. Not commercially available.	[41]
Technologies based on Size/Density	ISET	Fast processing time. Label-independent isolation.	Low specificity. Limited studies.	[16, 29]
	OncoQuick		High leukocytic background. Combination with other enrichment methods needed.	[9, 50]
	Spiral Microfluidics	2.5-3 log WBC depletion. Enrichment of viable MelCTCs.	Low specificity. Limited studies.	[4]
Cluster Isolation	Cluster-Chip	Marker independent isolation. Potential study of tumor-immune system interactions.	Lack of biological characterization and clinical significance. Not commercially available.	[53]

MelCTC Detection	Advantages	Disadvantages	References
Immunocytochemistry	Individual cell analysis. Common protocols used. Low cost.	Time consuming. Low sensitivity. Biased CTC detection.	[4, 51]
Flow Cytometry	Automated quantification. Provides multimarker information.	Marker expression-dependent; requires previous enrichment; limited number of markers.	[22, 25, 33]
qRT-PCR	Cost effective. High sensitivity.	Biased CTC detection. Unclear specificity. Not quantifiable.	[4, 16, 39]
ddPCR	High specificity. Cost effective.	Sensitivity compromised by WBC background. Analysis limited to prior knowledge of target mutation.	[45]

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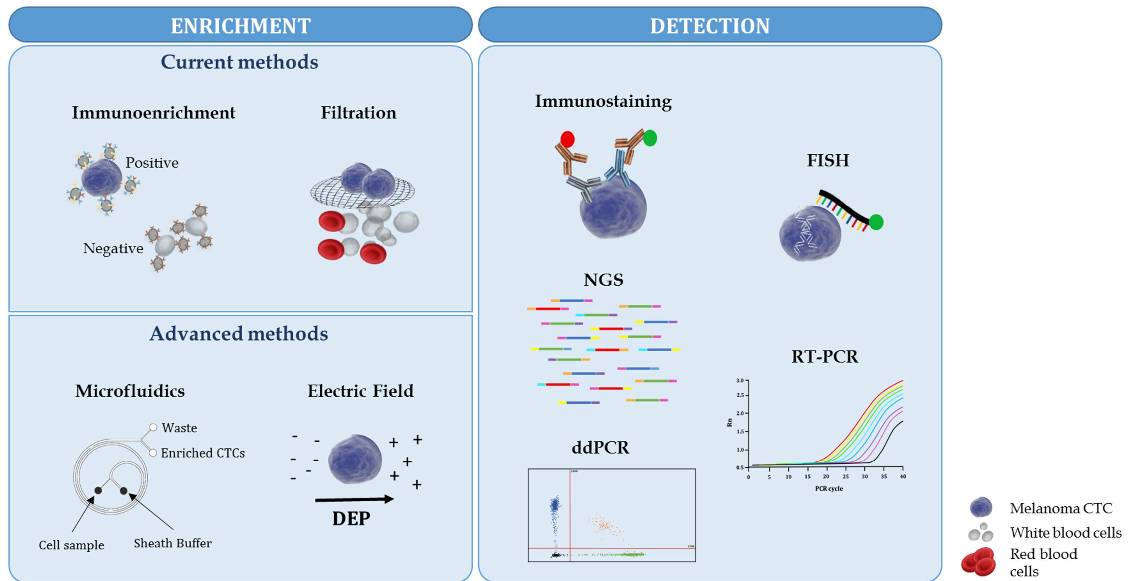

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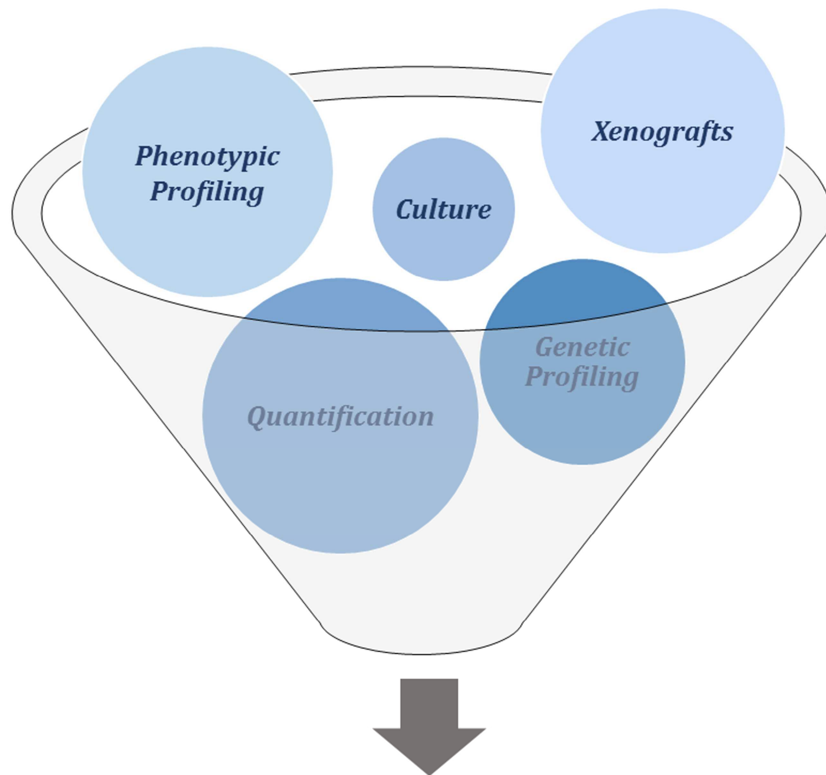

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Highlights:

- This review discusses Melanoma CTCs (MelCTCs)
- MelCTCs are promising liquid biopsy biomarkers in melanoma
- They are heterogeneous with no common marker for their detection, unlike epithelial cancers.
- Label-independent methods are preferred for isolation of diverse MelCTCs.
- Identification of MelCTC subtypes provides significant clinical utility.

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