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# Circulating Melanoma Cells in the Diagnosis and Monitoring of Melanoma: An Appraisal of Clinical Potential

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## Abstract

Circulating melanoma cells (CMC) are thought to be the foundation for metastatic disease, which makes this cancer especially lethal. Cancer cells contained in the primary tumor undergo genotypic and phenotypic changes leading to an Epithelial-to-Mesenchymal transition, during which numerous changes occur in signaling pathways and proteins in the cells. CMC are then shed off or migrate from the primary tumor and intravasate into the vasculature system. A few CMC are able to survive in the circulation through expression of a variety of genes and also by evading immune system recognition to establish metastases at distant sites after extravasating from the vessels. The presence of CMC in the blood of a melanoma patient can be used for disease staging, predicting metastasis development and for evaluating the efficacy of therapeutic agents. Overall survival and disease-free duration can also be correlated with the presence of CMC. Finally, analysis of CMC for druggable therapeutic gene targets could lead to the development of personalized treatment regimens to prevent metastasis. Thus, the study of CMC shows promise for the detection, staging, and monitoring of disease treatment, as well as determination of prognosis and predicting overall disease free survival. These are the areas reviewed in this article.

## Introduction

Circulating Melanoma Cells (CMC) are cancerous melanocytes that dissociate from the primary tumor and circulate through the blood stream, forming the basis of metastatic disease (1, 2). CMC lose epithelial junctions and break off from solid tumors. Cells with high metastatic potential enter the blood stream or intravasate into vessels and circulate in the vasculature to reach tissue areas fit for metastasis development (3). In order to establish metastatic tumors, extravasation or moving out of the vessels, which can be facilitated by interactions with neutrophils or other immune cells (4), must occur, enabling the cells to invade susceptible tissues (3). The study of CMC shows much promise in the detection, staging, and monitoring of disease treatment, as well as determination of prognosis and predicting overall disease free survival (5). These are the areas focused on in this review.

## CMC Formation

Prior to detachment from a solid tumor, cancerous melanocytes undergo the Epithelial to Mesenchymal Transition (EMT) during which epithelial cellular adhesion properties change (6, 7) (Figure 1). For example, epithelial cells express CDH1/Epithelial Cadherin, a cell-

surface protein ligand connected via cytoplasmic tail to α-catenin and β-catenin to the actin cytoskeleton. Through this connection, close intercellular junctions are typically formed among epithelial cells. Expression of vimetin, an intermediary filament regulated by the nuclear protein PARP-1, is associated with the induction of the Epithelial-to-Mesenchymal Transition (8). Subsequently, epithelial cells begin secreting EGF, FGF, and HGF (epithelial, fibroblast, and hepatocyte growth factors) via intracellular transduction pathways (1, 9–11). Additionally, changes occur in the MAPK pathway and the increased expression of SNA1 (Snail and twist), Wnt, Notch, SPARC, and Hedgehog (12–16). After interacting with the secreted factors, early stage melanocytic lesion cells cease expression of CDH1, and begin expressing CDH5/Non-Epithelial Cadherin, a calcium-dependent adhesion molecule, which binds the cells less tightly than does the normally expressed Epithelial Cadherin (1, 9–11, 17). The expression of CDH5 leads to the loss of characteristic epithelial intercellular adhesion by reduction of gap junctions, desmosomes, and other adhesion molecules (3, 11, 18).

As a consequence of the EMT, melanocytic lesion cells take on characteristics of motile mesenchymal progenitor cells (19), allowing for movement and dermal invasion (3, 20). Dermal invasion is stimulated by the increased activity of Wnt5a and the activation of PKC causing cytoskel et al changes that enhance cell motility (21). Melanocytic lesion cells are attracted to the lymph or blood vessels by macrophage-mediated secretion of cytokines, chemokines, and growth factors (22). The cells then invade, or intravasate, into the lymph system or blood stream (Figure 1) through passive or active processes. Passive intravasation requires little expenditure of energy as the invading melanoma cell is sloughed off from the tumor due to increased blood flow and/or low levels of CDH1 (23). Actively-induced intravasation occurs for example when melanoma metastatic proteins NEDD9 and DOC-3 are expressed by the cell, leading to the activation of Rac18, causing changes in the actin assembly within the cell promoting migration into vessels (23). Other proteins can also be involved in this process. Intravasation through active means can confer better metastatic ability, as the cells are more likely to survive in the blood stream (Figure 1).

Although $4\times10^6$ CMC can be shed from primary tumors daily (roughly 1 million per gram of tumor tissue), only 1–3 at best, are viable in the circulation (1, 24, 25). Most cells undergo necrosis or apoptosis as a result of the sheer stress occurring in the blood stream or from starvation or from immune system intervention (26, 27). Certain metastasizing cells are resistant to apoptosis due to the deregulation of the PI3K/Akt pathway, which interferes with the regulation of apoptotic genes (28). Specifically, increases in the phosphorylation of Akt3 and PRAS40 have been associated with the progression of melanoma and resistance to apoptosis (29, 30). Inhibition of either Akt3 or PRAS40 resulted in increased tumor cell apoptosis and susceptibility to apoptosis-inducing drugs (29, 30). Also contributing to the PI3K/Akt pathway deregulation is phosphatase and tensin homologue (PTEN), a tumor suppressor gene (29, 31). The loss of PTEN functioning in tumor cells allows for the deregulation of genes within the PI3K/Akt pathway leading to tumor progression. The introduction of functional PTEN into melanoma cells can inhibit tumor formation by increasing susceptibility to apoptosis (31). Many other proteins or signaling pathways are also utilized in the evasion of apoptosis by CMC, including a decrease in the expression of death receptors, increased expression of metalloproteins, increased secretion of growth

factors, and overexpression of anti-apoptotic proteins, such as BCL-2, BCL-XL, and FAK (focal adhesion kinase) (28, 32, 33). In addition, CMCs can avoid detection by natural killer cells, which induce apoptosis by targeting the NKG2D ligand expressed on the surface of certain cells. CMC internalize the NKGD2 natural killer cell target ligand in order to evade damage from natural killer cells (34).

Those cells that are able to defy the body's natural defense systems can be the most actively dividing and fit CMC (35). These cells are able to survive in the circulatory system and may aggregate to form microemboli or clumps of metastatic cells protected by a collection of surrounding of platelets and/or leukocytes (1, 36, 37) (Figure 1). Due to the sheer size of microemboli, they can become entrapped in small capillaries and CMC within the mix subsequentially invade into susceptible tissue to establish metastases (34, 38).

Prior to invasion into tissues, extravasation of single CMC out of the vascular system must occur (Figure 1). Often, single CMC capable of metastasis secrete IL-8 (interleukin-8) to facilitate extravasation (Figure 2). IL-8 attracts tethering neutrophils to the melanoma cells, and through interaction of ICAM-1 receptors on melanoma cells and $\beta_2$ Integrins on neutrophils, the two cells become physically connected (4). Similar interaction occurs between the neutrophil-melanoma complex and the endothelial cells lining the vessels through binding of sailyl carbohydrates on the membrane of neutrophils and E-Selectin on the surface of the endothelial cell. This results in tethering of the melanoma cells to the endothelial cell, thereby anchoring the CMC in place, which enables growth or invasion into the surrounding tissue, subsequently leading to metastatic tumor establishment (Figure 2) (4).

Vascular development, or angiogenesis, is necessary for the continued expansion in the size of a tumor and it is this process that might free certain CMC through passive means. The expression of VEGF (Vascular Endothelial Growth Factor) and HIF (Hypoxia-inducing factor)-1α induce angiogenesis for the maintenance of cells within tumors (39). Proper local vascular patterns and tumor cell-microenvironment interactions can enhance the fitness of a region for CMC invasion and tumor growth (5).

The cellular markers for CMC identification are similar to those of tumor cells, which include a high nuclear to cytoplasmic ratio (40, 41) and visible nucleoli (40). In addition, as occurs with the clonal nature of tumors, CMC show great heterogeneity in ligands expressed on the cell surface and proteins within the cells themselves (42). This high level of diversity creates a major obstacle in identifying those cells to target in order to inhibit the development of metastatic lesions, and will be an important area of research in the future.

## Detection of CMC

The rarity of CMC in the blood stream (1–3 CMC/~5 billion blood cells) and the lack of current technology to isolate every CMC will require further technological advancements for the field to reach its full potential (1). A very small number of CMC, roughly 1–3 cells can be found in 5 billion blood cells, and as such, very specific and stringent means of detection are necessary to isolate CMC (1). Furthermore, CMC can acquire new genetic mutations from the tumor of origin and are prone to phenotypic drift, which contributes to cellular

heterogeneity (35, 43–46) and accounts for the high variation among markers expressed by these cells. Few specific ligands linked to CMC survival have been identified, and fewer still have been exclusively linked to CMC metastasis (40). Heterogeneity of cell surface markers complicates the isolation of all CMC as capture technology would have to utilize a large variety of markers with high specificity to isolate all cells. Unfortunately, the majority of methods currently available lack the specificity to isolate an accurate number of CMC or those CMC specifically leading to metastasis development (47). Separation of CMC from red blood cells is rather simple as red blood cells can be lysed (48). However, distinguishing CMC from leukocytes or stray normal epithelial cells is more complex as many of these cells are caught by CMC-detecting beads and chips or adhere to the entrapped CMC (5). Leukocytes are difficult to lyse without affecting CMC survival or capture and cannot be separated via centrifugation, filtration or other current technologies (49). Specific selection for leukocytes via immuno capture using leukocyte surface ligands such as CD45 (negative selection) (48) or immuno capture using CMC-specific ligands, such as Melan-A/MART-1 or MAGE-3 (positive selection) (50) can be used as a means of separating metastatic cells from leukocytes. However, even using these approaches it is impossible to eliminate all of the leukocytes.

In recent years, many advances have been made in the development of CMC isolation technologies (Table 1). Most approaches utilize antibody-coated immunomagnetic beads or microchips to screen for multiple markers, including Melan-A/MART-1, Tyrosine, gp100, CD146, and MAGE-3, to account for variability among CMC (16, 50). Analysis methods frequently utilize RT-PCR (Real Time Polymerase Chain Reaction) analysis, combined with fluorescent imaging selection or immunocytochemistry to identify CMC or targets indicative of metastatic disease (5, 51, 52). Associated with these advances are increasing specificity, higher CMC yield during collection, as well as identification and utilization of new markers for CMC isolation. Cells isolated using these approach are then used for prognostic as well as diagnostic purposes (5).

## Functions of CMC

Circulating melanoma cells have potential for pinpointing patients who are prone to metastasis and for monitoring the progression of disease as well as efficacy of therapies (53). Detection of CMC can indicate impending micrometastases prior to clinically observable metastatic tumor formation (54). Specifically they can identify those patients prone to relapse after surgical removal of primary tumors (34). In these patients, CMC can be symptomatic of the presence of minimally residual disease before currently available clinical measures are able to detect tumor formation (55, 56). In early-stage patients (Stages 0-I), the observation of CMC can indicate a predisposition to develop distant metastatic tumors as the disease progresses (54). Two thirds of those who developed metastases or locally recurrent disease were identified as having CMCs in peripheral blood during the early stages of melanoma (57).

Since circulating melanoma cells are necessary, but not indicate metastasis development, CMC can be used to stage melanoma (50). However, the specificity and accuracy of the tools used for detection and the intermittent shedding of cells from tumors reduce the

reliability of CMC for melanoma staging as falsely negative results might not indicate that metastasis has not or will not occur (58). Despite the controversial results concerning CMC use in cancer diagnosis and prognosis, the majority of studies utilizing RT-PCR for CMC detection have indicated a strong correlation between the number of cells isolated from patient blood samples and the disease stage (52, 59, 60). Furthermore, the number of patients with detectable numbers of CMC was lower in Stage III (observed: 30%, expected: 60–70%) and stage IV (observed: 45%, expected: 100%) disease than was expected (58). However, an increase in the number of patients with CMC has been observed as the stage of disease increases (58).

There is also controversy over the reliability of CMC in gauging prognosis for overall survival time, disease-free survival, and the efficacy of targeted therapies. This lack of consensus stems from inconsistency related to the approaches used to capture and analyze these cells (61). There is no universally accepted method for the collection (i.e. immunomagnetic beads, CTC Chip, CellSearch, etc.), analysis (i.e. RT-PCR, immunocytochemistry, etc.), sensitivity or specificity of different collection methods. This causes discordant results stemming from variance among the protocols used in different studies. In addition, a plethora of markers (Tyrosine, Melan-A/Mart-1, gp100, MAGE-3, EpCAM) are used as identifiers for CMC with only cells containing those surface markers being isolated (61). In some studies multiple markers are used in detection of CMC; however, in others, only one marker, such as EpCAM was used, which eliminated collection of those cells lacking this marker (59). The propensity for accurate identification and isolation of CMC depends entirely upon the markers used. Therefore, antibodies common to normal cells can result in falsely positive detection caused by the capture of epithelial cells or leukocytes (50, 61). Similarly, falsely negative conclusions can result from using non-specific antibodies or inefficient capture methods (5). Finally, variety in age and disease stage, sample size, or the number of or time of collection of blood samples used for each patient could result in discordant results (59, 61).

Pretreatment CMC status strongly correlates with overall survival and disease free survival (47, 62–64). In multiple studies, including meta-analyses, CMC have been found to be relatively accurate gauges of prognosis for survival time (52, 65). In the Mocellin study in 2004, the presence of CMC in recently post-operative patients (within 12 months of surgical removal of solid tumor) reliably identified 83% of melanoma patients who were afflicted by minimally residual disease and relapsed within 8 months of the detection of CMC (61). In addition, CMC were found to be accurate predictors of overall survival (52). Metastatic melanoma patients who had <2 CMC in 7.5mL blood averaged an overall survival time of 12.1 months, whereas those measured as having ≥2 CMC had an average decreased overall survival time of 2.0 months (52).

## CMC in Personalized Therapy

Personalized therapy involves screening for a combination or set of genetic or proteomic changes, which can be associated with increased efficacy of particular chemotherapy or radiotherapy. Efficacy is based on the deregulated proteins expressed or genetic alterations occurring in the tumor of the patient. The specific drugs used are based on the cancer targets

or deregulated genes in the tumor cells or CMCs isolated from the patient (Figure 3) (66). Thus, drugs used are usually designed specifically for the patient receiving the therapy.

Currently, the avenues for personalized therapy is frequently based upon analyses of biopsies of primary tumors; however, in the future, this assessment may be based only on CMC isolated from patient blood (66). A continued limitation of the current approach is based on the heterogeneity of tumor cells, which hampers the design of effective personalized therapeutic regimens (42). A further complication could be that cells contained in a solid tumor grow and proliferate under different conditions than do CMC or cells undergoing the first steps of metastasis (43). Tumor cells can be limited by microenvironment in terms of space or available growth factors for proliferation (43). Furthermore, prior to undergoing the EMT, tumor cells are tightly packed and intercellularly bound, whereas CMC and those with metastatic potential circulate through the blood individually or in small microemboli (Figure 1). CMC, therefore, exist with fewer spacial restrictions on growth and proliferation. Drugs that target cells in the primary tumor might not affect CMC or metastatic cells as they may have lost epithelial ligands, such as those involved in the formation of cell adhesions, during the EMT (6, 7, 42).

Once isolated, the analysis of expressed surface ligands or deregulated intracellular proteins could be used for the development of personalized treatment. This approach would integrate therapies to specifically target CMC directly controlling metastasis development. The involvement of CMC in the design of targeted therapies to inhibit metastasis development is outlined in Figure 3. However, a complication of this approach is the heterogeneity of expressed markers in CMC and the aforementioned differences between these cells and those bound in tumors. This problem could cast doubt on a drugs' efficacy for killing both CMC and tumor-bound cells (47). As such, information obtained from both biopsies of solid tumors and analysis of CMC might be the best approach to eliminate these concerns when designing personalized therapies.

The role of CMC in monitoring the effects of targeted therapy has also been a subject of much controversy. Many studies investigating the change in the number of CMC isolated from patient blood samples and the success of targeted therapies in treating patients' disease have resulted in inconsistent conclusions (47, 55). These discrepancies likely stem from the aforementioned lack of a standardized isolation method, an approach for analysis of CMC, variation in the stages of disease of the test groups, and frequency of collection of blood samples (61).

The isolation of CMC resistant to drugs is a non-invasive approach for early detection of drug resistance (67, 68). Genetic mutations conferring drug resistance would be predicted in the population of CMC within the blood stream. These CMC would likely be the most fit, resilient, potentially drug resistant cancer cells in a patient (69–71). The separation and analysis of these drug-resistant CMC could reveal novel mutations, indicating the impending doom of a treatment regime. If this strategy was successful it might be a significant advance for cancer treatment and the prevention of recurrent resistant disease (53).

## CMC Potential for the Prevention of Metastasis

Increasingly, CMC presence is an important target for the prevention of metastasis. The identification of CMC in patient blood samples during the first year after surgical tumor removal has an 83% correlation with the recurrence of disease within 8 months of the first detection of CMC (50). Additionally, the detection of CMC in blood samples in the early stages (0-I) of melanoma development is indicative of patients with a high risk of developing metastasis (52). Adjuvant therapy and additional treatment can then be implemented for those who will most likely be affected by metastasis. Targeting CMC for personalized therapy using markers found in cells isolated from patients' blood streams appears to be a promising means of preventing metastasis (Figure 3).

Finally, targeting the genes encoding proteins involved in metastasis development for treatment has potential to thwart the progression of metastasis. Steps within the Epithelial to Mesenchymal Transition, such as the conversion of CDH1 to CDH5, leading to the loss of epithelial intercellular adhesion properties facilitating dermal invasion, intravasation, circulation and extravasation, could be targeted in CMC. The retention of cellular junctions would potentially disallow cells from breaking off from the tumor, thereby preventing metastasis (42). In addition, reduction of IL-8 secretion by invading CMC cells could reduce neutrophil-assisted extravasation (Figure 1) by 25–60%, subsequently reducing metastasis development by ~50% (4). In addition, many proteins associated with the process of metastasis, including SNA1 (snail and twist), Hedgehog, and Wnt (12–15), as well as anti-apoptosis metalloproteins, BCL-2, BCL-XL, and FAK (focal adhesion kinase) could all interfere with metastasis and lead to CMC death before metastasis formation (28, 32, 33).

## Future Directions in the Use and Detection of CMC

A standardized method of isolation and analysis must be established for those CMC that have the potential to metastasize. The identification of metastasizing CMC is clearly necessary for the future development of this research area and the success of targeted therapies (Figure 3). Millions of CMC are sloughed from tumors daily and enter into the blood stream; however, only very few of these cells are able to survive and establish metastases (Figure 1) (24, 25). Current CMC methods try to isolate all identifiable CMC, including those cells unable to survive in the blood stream (34). Designing a treatment regimen targeting all possible markers found on isolated cells would be far too expensive and possibly toxic due to the number of drugs necessary and possible interactions among these drugs. However, if a marker associated with the ability to survive while circulating or to establish metastatic lesions is identified, only cells expressing this ligand might need to be isolated and targeted, as cells not expressing this factor would not metastasize and would thus be irrelevant. This would be a huge advance in cancer treatment as it is metastasis that kills patients.

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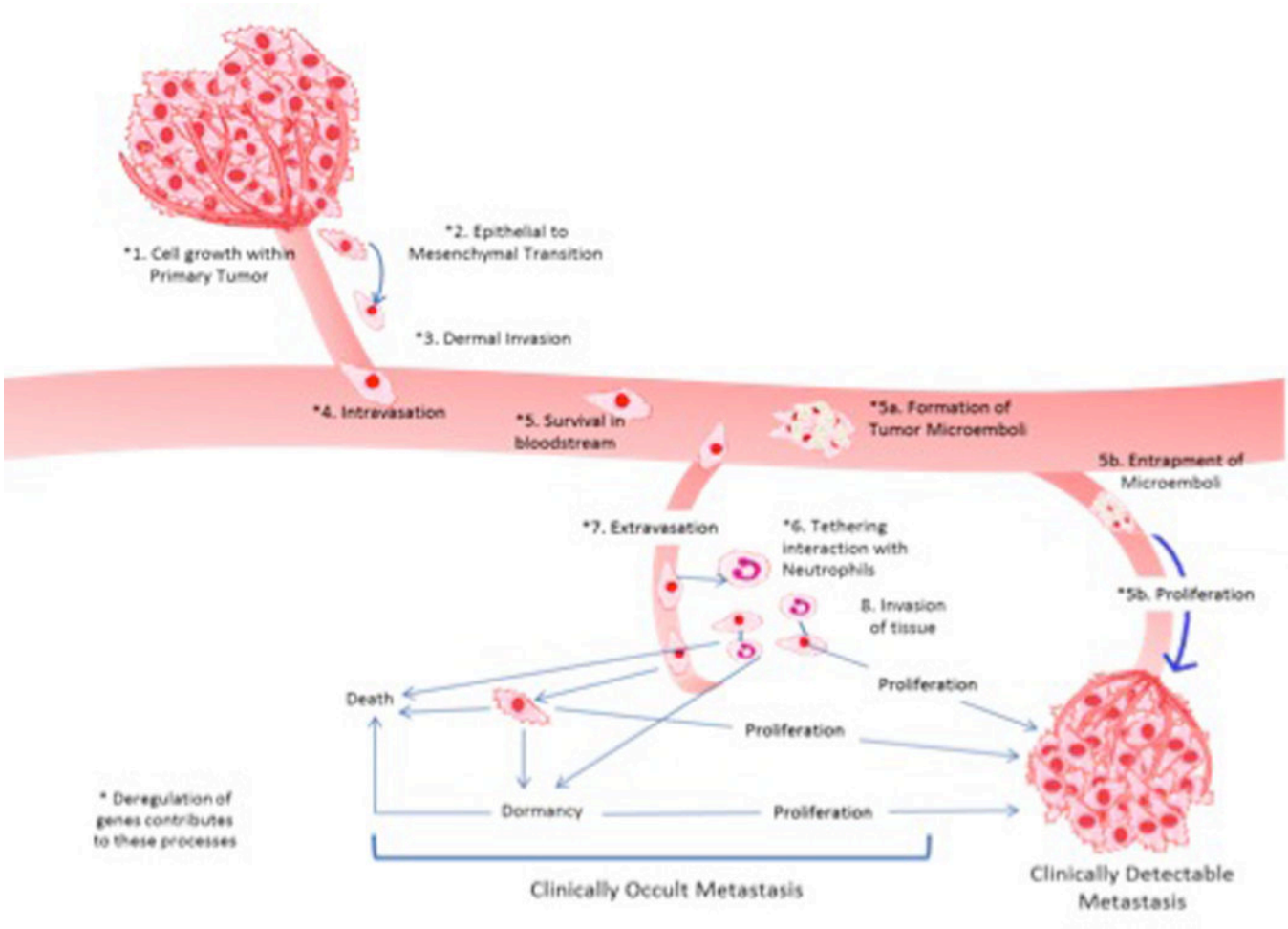


**Figure 1.**

Path of a metastasizing melanoma cell, from separation from the primary tumor through the establishment of a metastatic tumor from circulating melanoma cells.

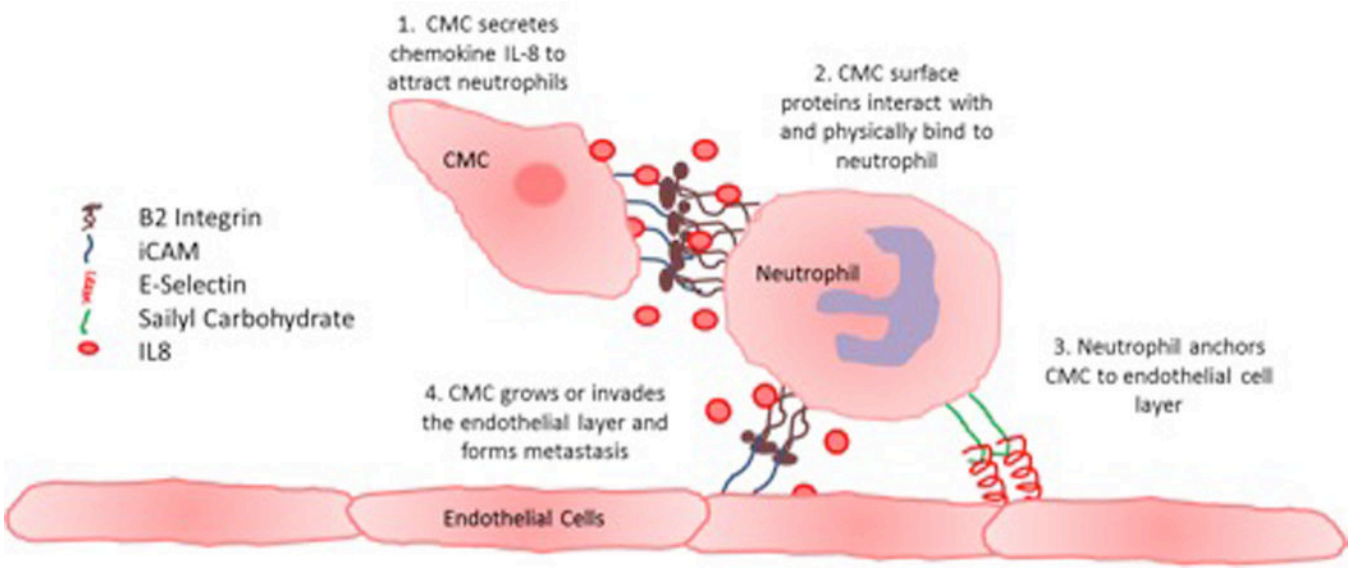


**Figure 2.**

Extravasation of circulating melanoma cell through interaction with neutrophil and tethering of the CMC to the vessel endothelial cell layer to promote metastasis.


**Figure 3.**

Idealized design of targeted personalized therapeutic regimen based on the isolation of only metastasizing CMC and selection of therapies against targets in these cells.

## Table 1

The process, sensitivity, and methods of analysis associated with selected methods of isolation of circulating melanoma cells.

| Isolation Method | Means of Isolation | Detection/Analysis | Sensitivity |
|---|---|---|---|
| Density Gradient Centrifugation(72) | Separation by cellular density | Immunocytochemistry(73) | 1cell/mL |
| Immunomagnetic Melanoma Cell Enrichment | Immunomagnetic bead (antibody coated) separation | Immunocytochemistry<br>RT-PCR | 1 cell/mL<br>1cell/mL |
| CellSearch | Anti-EpCAM ferrofluid | Removal of CD-45 expressing leukocytes, anti-CK antibodies and DAPI staining for automated fluorescent microscopy(5) | 1-0.5cell/mL |
| ISET (Isolation by Size of Epithelial Tumor Cells)(74) | Separation of cells by size (tumor cells larger) | Cytopathological Analysis<br>RT-PCR(75) | 1–3cell/mL(49) |
| RARE(76) | Leukocyte removal | RT-PCR | 1cell/mL |