Isolation and Characterization of Human Breast Circulating Tumor Cells via Whole Blood Volume Apheresis

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To my wife and daughters,

my parents, my brothers and sisters

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Contribution of authors

- The student contributed to all the chapters of the submitted thesis
- Dr. Richard Kremer co-contributed to the results and discussion section.

ABSTRACT ENGLISH

Introduction: In breast cancer (BC), metastasis is the major determinant of poor outcome, a highly organized, non-random and organ selective process. Most patients undergo surgery and also receive other treatments (hormone therapy, radiation or chemotherapy) before or after surgery. Drug resistance and tumor relapse are the main potential problems. Metastasis seeds may be already present at diagnosis as isolated tumor cells within lymph nodes, bone marrow or in the peripheral circulation as circulating tumor cells (CTCs). CTCs hence, are released together with circulating tumor DNA (ctDNA) to the peripheral blood circulation. The current challenge is the collection and characterization of CTCs and the more important subtype, cancer stem cell-like cells (CSCs).

Objective: The main objective of this study was to use and study CTCs heterogeneity using a specific methodology for isolation, characterization, and molecular analysis.

Methodology: Whole blood apheresis was used for isolation and enrichment. To analyze CTCs heterogeneity flow cytometry (FCM) was used with specific antibodies for the desired markers and then create and optimize platforms for CTCs identification. Subsequently, EpCAM positive CTCs and EpCAM negative CSCs subpopulations were isolated and collected with a technology using immunomagnetic particles coated with EpCAM mononuclear antibody. Finally, flow cytometry was used to compare the EpCAM positive (CTCs) and negative (CSCs) cell populations isolated from breast cancer patients.

Results: We successfully collected and isolated CTCs from 20 patients with breast cancer, representing all different subtypes. There was a much higher concentration and quantity of CD45(-) in patients compared to healthy controls. Gating CD45(-) cells, we also noted that patients had a higher count of EpCAM(+)cells. Two patients had EPCAM (-) CD24 (+) cells compared to healthy control which have been reported as CSC-like features. In addition , it was noted that most patients also exhibited a population not seen in healthy individuals where cells expressed both CD24(+) and CD44(+) cell surface markers.

Conclusion: Overall, our new methodology was successful in characterizing patient-specific EpCAM negative and positive CTCs indicative of heterogeneity. Our proposed platform is, to our knowledge, unique and at present the only one capable of examining in detail these rare populations particularly the EpCAM negative CSCs subpopulation. Current knowledge indicates that CSCs should be the prime target for therapeutic intervention and development of new drugs or drug combination. Future studies will incorporate further characterization of CTCs.

ABSTRACT FRENCH

Introduction: Dans le cancer du sein, les métastases sont le principal facteur de résultats médiocres, un processus organisé et sélectif pour un organe. La plupart des patients subissent une intervention chirurgicale et reçoivent également d'autres traitements (hormonothérapie, radiothérapie ou chimiothérapie) avant ou après la chirurgie. La pharmaco-résistance et la rechute sont les principaux problèmes dans le processus métastatique. Les semences de métastases peuvent être déjà présentes au moment du diagnostic en tant que cellules tumorales isolées dans les ganglions lymphatiques, la moelle osseuse ou dans la circulation périphérique en tant que cellules tumorales circulantes (CTC). Les cellules tumorales en circulation sont donc des fragments d'ADN tumoral qui sont libérés dans le sang et se propagent dans la circulation sanguine périphérique. Le défi actuel est la collection et la caractérisation des CTC et du sous-type plus important, les cellules ressemblant aux cellules souches du cancer (CSC).

Objectif: L'objectif principal de cette étude était de prouver et d'étudier l'hétérogénéité des CTC à l'aide d'une méthodologie spécifique d'isolement, de caractérisation et d'analyse moléculaire.

Méthodologie: L'aphérèse du sang a été utilisée pour l'isolement et l'enrichissement. Pour analyser les CTC, la cytométrie en flux (FCM) a été utilisée pour trouver des anticorps appropriés pour les marqueurs souhaités et pour créer et optimiser des plateformes d'identification de CTC. Les CTC positifs pour EpCAM et des sous-populations négatives de CSC ont été isolés et collectés à l'aide d'une technologie utilisant des particules immunomagnétiques revêtues d'anticorps mononucléés EpCAM. Enfin, la cytométrie en flux a

été utilisée pour comparer les populations de cellules positives pour EpCAM (CTC) et négatives (CSC) isolées des patientes atteintes d'un cancer du sein.

Résultats: Selon les résultats, nous avons collecté et isolé avec succès des CTC de 20 patientes atteintes d'un cancer du sein, de tous les sous-types. La concentration et la quantité de CD45 (-) étaient élevées chez les patients que chez les contrôles. Gating les cellules CD45 (-), nous avons également noté que les patients avait un nombre plus élevé de cellules avec EpCAM (+). Chez deux patients, par rapport au contrôle, des cellules EPCAM (-) CD24 (+) ont été notées, ce qui correspond à des caractéristiques analogues à celles du CSC. En plus de cela, il a été noté que le patient présentait également une population non observée chez des individus en bonne santé, dans laquelle les cellules exprimaient à la fois CD24 (+) et CD44 (+).

Conclusion: Notre nouvelle méthodologie a permis de caractériser l'hétérogénéité des cellules tumorales négatives et positives d'EpCAM spécifiques au patient. Notre plateforme proposée est, à notre connaissance, unique et actuellement la seule capable d'analyser en détail cette population rare, en particulier la sous-population de CSC négatifs pour EpCAM. Les recherches actuelles indiquent que les CSC devraient être la cible principale des interventions thérapeutiques et du développement de nouveaux médicaments ou de nouvelles combinaisons de médicaments. Les futures études intégreront une caractérisation plus poussée des CTC.

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LIST OF ABBREVIATIONS

AIB	Amplified in breast
BC	Breast cancer
BM	Bone marrow
BM-DTC	Bone marrow disseminated tumor cells
CAF	Cancer Associated fibroblast
CEA	Carcinoembryonic Antigen
CK	Cytokeratin
CTC	Circulating tumor cells
DNA	Deoxyribonucleic Acid
DTC	Disseminated tumor cells
EMT	Epithelial mesenchymal transition
ER	Estrogen Receptor
ESA	Epithelial specific antigen
FACS	Fluorescence-Activated Cell Sorting
FCM	Flow cytometry
FDA	Food and Drug Administration
FFPE	Formalin-Fixed Paraffin-Embedded
HER2	Human epidermal growth factor receptor 2
ICC	Immunocytochemistry
IHC	Immunohistochemistry
ISET	Epithelial tumor cell isolation
LN	Lymph nodes
MACS	Magnetic-activated cell sorting
MUHC	McGill University Health Centre
OS	Overall survival
PAX2	Paired box gene 2
PBMCs	Peripheral blood mononuclear cells
PCR	polymerase chain reaction
PFS	Progression-free survival
PR	Progesterone receptor
REB	Research Ethics Board
RI-MUHC	Research Institute-McGill University Health Centre
RNA	Ribonucleic Acid
RVH	Royal Victoria Hospital
TN	Triple-negative breast cancer
WBC	White blood cells
WHO	World health organization

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I. INTRODUCTION

In breast cancer, the major determinant of poor outcome is metastatic disease, a highly organized, non-random and organ selective process (1). Most patients with localized breast cancer undergo surgical resection of the primary tumor. Depending on the established histology and patients' characteristics adjuvant therapy might be offered to reduce the risks on recurrence; however, one of the problematic issues about breast cancer is drug resistance and tumor relapse since not all patients respond equally to the therapies available. Some patients subsequently relapse at distant sites probably because of undetected spread of tumor cells in the circulation during the primary treatment (2). The seeds of these metastases may already be present at diagnosis as isolated tumor cells within lymph nodes and bone marrow or in the peripheral circulation as circulating tumor cells (CTCs).

Therefore, CTCs are now considered metastatic precursors, where their potential roles as risk predictors of metastatic relapse and treatment monitoring are being studied. Also CTCs have the potential of acting as a therapeutic target for prevention of cancer metastasis. However, breast cancer is a heterogeneous disease, including multiple entities associated with different histological and biological features, clinical presentations and responses to therapy. Because of this heterogeneity there is a need to continuous treatment monitoring. "Liquid biopsies" represent the best options for real time monitoring and avoid the risks and cost of serial biopsies.

A large number of single blood-based biomarkers are now available and numerous gene expression patterns can be found in circulating mRNA, CTCs and Cancer Associated Fibroblast

(CAF). While a variety of biomarkers are known, their potential clinical value is still being investigated. In the last decade the studies on CTCs focused mainly on technology development for capturing, characterizing and isolating CTCs. They have proved to be important prognostic and monitoring indicators in the newly developed area of precision medicine.

The main methodologies for detection and isolation of CTCs can be broadly divided in three groups: 1) Antibody-based methods for CTC detection and isolation which rely on the universal epithelial marker EpCAM that is not present on white blood cells; 2) Isolation of CTCs based on their physical properties including density, size, mechanical plasticity, and dielectric properties; and 3) Nucleic acid–based detection of CTCs which identifies specific DNA or mRNA molecules that serve as markers to indirectly detect the presence of CTCs. At the RNA level, RT-PCR analyses have been applied either to unpurified plasma nucleic acids or more commonly to enriched CTC populations. Specifically, studies have explored cytokeratin-19 mRNA detection in breast cancer.

There are controversies regarding which methods are the most appropriate, not only because the different techniques for CTC detection and isolation use a variety of CTC markers, and CTC cut-off points, but also for the reliability of measuring CTC in small volumes of peripheral blood. There are indeed conflicting reports in CTC determination using blood volumes ranging from 1-10mL (1). Collecting larger blood volumes to characterize CTCs more accurately therefore appears as a rationale option. Apheresis is a technique that allows collection of all nucleated cells from the blood, i.e. white blood cells and CTC, using a centrifuge machine that separates these cell fractions and returns the rest of the blood components to the patient.

In this study, we used whole blood apheresis to isolate CTCs. CTCs heterogeneity used mainly flow cytometry (FCM) with specific antibodies for the desired markers and as a result create and optimize a unique platform for CTCs identification. Subsequently, EpCAM positive CTCs and EpCAM negative CSCs/EMT subpopulations were isolated with a technology that using immunomagnetic particles coated with EpCAM monoclonal antibodies. Finally, gene expression microarrays were used to compare the EpCAM positive (CTCs) and negative (CSCs/EMT) cell populations isolated from the four subtypes of breast cancer using flow cytometry and PCR.

Our hypothesis is that CTCs due in part to their heterogeneity and metastatic potential, have a different gene expression profile compared to non-metastatic breast cancer cells.

II. LITERATURE REVIEW

2.1 Evolution of breast cancer

Breast cancer (BC) is the most frequently diagnosed malignancy in women (3, 4) in both developed and developing countries, accounting for more than half of all breast cancer cases (5). BC is a heterogeneous disease and it comprises multiple entities associated with different histological and biological features, clinical presentations and responses to therapy. The two most common histologic types of invasive breast cancer are ductal (originated from ducts), and lobular carcinomas (originated from lobules), which account for approximately 75 and 15% respectively of all cases (6). Management of this disease has improved over the last years, with the current 5-year survival of early BC being quite high (between 80 and 92%) and the survival rate decreasing tremendously when the cancer becomes metastatic (7). According to the Canadian Cancer Society Statistics in 2012, BC is the most prevalent cancer in Canadian women and the second leading cause of cancer deaths in women.

The reference book from the WHO clusters breast cancer into 17 different types according to their microscopic appearance (8) while the molecular classification of BC is becoming a new standard (9, 10). However, clinicians still rely on the clinico-pathological features and tumor markers including the estrogen receptor (ER), the progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2/neu) (10). ER, PR, and HER2/neu classification is reliable, inexpensive and is routinely available providing information for therapeutic decisions (11). There are eight different combinations of ER, PR, and HER 2 that are associated with differences in demographics, survival, and tumor characteristics providing a risk category

(12).Using gene expression profiling the classification is as follows: luminal A, luminal B, HER2 overexpressing, and triple-negative (TNBC) breast cancers (13). TNBC lacks the expression of the ER, PR, and HER2, and is associated with a more aggressive phenotype lacking targeted therapy (Diaz 2007). Tumor cell characteristics are also a very important factor to guide treatment strategies for patients that had developed metastatic breast cancer (14, 15).

The 15th St. Gallen consensus (2015) had highlighted the extensive genomic analysis of breast cancers disclosing four distinct groups. This study published in *Nature* (16) (The Cancer Genome Atlas Network 2012) analyzed primary breast cancers by genomic DNA copy number arrays, DNA methylation, exome sequencing, mRNA arrays, microRNA sequencing and reverse phase protein arrays; providing insights related to the four classes of breast cancer mentioned before (luminal A, luminal B, HER2 overexpressing, and triple-negative). The Cancer Genome Atlas Network also discovered that somatic mutations in only three genes (*TP53*, *PIK3CA* and *GATA3*) occurred at > 10% frequency across all breast cancers, but there were also many subtype-associated and novel gene mutations-associated breast cancers. These subtypes can be defined by multiparameter molecular tests such as Pam-50 (17). This classification by molecular subtypes might be useful when discriminating between patients who will or will not benefit from a particular therapy. However, this type of analysis is not yet widely available for economic reasons, and tumor markers are still the most widely used to classify breast cancers.

Receptor status of breast cancer is very pertinent for selection. ER/PR positive cancer cells express estrogen receptors/progesterone receptors on their surface. Upon binding of estrogen to the ER, it will elicit a cascade of signaling pathways that lead to growth and tumor progression.

HER2, when over amplified contributes to the immortality and growth of cancerous cells (18). Most patients will undergo surgery but also receive additional treatments (hormone therapy, radiation or chemotherapy) before or after surgery (4). How women respond to therapies depends in large part of their tumor gene expression pattern and their position in the cancer genome (19). Nowadays with the rapid development of biotechnologies, the genomic characteristics of breast cancer have been extensively studied and a new generation of biomarkers has emerged. It also involves the discovery of genetic alterations responsible for the initiation and progression of human breast cancer (20). An important factor that should be considered when treating ER+ and HER2+ patients is the ratio of two transcription factors, AIB 1(amplified in breast)/PAX2 (paired box gene 2). The ration of PAX 2 to AIB1 can be predictive of the efficacy of tamoxifen in breast cancer treatment. On the other hand, patients with TNBC are normally treated with a combination of surgery, chemotherapy, and radiation therapy since there is no a targeted therapy available for this type of cancer (21). Hence, treatment options for breast cancer at the moment focus on tailoring therapies based predominantly on breast cancer classification.

2.2 Discovery of circulating tumor cells (CTC)

The molecular characterization of tumor cells has already translated into possible predictions of survival and treatment efficacy. However, because of breast cancer heterogeneity there is a need to constant monitoring during and after treatment, which involves repeated high-quality biopsies from different metastatic sites at different times that cannot always be obtained once the primary tumor has been removed (8, 22). This also is very challenging in regards to cost, pain, and location of metastatic disease, and potential risk for the patient. Therefore, liquid biopsies or

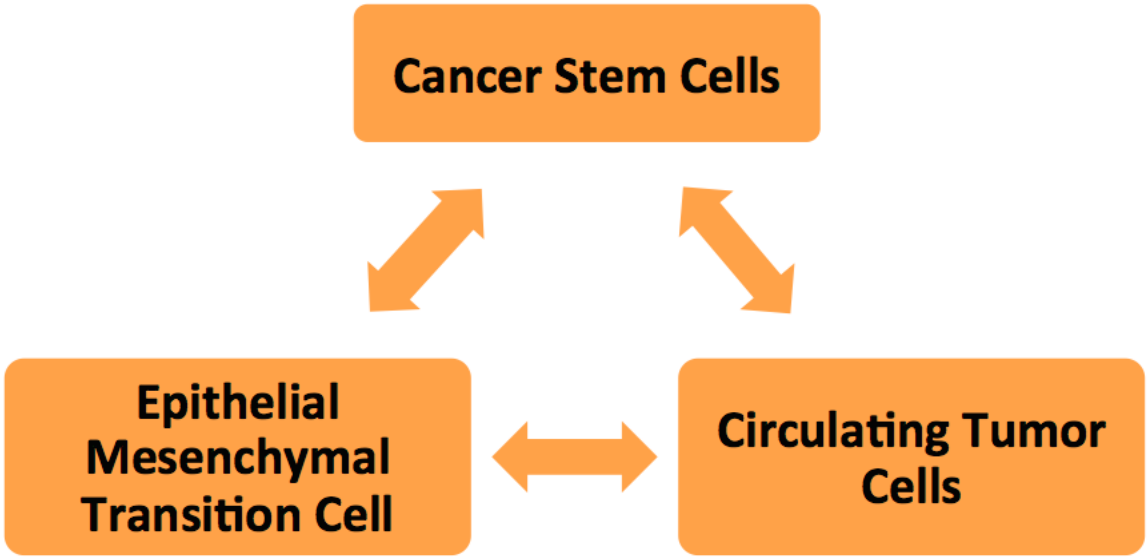
blood-based biomarkers have opened a whole new perspective for real-time monitoring of breast cancer (20).

A large number of single blood-based biomarkers can be distinguished, the most common of these are the soluble proteins known as HER2, Carcinoembryonic Antigen (CEA), Cancer antigen 15-3, and MUC1 (12). Additionally, many gene mutation patterns can be found in circulating mRNA or free circulating tumor DNA (ctDNA), CTC's and Cancer Associated Fibroblast (CAF) providing the information needed (23). While a variety of biomarkers are known, their usefulness has been reevaluated in recent years in order to select those that can provide information regarding treatment targeting, monitoring, and prognosis of the disease.

CTCs hence, together with ctDNA, are released into the peripheral blood circulation from the primary tumor (24-26). These ctDNA fragments carry tumor-specific sequence alterations representing a variable and very small portion of the total circulating DNA at early stage of the disease and increase progressively during tumor progression. CTCs are defined as nucleated cells (DAPI+) expressing cytokeratin (CK+) but lacking expression of the white blood cell marker CD45(-) (27). The cell surface adhesion molecule EpCAM is widely used in combination with cytokeratin staining to positively identify CTCs in the blood compartment. However, this strategy excludes the presence of EpCAM negative, CD45(-) and CK negative (CK-) precursors cells or cells in the epithelial mesenchymal transition (EMT) phase known to express low levels of these markers (28). EMT is a process in which epithelial cells lose cell-to-cell adhesion through down-regulation of epithelial E-cadherin and up-regulation of mesenchymal N-cadherin thereby promoting invasion through the extracellular matrix permitting the released cells to

invade their metastatic targets. Biological processes during CTC dissemination and metastasis such as EMT are now being examined in the clinical setting.

Figure 1: The co-existence and relationship between different cancer subpopulations.



In several types of cancer, CTCs can be detected in early stages and late stages of the disease as well (29). The precise enumeration of CTCs in the peripheral blood as surrogate of the dissemination process is available now because of the international standardization that happened in the last years (30, 31). Furthermore, the CTC count at different time points during treatment could be used as a marker of treatment response (32). Many studies clearly support the usefulness of CTCs as prognostic indicators in several types of cancer including breast (33, 34), prostate (35) and colorectal (36). Using the CellSearch (Veridex) method, detection of over 5 CTCs/7.5 ml of peripheral blood before treatment in a large series of breast cancer patients was associated with a worse prognosis (37). Between 10% and 30% of women with stage I to III

breast cancer, and 50% to 70% of patients with metastatic breast cancer have detectable CTCs (38).

Advances in single-cell genomic profiling have improved the ability to analyze CTCs for 'actionable' aberrations and emerging resistant cells. Another exciting development would be the possibility of monitoring patients longitudinally with the objective to adapt chemotherapeutic regimen as changes occur in the patient's status particularly in the context of acquired drug resistance. A meta-analysis by Wang et. al. (39) was conducted to determinate the prognostic value of HER2-positive circulating tumor cells; HER2 has been reported to be overexpressed in about 25% of primary breast cancers, being associated with aggressive tumors, poor prognosis, and resistance to therapies (40). These researchers concluded that HER2 positive CTCs are associated with worse overall survival (OS), and that such patients may benefit from more aggressive or targeted therapies (39). In a similar way, the presence of CTCs has been associated with poor prognosis in patients with early stage BC as well as with metastatic BC (41, 42).

The monitoring of treatment response is very important to prevent continuing ineffective therapies, unnecessary side effects, and to analyze the benefit of novel therapeutics. Treatment response is assessed mainly with the use of serial imaging, but these measurements sometimes had failed to detect changes in the tumor. For this reason, there was a need to develop biomarkers that could achieve a high level of sensibility and specificity. Is a decrease in CTCs a marker of therapeutic response? Several studies seem to indicate that CTCs enumeration tend to decrease in response to effective treatment (43-51). However, because of the low number of CTCs isolated, a direct demonstration of the effect of chemotherapeutic agents on isolated viable

cells has not yet been achieved. Particularly interesting would be the isolation and characterization of these cells in early localized or invasive cancer. New technologies are now arising that will allow full characterization of CTCs providing new possibilities for their clinical utility.

2.2.1 CTC Markers

Epithelial-mesenchymal transition or EMT is a process in which cells lose their epithelial characteristics, such as cell to cell adhesion, apical-basal polarity and acquire mesenchymal proprieties such as invasiveness, resistance to apoptosis and mobility (52). EMT facilitates cell migration and invasion because it makes weaker cell-cell cohesion increasing ECM degradation which in turn modifies the cellular cytoskeleton. Cytokeratins (CKs) have become one of the best CTC markers for epithelial tumors. In addition, a variety of markers are now available to examine tumor heterogeneity as indicated in Table 1.

Table 1: CTC Markers (52).

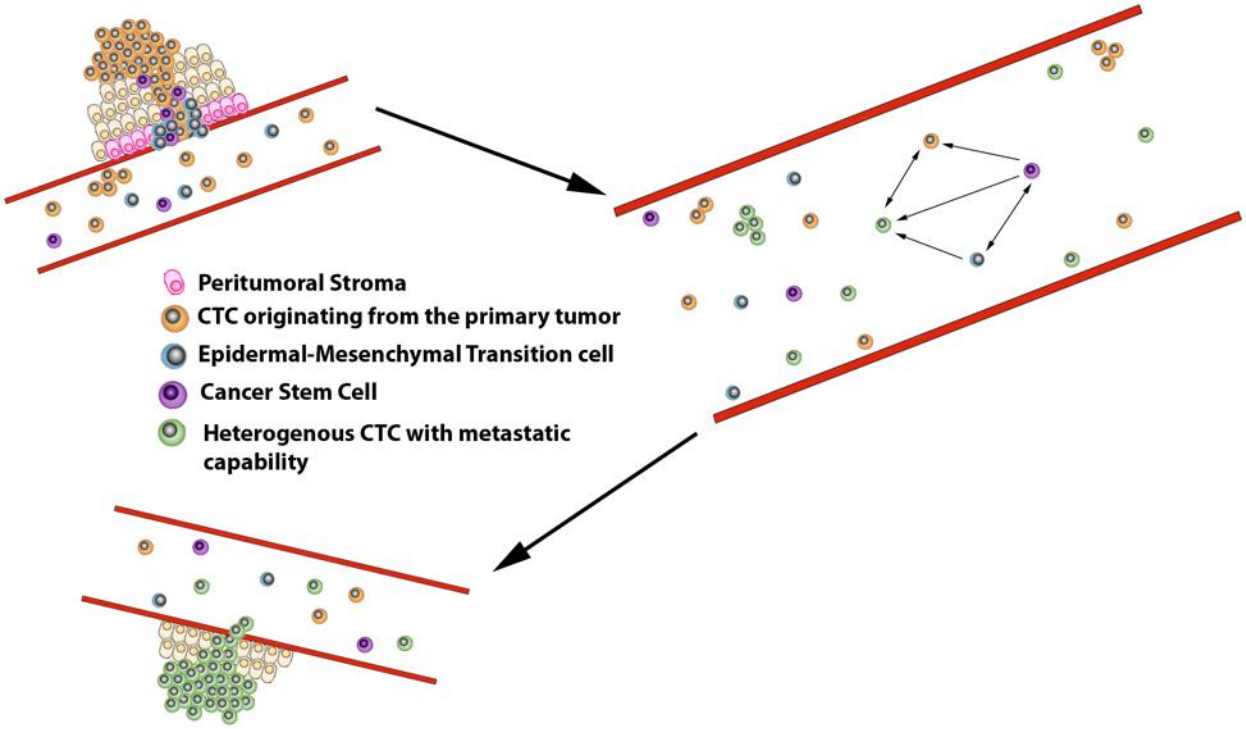
Markers used with cytometric techniques	**Markers used with nucleic acid techniques**
CK	ANKRD30A (ankyrin repeat domain 30A)
EPCAM	B305D (antigen B305D)
ERBB2	b-HCG (chorionic gonadotrophin)
uPAR (plasminogen activator receptor)	b-HCG (chorionic gonadotrophin)
CTS	c-MET (proto-oncogene met)
MUC1	CEA
IGF-IR (insulin-like growth factor 1 receptor)	CKs
	EGFR

	EGP2, epithelial glycoprotein 2
	EPCAM
	GABRP, GABA A receptor pi
	GalNAc-T (UDP-N-acetyl-D-galactosamine)
	ERBB2
	MAGEA3
	MUC1
	MUCL1 (mucin-like 1)
	PIP (prolactin-induced protein)
	PTHrP (parathyroid hormone receptor protein)
	SPDEF (SAM pointed domain containing ETS transcription factor)
	TTF1 (trefoil factor 1) TTF3 (trefoil factor 3)
	SCGB2A1
	SERPINB5
	BIRC5
	miRNA

2.3 CTCs in breast cancer

Tumour heterogeneity is defined in both “space and time” (53). Distinct regions of the same primary tumour and their respective metastases exhibit clear differences in their genomic architecture (54). The hallmarks of cancer constitutecand be classified into six different biological behaviors that a tumor acquires during its course of development. These include: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Figure 2) (55).

Figure 2: Mechanism of cancer cell dissemination during metastasis: (a) Primary tumor shedding cells into the blood circulation is comprised of CTCs, EMTs and CSCs. (b) Plasticity of the different cancer cell types showing the interrelationship between the 3 cell types described in figure 1. (c) Homing of tumor cells at the metastatic site.



The factor that contributes to these different capabilities is the genomic instability of the tumor that generates genetic diversity. Fortunately, different anti-cancer drugs exist which target these pathways. While anti-cancer drugs are promising in their proposed mechanism of action to eradicate tumor cells, they can also cause unwanted side-effects that might be more damaging than the cancer itself. The role of CTCs in metastasis biology as well as the subset of CTCs with tumour-initiating capacity has also been studied (56).

More than a couple of thousands of studies on CTCs have been published in the last 15 years, covering topics from technology development for obtaining, characterizing, and isolating CTCs (11, 57, 58) to biomarker efficacy (22, 23), disease prognosis (14, 59-61), and therapeutic tailoring (7, 15, 62, 63).

Biological biomarkers are becoming important prognostic tools in predicting disease progression and also to assess how the patient will respond to a specific treatment. Improvements have been made to select patients that better respond to certain therapies than others. For example, EGFR monoclonal antibody therapy is used in patients with KRAS gain of function. Unfortunately, despite its higher specificity, only 20% of patients are sensitive to this treatment. Among the various reasons for this failure the predominant one is that therapies are all based on the primary tumor phenotype.It has been proposed that **"**Analysis of metastatic sites is better indicative for the actual tumor load and its underlying biology**"** (64). However, obtaining serial biopsies from metastatic patients is an invasive and painful procedure. This is where CTCs potential could be regarded as an excellent alternative to organ biopsies; CTCs can be obtained and characterized repetitively serving as a liquid biopsy of disease progression and metastases.

2.4 Clinical relevance of CTCs in breast cancer

The development of personalized medicine for cancer treatment depends on the identification of the molecular drivers of their disease (57). Many studies conducted on CTCs have validated their prognostic significance (59). In a study conducted by Braun et al., approximately 30% of women with primary breast cancer have disseminated tumor cells in the bone marrow (65) and the 10 year follow up of these patients revealed a significantly decreased overall survival, when

compared to patients without disseminated bone marrow tumor cells(DTCs). Because dissemination mostly occurs through blood, CTCs shed into the vasculature may become disseminated tumor cells when they invade the bone marrow. Moreover, the presence of DTCs in bone marrow is associated with higher tumor stage and worse differentiation. Another study conducted by Wiedswang et al. demonstrated that the persistence of DTC from breast cancer patients after adjuvant therapy is predictive for disease recurrence (26). Other studies have also validated the usefulness of CTCs as prognostic indicators in different types of cancer such as breast (66-69) prostate (70) and colorectal cancers (36).

A systematic review conducted by Kathami et. al. in 2017 reported the published studies evaluating the association of CTC's enumeration and molecular identification with clinico-pathological characteristics and outcomes of breast cancer. They found several articles showing that monitoring CTCs levels facilitates the prediction of treatment efficacy (4, 34); the authors checked CTCs at baseline, through the first weeks of treatment, and after treatment in relation to the following three classifications: progression-free survival (PFS), overall survival (OS) and relapse. CTCs in this systematic review included mostly CTCs with cytokeratin positive (CK) and CD45 negative phenotype . Kathami et. al. found that the cut-off point of five or more CTC per 7.5mL of blood during therapeutic monitoring can accurately predicts prognosis in metastatic breast cancer (4, 71)and that patients having decreased responses by their immune cells in comparison with patients with 5 CTCs or less (4, 72), concluding that CTCs enumeration is a strong prognostic factor for overall survival in metastatic breast cancer (59). Additionally, CTCs are a powerful predictive of survival in all metastatic breast cancer subtypes excluding HER2 positive patients who have received targeted therapy. In the 21 studies included in the systematic

review the most common molecular marker were the proto-oncogene Neu (HER2) alone or in combination other markers such as epithelial cell adhesion molecule (EPCAM), PR (or also known as NR3C3 or nuclear receptor subfamily 3, group C, member 3), and ER.

However, there are conflicting results regarding the prognostic value of CTCs and their usefulness for treatment monitoring. A meta-analysis conducted by Fei et. al (2014) has reported that a decrease of the CTC count after cancer treatment in breast cancer patients did not indicate an improved response to treatment (73). This is in contrast to what other studies had reported, including the conclusion of the systematic review and meta-analysis by Lv et. al (2015) which proposed that HER2 positivity could be a significant factor for the presence of CTCs, that CTCs have a significant prognostic value for metastatic cancer patients, and that CTCs should be continually monitored to guide treatment particularly for patients with HER2 positivity (74).

The clinical validity of CTCs in patients with TNBC has also been analysed in a systematic review by Lu et. al. (2016). In this meta-analysis they found strong evidence that detection of CTCs in the peripheral blood is an independent prognostic factor for poor survival outcome. This study therefore strongly supports the effective and promising predictor value of CTCs in TNBC patients, regardless of whether CTCs had been detected in an early stage or not (21).

Finally, Yan et. al conducted a meta-analysis in 2016 in which CTCs were measured before and after treatment in breast cancer patients. They also estimated the effect of therapy on CTC reduction during therapies the patients were taking. The effect on CTC reduction for each different treatment was investigated separately through subgroups of patients with different

molecular subtypes. Overall CTC-positivity was significantly decreased following therapies. The CTC status was classified by different methods and markers in the 50 studies analysed (for a total of 6712 patients). The CTC-positivity rate was reported in all of the studies, and the different cut-off values of CTC count were established as follows : i.e. ≥ 5 CTCs/7.5 mL, ≥ 1 CTCs/7.5 mL, etc. (75). The studies included in the meta-analysis also included RT-PCR analysis of various markers using different expressions thresholds of epithelial genes such as EpCAM, CK18, and CK19. The study also classified the therapeutic methods into: neoadjuvant setting, adjuvant setting, metastatic setting, surgery, and combination therapy. The differentiation of ER, PR and HER2 expression is currently the basis for clinical management, therefore it is well known now that different subtypes of breast cancer are associated with distinct malignant phenotype and response to therapy. The current meta-analysis by Yan et. al. assessed the effects of therapies on reduction of CTC's in different subgroups: HER2 positive, HER2 negative, and TNBC. Their findings pointed out that CTC-positivity rate decreases following different treatments, including neoadjuvant treatment, adjuvant treatment, metastatic treatment, and combination therapy, but not after surgery. Also the CTC-positivity rate decreased after therapies in the HER2 -positive or -negative patients, but not in TNBC (75).

Hence, CTCs can be a valuable tool in predicting patients' response to therapeutic regimens. In fact, the presence of CTCs after therapy might reflect a failure of systemic therapy (76). CTCs clinical validity has been demonstrated at the metastatic stage in many cancer types (59, 63, 77). Moreover, RT-PCR detection of CK19 positive CTC after adjuvant therapy in early breast cancer patients is reflective of resistant residual disease (78).

2.5 Current CTC definition

In this thesis, we will use the term CTCs to describe CD45 (-), EpCAM (+), CK(+) cells. The term CSCs will refer to the combined population of Cancer Stem Cells and EMT. CSCs also known as tumor initiating cells or progenitor-like cells, have been characterized as CD45(-), EpCAM(-), CK(-), CD24(-), CD44(+) and EMT are described as CD45(-), EpCAM(-), CK(-), Vimentin(+), E-Cadherin(-) and N-Cadherin(+) cells. CTCs have presumed important biological properties and their presence correlates with the metastatic process (43, 79-86), however, their baseline level is unrelated to tumor size (87).

Once in the circulation, CTCs can evade immune detection and could extravasate into microvessels of target tissues such as lymph nodes, bones, liver, brain, and lungs (88). In fact, the formation of a metastatic lesion could depend on the CTC's ability to adapt, survive, and induce neoangiogenesis in the invaded tissue (89).

The cancer phenotype, hence, represents a distinct biological component related to prognosis, recurrence and possibly therapeutic response (sensitivity and drug resistance) of cancer patients. Interestingly, although CTCs do not correlate with the primary tumor size, they do correlate well with the extent of skeletal metastasis suggesting a possible link between the presence of CTCs and bone metastasis. In this context, it is interesting to note that the development of bone metastasis correlates with the presence of DTCs in the bone marrow and that DTCs number correlate strongly with CTCs number (90-99) suggesting that the bone marrow may be a reservoir for the development of metastasis within and outside the skeleton. CTCs count is also correlated to relapse in the same anatomical region as the primary, this suggests that the

dissemination of breast cancer cells in the body might be also associated with a "tumor self-seeding" mechanism, promoting tumor growth, angiogenesis, and stromal recruitment through seed-derived factors (100).For example, it has been shown that breast cancer cells recirculate from distant sites back to the primary site (31). This mechanism has only been demonstrated in experimental models (101) and with bone marrow tumor cells (102).

The exact composition of CTCs remains elusive but it is now established that they represent a very heterogeneous population that needs to be better characterized (103, 104). The potential applications of CTCs are just emerging and include but are not limited to their use as diagnostic and prognostic markers (33) and potentially to identify new targets in cancer metastasis.

III. CURRENT METHODOLOGIES

As of today , there is no gold standard to refer to, as there is no optimal platform that has yet enabled detailed molecular and functional characterization of CTCs. Therefore, there is a great need to isolate this rare population in sufficient quantity, purity and in a viable state to conduct such studies. The most important technical challenge for CTC research is their rarity in the peripheral blood of cancer patients, being estimated on average of just one CTC per ~10^7 white blood cells (WBCs) per milliliter of blood. Most of the new technologies that have been developed use a combination of "enrichment" and "detection" (57, 105). The CTC enrichment is a process for capturing the rare tumor cells in a vast group of normal white blood cells. This can be achieved either according to the physical properties of the cells, for example density, size, etc.; or their biological features, for example, their tumor cell surface maker expression as discussed earlier (57, 106). Positive identification of CTCs is commonly achieved using immunostaining and microscopy. PCR methods (59) will also be discussed in the following sections.

The potential applications of CTC analyses are extraordinarily promising, however, the development of appropriate and reliable technological platforms for this rare population needs further development. Appropriate interpretation of the analyses reported in CTC studies requires ta thorough understanding of the technical limitations of the analysis. In recent years, multiple methods have been reported for detecting CTCs, such as the CellSearch™ system, RT-PCR, Magnetic-activated cell sorting (MACS), and the ChromaVision Medical System (107). Among these the CellSearch™ system (Veridex, Raritan, NJ, USA) is the most commonly used and is

the only method approved by the Food and Drug Administration (FDA) for CTC detection and enumeration based on the expression of EpCAM but this method has a low sensitivity (108). This semi-automated system has contributed considerably to the development of CTC studies and enumeration, however, there are a number of limitations that we will discuss in the following sections. In the meta-analysis by Yan et. al (75) the authors analysed CellSearch^{TM} and other methods such as RT-PCR (which determine the CTC by detecting the mRNA expression of epithelial markers such as EpCAM or CKs).

Recently innovative methods have been developed to identify and quantify CTCs in blood samples using a biosensor field and microfluidic chip (109, 110). These technologies bring new insights for tracking metastatic breast cancer using novel enrichment and isolation platforms (111).Another technology called functional cell separation method called collagen adhesion matrix (CAM) assay has recently been described to improve the enrichment and identification steps (4). In the following sections we will discuss the most common technologies available for isolation and characterization of CTCs.

3.1 Detection and Isolation Techniques

Detection is the direct or indirect identification of CTCs in a sample and isolation refers to the efficient separation of CTCs from all other cells. According to the review by Esmaeilsabzali et. al. (2013) where the authors classified the detection and isolation techniques currently available, the performance of a typical CTC detection and isolation system should ideally be evaluated using the following parameters (107):

- "Recovery rate (sensitivity): the ratio of detected or isolated CTCs to all CTCs present in a sample. It is also necessary to determine the smallest number of CTCs per sample that can be detected or isolated. This is particularly crucial for the early diagnosis when the number of CTCs in the peripheral blood is often low.
- Purity rate (specificity): the ratio of detected or isolated CTCs to all detected or isolated cells from a sample.
- Enrichment rate: the ratio of CTCs to blood cells before and after CTC enrichment or isolation.
- Throughput: the speed by which the sample is processed. The throughput is mainly reported as the number of cells processed per unit time.
- Viability: the percentage of viable CTCs to all isolated CTCs from a sample (107)

3.1.1 Antibody-based methods for CTC detection and isolation

The most widely used methods are positive selection methods based on antigen capture on the surface of CTCs using antibodies bound to magnetic particles. They rely on the universal epithelial marker EpCAM that is not present on white blood cells (112, 113). The CellSearch (Veridex) method is the only FDA approved method and uses positive selection by anti-EpCAM antibodies followed by cytokeratin histological confirmation (114). Non-specific background of white blood cells is eliminated using CD45 staining (a cell surface marker of white blood cells).

However, this method suffers from several drawbacks, including low sensitivity of detection (less than 40% of patients with chemotherapy naive advanced cancer are tested positive), as a minimum of 4 CTCs/ml of blood (50) is necessary to positively identify patients. This lack of

sensitivity is mainly due to the process of EMT on circulating CTCs, which leads to the loss of epithelial markers such as EpCAM, and also leads to over expression of N-cadherin, and cytoskeletal alterations. Additionally, it produces phenotypical and structural changes leading to an increased motility and invasiveness (115). Hence, EpCAM-based tests are unable to detect CTCs with low or absent EpCAM expression and other CTCs, including CSCs and CTCs undergoing EMT, all of which have been identified in early and metastatic breast cancer patients (11, 116). Furthermore, EpCAM-based examinations make it difficult or even impossible to detect cancer associated macrophagen-like cells (CAMLs) which are specialized phagocytic myeloid cells found in the peripheral blood of breast cancer patients with solid tumors (117). Due to these limitations, other approaches have also been proposed including nucleic acid detection methods and isolation methods based on physical characteristics of CTCs (43).

Negative selection methods using antibodies against the cell surface marker CD45 to eliminate white blood cells (leaving behind CTCs irrespective of their cell surface markers) have also been proposed. In theory, this approach would permit isolation of both EpCAM positive and negative cells, including CSCs and EMT, which harbour little or no expression of this epithelial marker. However, reports using this approach have had limited success (118-121) in part due to the low yield of CTCs using small amounts of peripheral blood. Nevertheless, single-step enrichment platforms make it difficult to isolate pure CTCs and result in persistent leukocyte contamination. Antibody capture systems always enrich a proportion of leukocytes physically trapping these cells amongst the magnetic beads and filtration devices cannot exclude all leukocytes due to natural variation in leukocyte size. On the other hand, detection and isolation of CTCs based on

physical properties have potential value because of its relative simplicity, detection irrespective of cell surface markers, low cost and amenability to high throughput screening (122-124).

Additional cell surface marker–based CTC detection approaches are commonly used including standard flow cytometry (FACS) and the MagSweeper that is coated with an antibody to EpCAM (125).Overall, negative selection using an antibody against the CD45 surface marker (by removing the leukocytes, thereby leaving residual CTCs) is preferable.

3.1.2 Nucleic acid–based detection as indirect measures of CTCs

Free DNA and RNA circulating in plasma from patients with cancer studies have suggested a link between the presence of CTCs and the detection of free tumor-derived DNA in serum/plasma of prostate cancer patients. This method identifies specific DNA or mRNA molecules that serve as markers to indirectly detect the presence of CTCs. Specific primers are employed in PCR12 to target known DNA or mRNA molecules that are extracted from an enriched sample and are supposedly associated with CTCs genes (107). These genes either code for tissue-organ-, or tumor-specific proteins or polypeptides.

As the authors point, the nucleic acid-based approach offers the highest sensitivity for indirect measurement of CTC using commercially available reverse transcription RT-PCR kits as the most common methodologies. However, the origin of these nucleic acids include products of necrotic cells in tumor deposits, tumor-derived exosomes, or lysis of CTCs in the bloodstream (58).Attempts to overcome these non-specific components have been made by isolating nucleic acids from cell-free plasma or purifying first the nucleated cells shadowed by lysis. Another

major limitation relates to the false-positive signals could originate from small number of non-cancerous cells that have entered the circulation due to inflammation, invasive diagnostic biopsies, or during the surgery (58).

Other studies have tried to overcome these limitations by inferring the presence of CTCs through the detection of tumour-specific mRNAs from enriched samples. At the RNA level, RT-PCR analyses have been applied either to unpurified plasma nucleic acids or more commonly to enriched CTC populations. Specifically, researchers have focused on cytokeratin-19 mRNA detection in breast cancer (57) and tumor-specific methylation patterns of breast cancer metastasis suppressor-1 promoter (126). Ideally, in order to obtain a pure CTC sample one needs to apply a second isolation step following CTC enrichment, for example by physical micromanipulation.

All in all the approaches based on detection of released DNA or RNA (127-129) from CTCs lack specificity and rely on PCR based detection of rare cells among large quantities of white blood cells resulting in many false negative or positive tests (130). Hence, the challenges of these approaches include the frequency of both false-positive and false-negative PCR products.

3.1.3 Isolation of CTCs based on their physical properties

Isolation of CTCs can also be based on their physical properties including density, size, mechanical plasticity, and dielectric properties. CTCs have a larger size (~20–30μm) compared to that of white blood cells (~8–12μm) and the even smaller red blood cells and this characteristic is the foundation for size-based isolation which rely on this differences, for

example membrane filters, size/deformation-based microfluidic chips, and size-based hydrodynamic methods (107). Isolation of CTCs using this approach has been applied using several different filtration-based approaches, such as isolation by size of epithelial tumor cells and microelectromechanical systems (58). Furthermore, size-based isolation of CTCs has been done using track-etched polycarbonate filters, for example the ones commercialized by ISET (Rarecells, Paris, France) and ScreenCell systems (ScreenCell, Paris, France) for the isolation of fixed and live CTCs, respectively (76). The filter comprises a porous membrane which contains several randomly distributed 8-μm-diameter holes that allow blood constituents to cross but capture the supposedly larger CTCs. Cells often remain intact, allowing for their subsequent morphological or molecular analysis (107). However, the distribution pattern of the pores could result in the fusion of pores to form escape routes for CTCs. Technically, this problem could be resolved by having a distribution and geometry of the pores established.

These platforms relying on cell size and density are flawed by the inherent heterogeneity of CTCs, which vary widely in size within and between patients. These methods will need further extensive validations using known comparators but are unlikely to be useful in the absence of extensive and parallel characterization using antibody-based approaches described above. They may, however, be used as a filtering step prior to more specific antibody-based isolation methods. Recently, another solution as been proposed, using an antibody-mediated size-based approach (131). To address the size heterogeneity of CTCs, cancer cells have been labeled with micro beads that specifically attach to surface antigens (i.e. EpCAM). This approach increases the size of CTC by creating a layer of microbeads on the surface, in this way a clear cut-off is set

for the size of non-target cells. However, uniform surface labeling of target cells will be very difficult to achieve in practice (107).

CTCs are also dielectric particles, which means that they are neutral electrically but they can be polarized. Using this feature, researchers had used an electric field such as DC or AC, generating electric dipoles moments on the cells; depending on their phenotype, morphology, and physiological state, the cells will have different properties. This is also called electrokinetic isolation of CTCs (106). The cells CAN then be exposed to this electric field and experienced different electrokinetic reactions.

3.1.4 Additional innovative CTC detection strategies

A more recent and interesting approach is a CTCs chip developed by Stott et al. (132) which is comprised of multiple chambers coated with anti-EpCAM antibodies and is amenable to enumeration and analysis of around 50 CTCs/ml (total of around 100-200 CTCs/chip using 2-4ml of blood). This technology permits longitudinal monitoring and is expected to allow limited molecular characterization. Characterization by flow cytometry has also been reported using few ml of collected peripheral blood (133-135) but its applicability is limited by its sensitivity to detect the low number of cells present in a small blood sample. The MagSweeper which is an alternate magnetic bead method coated with anti-EpCAM antibodies is another available platform (125). An automated technology called the DEPArray also uses a closed system of moveable electrostatic cages to move and capture the cells, this has been used for isolation of single or groups of CTCs. Additionally, a number of cell based scanning techniques are also

emerging using fiberoptic or laser scanning (86, 124, 136) for cytological evaluation. However, these methods will also need further careful validation.

Finally, according with Yan et. al (2016) it is probable that CTC detection methods based on different approaches would enumerate CTCs differently in the same patients, particularly the ones with non metastatic breast cancer. A recent study by Ignatiadis et. al (2014) evaluated the inter reader agreement of 22 different readers from 15 laboratories comprising 15 centers from Europe and the United States, and 8 readers from Veridex laboratories, using non metastatic and metastatic breast cancer samples. The study reported a median agreement of 92 % between the academic readers and laboratories in metastatic cancers but also found a reduced agreement in non-metastatic breast cancer patients with low CTC counts. The study concluded that it was important to establish a consensus guideline for image interpretation for CTC detection in non-metastatic breast cancer. In addition, the inconsistency of cut-off values to determine CTC-positivity between methodologies is a limitation for actual application of CTCs in clinical setting.

Mansoori et. al. illustrates the different methods for identification and isolation of CTCs as follows (137):

1. Immunomagnetic enrichment: this is a method based on the different specific surface antigen on ephitelial tumor cells, using both positive and negative selection. The CTC can be selected via magnetic beads when they are coupled to antibodies directed to specific markers (CK, EPCAM, ERBB2, etc.). One technique with this approach is the magnetic-activated cell sorting (MACS). MACS uses surface antigens (CD molecules) for cell separation called immune-labeling superparamagnetic particles.These beads are

biodegradable, therefore it is not necessary to remove them from the cells after the separation process. The cells preserve their structure, function, and activity (52). The sensitivity of MACS is one cell per 0.3 mL requiring 5–15 mL blood. Another widely used example of the immunomagnetic separation is CellSearch™, a semi-automated method approved by the FDA which has been widely used for detection of CTCs in metastatic breast, prostate, and colorectal cancer.

2. Flow cytometry is a well-defined method for the identification of breast cancer stem cells (BCSCs) and CTCs. This method uses unique cell surface markers for its isolation, including epithelial specific antigen (ESA) and CTC specific markers (138). It is a laser- or impedance-based, biophysical technology employed in cell counting and biomarker detection, which allows individual characterization of rare cells. It is predominantly used to measure fluorescence intensity produced by fluorescent-labeled antibodies detecting proteins or ligands that bind to specific cell-associated molecules. Through this approach it is possible to quantify specific cell subpopulation, simultaneous examination of cell size, viability, DNA content, and intra-extracellular markers (139).Furthermore, CTC acquired by this method can undergo additional morphologic assessment and molecular analysis.

3. Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry. "It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon attachment of a conjugated antibody to

the cell surface which emits specific fluorescent lights that enables characterization of each cell type"(137).

4. Reverse transcription polymerase chain reaction (RT-PCR) is a variant of polymerase chain reaction (PCR) and is used to detect RNA expression and to measure its quality. Several RT-PCR methods of epithelium- or organ- specific expression can facilitate research of target genes relevant to the metastasis process (112).

5. Real-time polymerase chain reaction (real-time PCR): also known as quantitative polymerase chain reaction (qPCR) monitors the amplification of a targeted DNA molecule during the PCR.

6. Filtration is used to enrich CTCs through the size of the cells.Such method includes the epithelial tumor cell isolation (ISET) which uses a filter that allows passage of leukocytes and erythrocytes while entrapping large tumor epithelial cells. It uses 6-15mL of peripheral blood, and has an isolation efficacy of 80% for breast cancer cells with a sensitivity of one CTC per millilitre (52).

7. Density gradient centrifugation and immunocytochemistry is based on enrichment of CTCs using their density gradient by centrifugation in Ficoll Hypaque (GE Healthcare). Mononuclear cells(MNCs) are isolated and subsequently spun on glass slides. Density of MNCs is <1.077 g/mL, while the density of the rest of the cells is higher. Hence, separation is accomplished using different layers, this method has a sensitivity of one CTC per 4.6mL and uses approximately 15-30mL of blood. This method however lacks

specificity because of potential cross-contamination with polymorphonuclear cells. Centrifugation has to be performed immediately to prevent mixing layers.

8. Chip based methods: Microfluidics, immobilization and in situ hybridization (ISH) use special chips that combine microfluidics and immobilization of CTCs through binding of specific antibodies. For example, affinity-based chip (CTC chip) uses a microfluidic chip composed of 78,000 microposts (100 mm height and 100 mm diameter). It uses size-based separation of nucleated cells from whole blood in a microfluidic channel.

Table 2: Clinical relevance of specific CTC (circulating tumor cell) subsets (140).

Method for CTC Detection	**Subset of CTC Identified**	**Clinical Relevance**
CellSearch assay (Veridex)	$EpCAM^{+}/CK8^{+}/CD45^{-}$	A number of CTC ≥5 cells/7.5 mL at baseline and at the first follow-up represented an independent negative prognostic factor for OS and PFS.
	$EpCAM^{+}/CK^{+}/CD45^{-}$	An elevated CTC number before the second cycle of chemotherapy was an early predictive marker of poor PFS and OS.
	$EpCAM^{+}/CK^{+}/CD45^{-}/M30^{-/+}$	The presence of M30-negative CTC was associated with a decreased chance of survival in metastatic patients. Both a decrease in the total CTC number and an increase in the fraction of apoptotic CTC (M30-positive) represented a predictive marker.
	$EpCAM^{+}/CK^{+}/CD45^{-}/HER2^{\pm}$	Evidence that HER2-negative primary tumors could develop HER2-

Method for CTC Detection	Subset of CTC Identified	Clinical Relevance
		positive CTC during disease progression. The HER2 status of CTC could be a prognostic factor in MBC patients.
Adna Test Breast Cancer	MUCIN-1-EpCAM^{+}/HER2$^{\pm}$	HER2-positive CTC could be detected in HER2-negative primary tumors.
	MUCIN-1-EpCAM^{+}/Twist1$^{\pm}$/Akt2$^{\pm}$/Pl3Kα$^{\pm}$/ALDH1$^{\pm}$	CTC expressing EMT or stem cell-like markers were associated with poor prognosis and drug resistance.
	MUCIN-1-EpCAM^{+}/HER2$^{\pm}$/ER$^{\pm}$/PgR$^{\pm}$	The molecular profiling of CTC could predict the risk of recurrence and drug resistance.
DEPArray (Silicon Biosystems)	EpCAM^{+}/CK^{+}/CD45^{-}	The mutational analysis of the TP53 status of CTC showed the presence of heterogeneity between CTC and primary tumors. The presence of TP53 mutations, as assessed by next-generation sequencing performed on single-cell sorted CTC, could represent a negative prognostic factor.
	4 CD45-negative subsets: Epithelial-CTC: EpCAM-E-cadherin^{+} EM-CTC: EpCAM-E-cadherin^{+}/CD44-CD146-N-cadherin^{+} Mesenchymal cells: CD44-CD146-N-cadherin^{+}/EpCAM^{-}/E-cadherin^{-} Negative cells: EpCAM^{-}/E-cadherin^{-}/CD44^{-}/CD146^{-}/N-cadherin^{-}	The presence of CTC in EMT was associated with a poor prognosis. The study highlighted also a correlation between the clinicopathological features of patients and the different subsets of CTC identified.
	EpCAM^{+}/CK^{+}/CD45^{-}	The presence of activating PIK3CA mutations in CTC could predict resistance to anti-HER2 therapies.
Fluorescence in situ hybridization (FISH)	EpCAM^{+}/CK^{+}/CD45^{-}/HER2 amplification$^{\pm}$	Evidence that HER2-negative primary tumors

Method for CTC Detection	Subset of CTC Identified	Clinical Relevance
		developed HER2-positive CTC during disease progression, opening the way to targeted therapies.
Dual-colorimetric RNA-in situ hybridization	E-CTC: CK5-CK7-CK8-CK18-CK9-EpCAM-E-cadherin^{+} M-CTC: FN1-N-cadherin-SERPINE1-PAI1^{+}	The mesenchymal immunophenotype was associated with disease progression. Furthermore, CTC from patients with lobular breast cancers were predominantly epithelial-like, whereas those from the triple negative and HER2-positive subtypes were predominantly mesenchymal-like.
Fluorescence activated cell sorting (FACS)	EpCAM^{-}/HER2^{+}/EGFR^{+}/Heparanase^{+}/Notch1^{+}	Identification, on CTC, of a signature suggestive of metastatic competency to the brain.
EPISPOT (Epithelial ImmunoSPOT) assay	CK19^{+}/MUCIN-1^{+}	CTC releasing CK19 (CK19-RC) was correlated to an unfavorable clinical outcome.
ISET (isolation by size of epithelial tumor cells)	Size/CK7^{+}	Evidence that EpCAM-negative CTC could escape from the CellSearch analysis.
RT-qPCR	CK19 mRNA	CK19 mRNA-positive cells could be detected in both early-stage and metastatic breast cancer patients, suggesting the use of RT-qPCR for the continuous monitoring and quantification of circulating epithelial cells.
	EM-CTC: EpCAM^{+}/CD45^{-}/TWIST1^{+}/SNAIL1^{+}/ZEB1^{+} Cancer stem cell-like cells: EpCAM^{+}/CD45^{-}/ALDH^{+}/CD133^{+}	EM-CTC and cancer stem cell-like cells had a prognostic value in HER2-positive MBC patients treated with targeted therapies.

3.2 Apheresis

Apheresis is a standard clinical method used to isolate mononuclear cells (MNCs) from blood for various applications. Apheresis involves removing whole blood from the patient or donor and separate the blood into components (such as leukocytes or platelets). The remaining blood is reintroduced back into the bloodstream of the patient.In clinical practice apheresis is used for different purposes including removing specific blood components for donation, treatment for specific medical conditions, and removal of stem cells (Fischer, 2013). Apheresis is made possible by using a continuous density-based MNCs separation (density of 1.055-1.08 g/mL) of total processed blood (TPB), hence the procedure makes it possible to collect CTCs together with MNCs(141).

Apheresis using centrifugation procedures require blood flow rates of 50 - 150 mL/minute.(141). Also, some apheresis-based treatments require specific sequential blood exchanges cycles or continuous processing. The Canadian Apheresis Study Group has reported that 67% of 5,234 therapeutic plasma exchange (TPE) procedures could be completed with peripheral venous access alone with a very low frequency of complications.

IV. OBJECTIVES

5.1 Hypothesis

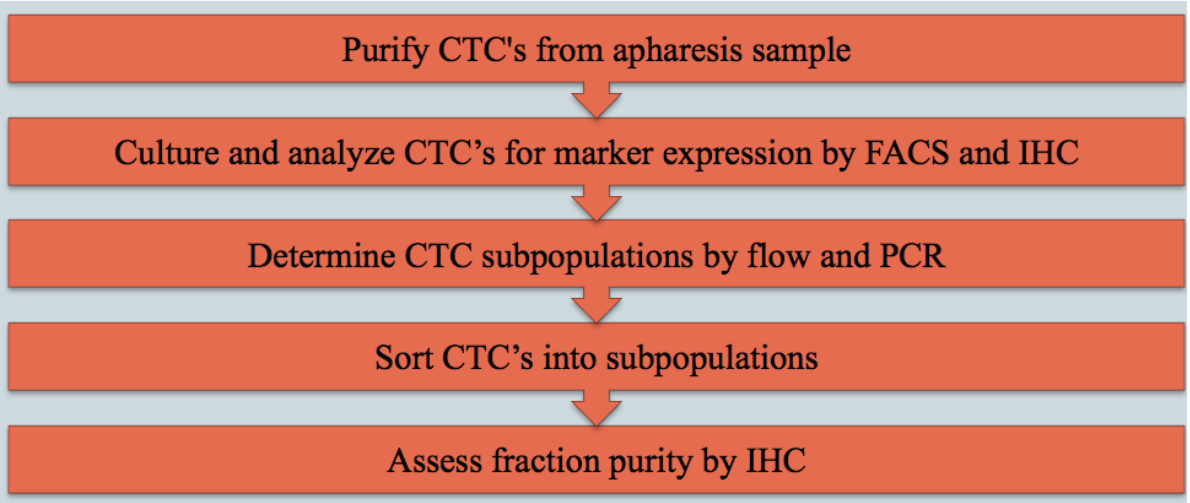
We hypothesize that CTCs due in part to their heterogeneity and metastatic potential have different expression profile compared to non-metastatic breast cancer cells.

5.2 Aims of study

1. To prove and study CTCs heterogeneity, Dr. Saleh will focus mainly on using flow cytometry (FCM), find suitable antibodies directed against the desired breast cancer cells markers to create and optimize a platform for CTCs identification. Cell populations will be analyzed on an LSRFortessa (BD Biosciences) at the MUHC flow cytometry core facility, using the software FlowJo. FCM will be used to gate the CD45 (-) cells and followed by separation CTCs and CSCs based on their EpCAM status.

2. Following characterization by FCM, EpCAM positive CTCs and EpCAM negative CSCs/EMT subpopulations will be isolated and collected using the FACS-Aria cell-sorting machine (BD Biosciences) that uses immunomagnetic particles coated with EpCAM monoclonal antibodies. Prior enrichment with leukocyte-specific CD45 conjugated monoclonal antibodies will be done to exclude the white blood cell population.

Figure 3: Steps required for full characterization and isolation of circulating tumor cells



V. MATERIALS AND METHODS:

5.1 Patient screening and sample collection

Breast cancer patients seen at the Breast Centre of the Royal Victoria Hospital were approached for consent (for sample collection, investigation of previously archived specimens and follow-up). Patients were enrolled between January 2011 til June 2012. 35 ml of peripheral blood was drawn from each patient. The first 5 ml of blood sampled was stored separately to avoid potential contamination with normal epithelial cells during venipuncture. 20 ml was used for CTC screening, while 10ml was banked for validation of any biomarkers identified in the course of this study. Eligibility criteria included diagnosis of metastatic breast cancer in patients above 18 years of age.

Peripheral blood screening for CTCs was performed via IHC directed against epitopes specific to epithelial cells, using a commercially available kit (EPIMET, AS Diagnostik, Germany). Full clinical, epidemiological and pathological information (tumor grade, ER, PR, Her2/neu status, histological subtype, etc.) for patients entered in this study was collected via chart review. All results were kept in a database in accordance with our institutional policies on patient record confidentiality. Regular chart review was used to detect changes in patient status. Patients found to be CTC-positive by peripheral blood screening were approached and offered whole blood volume aphaeresis. The eligibility criteria for the study are shown in figure 5. Patients who screened positive for CTCs were asked to undergo apheresis.

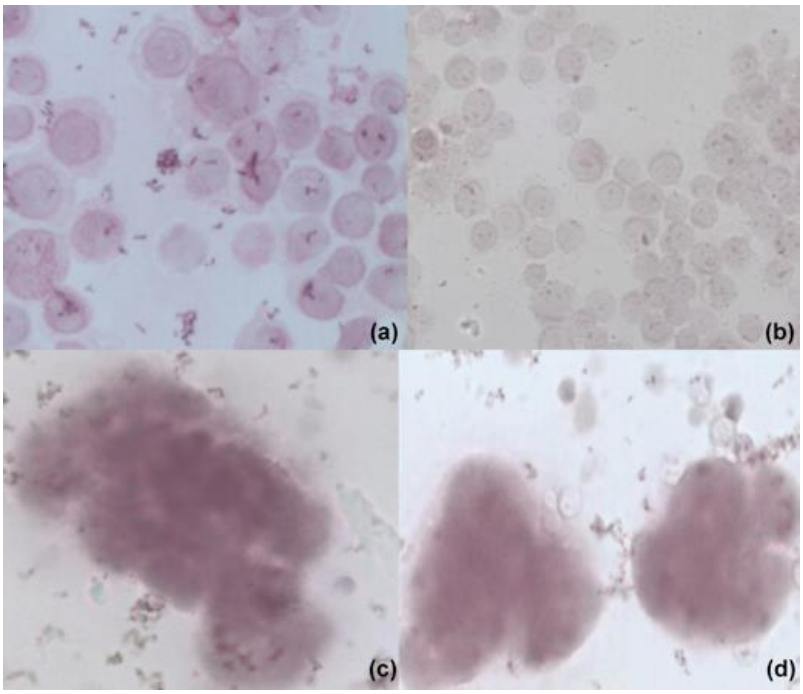
5.2 Healthy volunteers

Given that large volume collection for CTCs had not been reported in the literature, one concern would be the possibility of false positive results in our study. In order to better assess the validity of our methodology and the baseline background of cytokeratin positive cells in normal individuals , we approached 5 healthy women to obtain 25mL of peripheral blood. An informed consent, approved by the hospital's REB, was also obtained. The same screening methodology mentioned in this report was applied to the healthy volunteer's blood.

5.3 Screening with immunostaining for cytokeratin

Acetone-fixed slides were washed with PBS for 3 minutes for a total of 3 times and then stained with EPIMET Epithelial Cell Detection kit3 (EPIMET, AS Diagnostik, Gennany) according to manufacturer's instruction. Briefly, slides were incubated with anti-cytokeratin antibody Fab fragment conjugated with alkaline phosphatase or control antibody for 45 minutes at room temperature. After 3 washes with PBS, slides were incubated with alkaline phosphatase substrate for 10-20 minutes at room temperature. Another 3 washes with PBS were done. Slides were then sealed with aqueous mounting media (Dako, Burlington, ON, Canada) and cover slips (Fisher Scientific Canada). Stained slides were evaluated with bright-field microscope (Leica Microsystems Inc., Concord, ON, Canada). A cell was considered positive if it demonstrated pink/red cytoplasmic stain.

Figure 4: (a) Peripheral blood of a normal healthy volunteer spiked with MCF-7 showing positive cytokeratin staining; (b) Negative control MCF-7; (c) & (d) Patient's sample showing cytokeratin stained circulating tumor cells in clusters.



5.4 Isolation and enrichment of large amount of CTCs by apheresis

To isolate large numbers of CTCs, whole blood aphaeresis was used. As mentioned before, apheresis is a technique used routinely to sort and collect large amounts of cells from the blood circulation. An apheresis machine collects all nucleated cells from the blood (white blood cells and CTCs) by using a centrifuge, which separates the cells from the blood and returns the non-nucleated components (red blood cells and platelets) back to the patient. The apheresis procedure takes 3 to 4 hours to complete.

This procedure was carried out in a clinical aphaeresis facility at the MUHC, a routine procedure, under the supervision of experienced nurses and an attending clinical haematologist.

While this technique involves the insertion of a large-bore peripheral venous catheter, it has been shown to pose minimal risks to patients. No adverse events occurred in any of the patients subjected to the procedure.

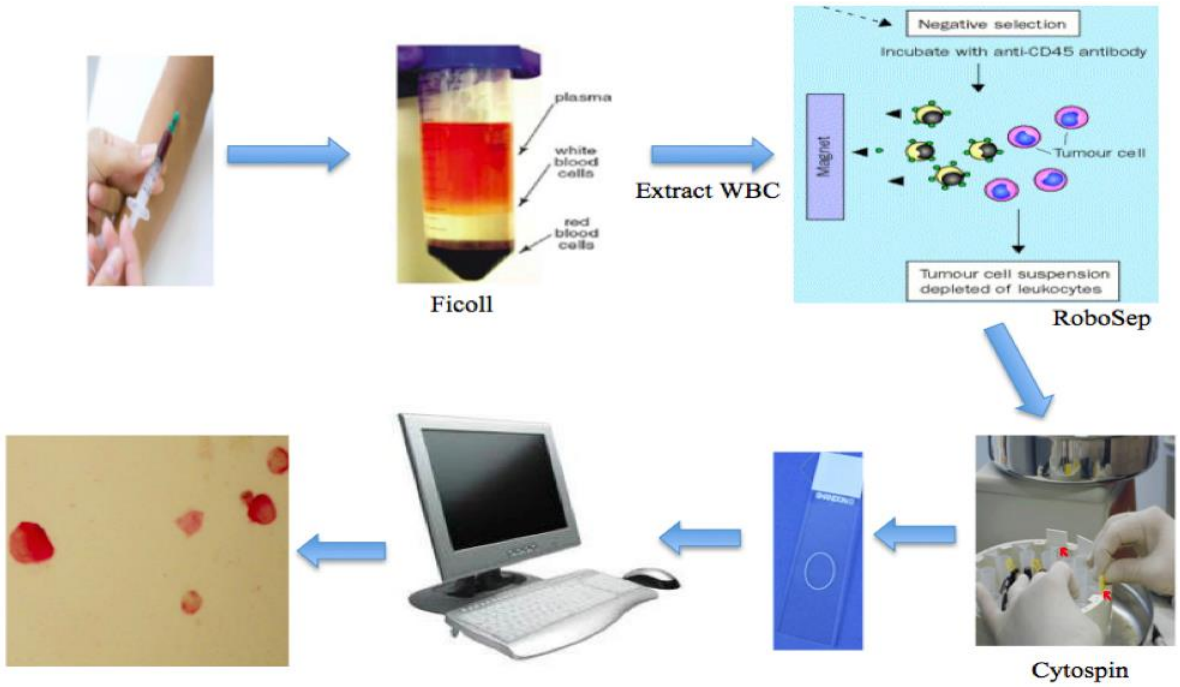
5.5 Peripheral blood mononuclear cells(PBMCs) collection using Ficoll Paque

Peripheral blood mononuclear cells (PBMCs) were collected using Ficoll Paque Plus (GE HealthCare, Mississauga, ON, Canada) according to manufacturer's instruction[1]). Briefly, anticoagulant-treated (EDTA or Sodium citrate) blood or apheresis product was diluted with equal volume of PBS (Wisent Bioproducts, St Bruno, Quebec, Canada) containing 2% FBS (Life Technologies, Burlington, ON, Canada). The diluted sample was layered over equal volume (for screening sample) or half volume (for apheresis product) Ficoll Paque Plus. Centrifugation was then carried out at 400 × g for 30 minutes at 18–20 °C. The PMBC layer was collected and diluted with PBS containing 2% FBS. Centrifugation was done at 400 × g for 10 minutes at 18–20 °C. Supernatant was removed and pellet was resuspended in appropriate volume of PBS. Cell count was performed with Z1 Particle COULTER COUNTER® (Beckman Coulter Canada, Mississauga, ON, Canada) and cell concentration determined.

5.6 RoboSep enrichment

PBMCs collected using Ficoll Paque were used for CTC enrichment (CD45 depletion). Cells (the whole PBMC fraction for Screening and $1x10^9$ PBMC from apheresis product) were resuspended in RoboSep buffer to $1x10^8$/ml and CD45 cells were depleted on RoboSep using a human CD45 depletion kit2 (Stemcell Technologies, Vancouver, BC, Canada) following manufacturer's instruction. CD45(-) cells were counted and cell concentration calculated.

*Figure 5: S*teps in the methodology mentioned above. (1) Apheresis is done then (2) Ficoll to extract the WBC, (3) cells are then processed by negative selection through the (4) (RoboSep) and then cytospined and placed on slides, cells are then either analyzed by Flow or cultured.



5.7 Cryopreservation

PBMCs from apheresis product were resuspended in freezing media to freeze down the unenriched fraction (cells not subjected to RoboSep CTC enrichment) at a concentration of $1x10^8$/ml and the enriched CD45- cells at at a concentration of $4\text{-}10x10^6$/ml. The cells were frozen first at -80°C for a few days and then transferred to liquid nitrogen tank for long term storage.

5.8 Immunofluoresence technique

CD45- cells obtained from screening sample or apheresis product were resuspended in PBS at $1x10^6$/ml and 0.1ml deposited per spot onto super frost plus microscopic slides (Fisher Scientific Canada, Whitby, ON, Canada) using Shandon cytofunnels (Thermo Fisher Scientific Inc., Waltham, MA, US). The funnels were centrifuged at 1000 rpm for 5min using Shandon cytospin 4 (Fisher Scientific Canada). The slides were air-dried overnight. They were then fixed with cold acetone for 10 minutes and air-dried for 30 minutes. The slides were subjected to immunostaining or stored at -80C.

5.9 Mammosphere protocol

A post-Robosep enriched aliquot of cells was seeded into a low-adherence 6 well dish with 3 ml of mammocult medium per well. Mammocult medium was supplemented with proliferation supplements, 4 ug/ml heparin, 0.48 ug/ml hydrocortisone, and 1% pen/strep. After 1 week of culture cells were "fed" with an additional 1 ml of mammocult medium per well. After another week (2 weeks total) pictures of cells were taken. To passage, the floating non-adherent cells and mammospheres were collected in a 50 ml tube, spun down, washed with PBS and counted under the microscope. The mammospheres were then trypsinized with 4 ml trypsin at 37 degrees Celsius for 10 minutes, pipetting up and down every 5 minutes. 16 ml of DMEM + 10% FBS+ 1% Pen/Strep was then added to neutralize the trypsin and the cells counted using a Coulter Counter. Cells can then either be collected for RNA (spun down and frozen at -80),used to prepare slides or transferred to a new culture dish for secondary mammosphere culture (plate P2). To prepare slides, an aliquot of cells is resuspended in PBS and 100 µl of the cells are put on a slide and left to dry overnight. The slides are then left at 4 degrees Celsius for storage. The P2

cells continued to be fed every week as for P1 mammospheres cultures and passaged every 2 weeks until they no longer grow.

5.10 Identification of cell populations by flow cytometry

In order to characterize the collected EpCAM positive CTCs and EpCAM negative CSCs our target was to first isolate the subpopulations by FCM them based on their phenotype and subsequently analyze the collected cells.

Given that there was no literature evidence on the use of FCM to analyze and isolate large numbers of CTCs, the concentration of each FCM antibody to be used was to be determined. We first started by titrating the concentration of each antibody to determine its appropriate concentration(See table 3).We validated this step using cells collected from one patient post-Robosep.

Following this validation procedure, we then used post-RoboSep enriched frozen aliquots from two patients (one with metastatic TNBC and another with ER (+) breast cancer) using three predefined platforms for FCM (Figure 6). Collected post-Robosep enriched apheresis products containing CTCs were incubated with different monoclonal antibodies (a specific amount of each antibody was added at 4°C for 30 min protected from light). Cells were then washed twice and analyzed by FCM on LSRFortessa (BD Biosciences) using the software FlowJo. Using FCM, we gated the CD45 (-) cells and separated CTCs and CSCs based on their EpCAM status.

1.1.1 The EpCAM positive fraction was then be subdivided into two main populations.

Panel 1 represents chemokine receptors expressed on tumor cells and attracted to the metastatic sites (target tissues) by their specific ligand. Their profile was subsequently correlated to the metastatic profile of the patient.

Panel 2 depicts receptors (ER, PR and Her2) expressed by breast tumors. A change in receptor status has previously been observed in CTCs when compared to the primary tumor. A change in receptor status might influence the course of the disease and could have important therapeutic implications. Her2 is expressed at the surface of cancer cells and could be assessed in live cells by flow. However, ER and PR are intra-cellular receptors and will require fixation prior to FCM. A combination of markers from panels 1 and 2 may be used as an alternative strategy.

1.1.2 The EpCAM negative fraction, containing the CSCs subpopulation, was further examined based on cell surface markers characteristic of breast CSCs including CD24, CD44 and CD133. This strategy is shown in panel 3 of figure 6. Chemokine receptors are also expressed on CSCs and their relevance to metastatic sites was also examined. It is important to note that the only markers linked to EMT are Vimentin and E-and N-Cadherin. These cell surface markers were used in an attempt to further isolate the EMT population from the CSCs in aim 2.1.

1.1.3 For validation purposes, the same procedures (1.1.1 and 1.1.2) were applied to the blood collected from healthy volunteers.

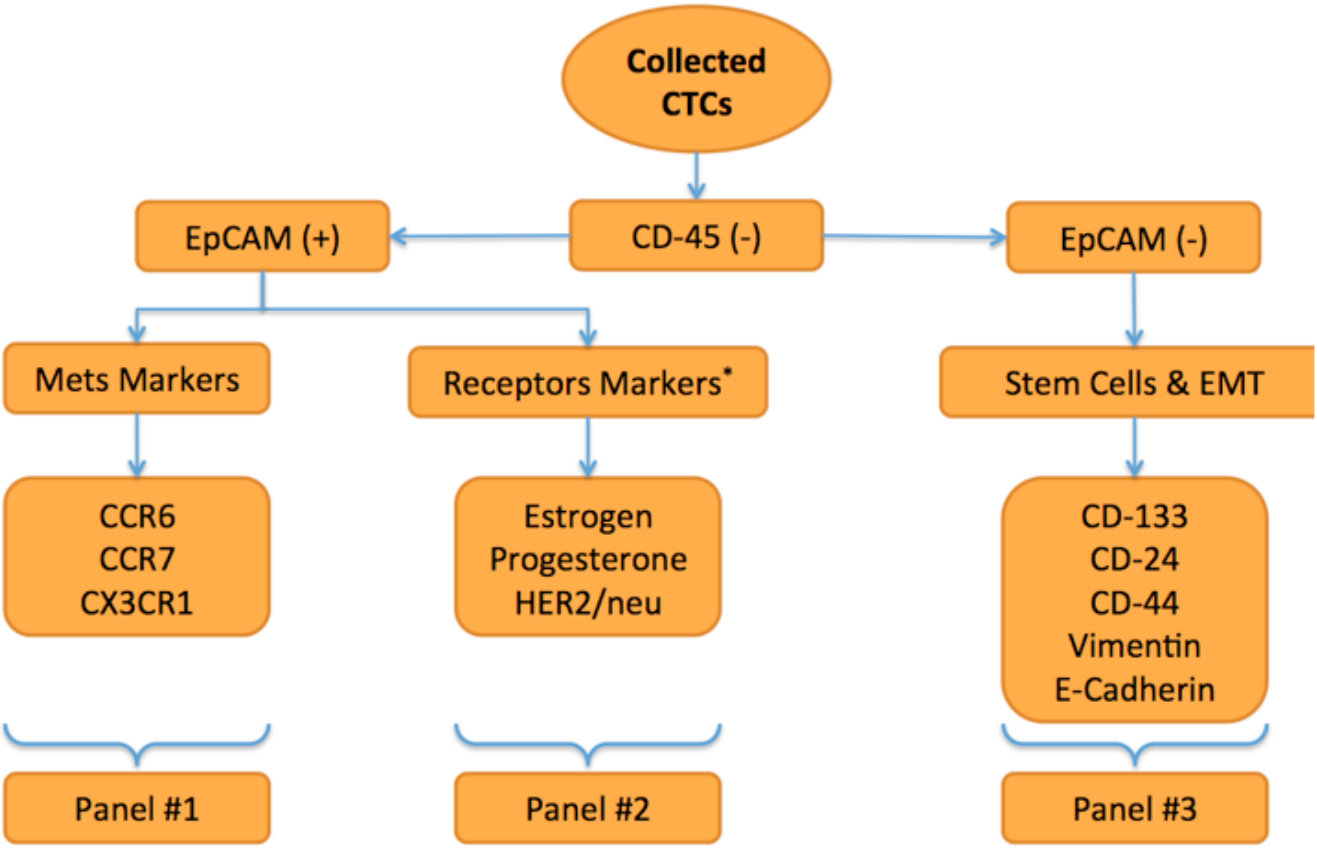


Figure 6: Different flow cytometry panels required for the characterization of the circulating tumor cells.

Possible Combination of Markers

Type / Format	1		2	3	4	5		6		7	8	9
	FITC	Alexa Fluor 488	PE	PerCP-Cy5.5	PE-Cy7	APC	Alexa Fluor® 647	APC-Cy7	APC-H7	AmCyan	BD Horizon V450	PerCP
EpCAM	X		X	X		X						
RANK			X									
CCR6	X		X	X	X	X	X					X
CCR7	X	X	X	X	X	X	X	X			X	X
CX3CR1	X		X		X							X
CXCR4	X	X	X			X	X	X				X
CD45	X	X	X	X	X	X		X	X	X	X	X
CD133			X			X						
CD24	X		X	X	X	X	X					X
CD44	X	X	X	X	X	X	X					X
Vimentin	X	X	X				X					
E-Cadherin	X	X	X			X	X					X
ER	X		X			X	X					
PR	X											
HER2	X		X			X						
CK 8	X	X	X			X						
CK 18	X	X	X			X						
CK 19	X	X				X						

Table 3: Possible marker combination using flow cytometry

VI. RESULTS

6.1 Screening and Isolation

27 patients were screened and consented to participate (Table 3). 7 dropped out post-screening due to refusal to proceed with a central line for the apheresis technique or due to progression of the disease. Half the patients were luminal A (10/20), 4 were triple negative, 1 Her2 positive and 5 Luminal B.

Table 4: Patient approached and total screened.

Event	Number
Number of patients consented	27
Number dropped	7
Number of screening done	30
Total Aphaeresis done	20
Early stage patients	7
Metastatic patients	13

Table 5: Patient characterization.

(Legend: IDC = Invasive ductal carcinoma; ILC = Invasive lobular carcinoma)

ID	Age	Status	Type	Estrogen Receptor		Progesterone Receptor		Her2 neu		Grade
				+/-	%	+/-	%	+/-	FISH	
BC0001	65	Metastatic	ILC	-		**ND**		0		II
BC0003	52	Metastatic	IDC	+	90	+	90	2+	No ampl.	II
BC0004	61	Metastatic	ILC	+	95	+	15	0		II
BC0005	29	Metastatic	IDC	+	90	+	30	1+		II
BC0006	82	Metastatic	IDC	+	>95	-		0		III
BC0007	66	Metastatic	IDC	+	40	-		**ND**		II
BC0008	43	Stage III	IDC	+	80-90	+	20-30	3+		II - III
BC0009	48	Metastatic	IDC	-		-		0		III
BC0010	55	Metastatic	IDC	+	80	+	30	1+		II
BC0011	67	Metastatic	ILC	+	90	+	60	0		II
BC0012	69	Metastatic	ILC	+	75-100	-		2+		III
BC0013	79	Metastatic	ILC	+	90	-		0		II
BC0016	55	Metastatic	IDC	+	Missing Data	+	Missing Data	1+		II
BC0017	47	Metastatic	IDC	+	90	+	90	2+	No ampl.	III
BC0019	68	Stage III	IDC	+	90	+	10	2+	No ampl.	II
BC0021	40	Stage I	IDC	+	90	+	90	2+	No ampl.	III
BC0022	43	Stage I	IDC	-		-		1+		
BC0025	58	Stage I	IDC	+	90	-		1+		II
BC0026	50	---	IDC	+	>95	+	20	2+	No ampl.	II
BC0027	55	---	IDC	-		-		-		

Table 6:Breast Cancer characterization of the consented and apheresed patients

Receptor type	Number
Triple negative	4
Her 2 (+)	1
ER(+)/PR(+)	10
ER(+)/PR(-)	5
TOTAL	**20**

6.1.1 Processing of the blood

All 20 patients had successful cell collection. We noted that most of the cells collected were white blood cells (WBC). Post=-apheresis, the number of cells per tube varied per patient with the mean being 6.262 x 10^6 cells per tube.

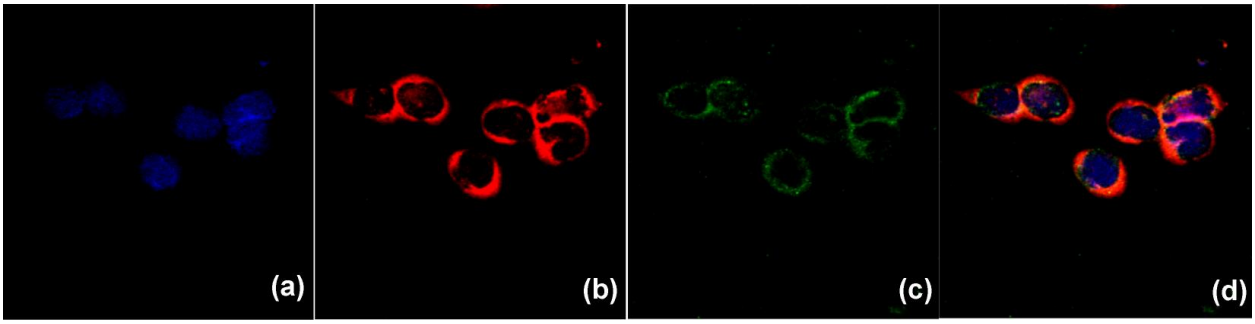
Table 7: Amount of tubes and cells collected of the patients who underwent apheresis.

Subject ID	Post-Ficoll ($1x10^8$/tube)	Post-RoboSep CD-45 (-)	
		Tubes	Cells/tube ($x10^6$)
BC0001	10	6	8.7
BC0003	20	7	2.07
BC0004	7	4	2.07
BC0005	20	2	9.9
BC0006	20	7	4.5
BC0007	13	6	8.03
BC0008	20	7	10.09
BC0009	20	6	9.72
BC0010	13	7	9.28
BC0011	20	8	6.4
BC0012	18	7	4.01
BC0013	20	5	3.8
BC0016	18	11	4.35
BC0017	18	7	7.4
BC0019	20	11	7.77
BC0021	18	9	6.2
BC0022	25	12	6.35
BC0025	18	15	4.6
BC0026	15	19	5
BC0027	17	31	5

6.2 Immunofluorescence

A sample of cells was then stained by immunofluorescence(IF) and analyzed by confocal microscopy to further characterize the CTCs. The image below depicts on CTCs collected from one of our patient. Here a cluster of CTCs co-stained positively for DAPI, ALDH1 and CK8(a marker of CTCs).Other patients yielded the same results while other patients co-expressed CK8 and other markers.

Figure 7: Immunofluorescence staining of collected circulating tumor cells that show positive staining for: (a) DAPI, (b) ALDH1, (c) CK8, (d) Merge.



6.3 Cells in culture

Cells where successfully cultured in Mammocult^{TM} medium(Stem Cell technologies, Vancouver, BC) in non-adherent plates to grow floating cells as mammospeheres and in human mesenchymal stem cell growth medium in adherent plates to obtain attached cells (figure 8).Note the appearance of mammospheres after 3 weeks of culture.

Mammospheres were then stained for DAPI, ALDH1 and CK8 and examined by confocal microscopy (figure 9). Note that the mammosphere shown stained positive for DAPI and ALDH1 but negative for CK8.Other mammospheres co-expressed CK8 and ALDH1 while others expressed other CTCs and CSCs markers(not shown).

Figure 8: Mammosphere culture of isolated circulating tumor cells grown in suspension in mammocult medium (StemCells Technologies): (a) Day 1 – 10x, (b) Day 5, regular O_2 – 10x, (c) day 14 – 20x (Please note the appearance of mammospheres indicated by the arrows that represent a collection of CSCs).

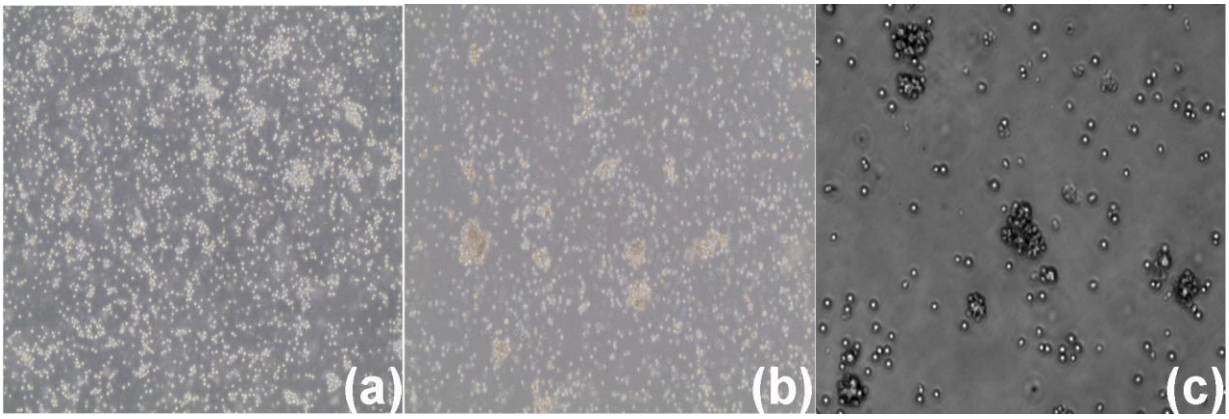
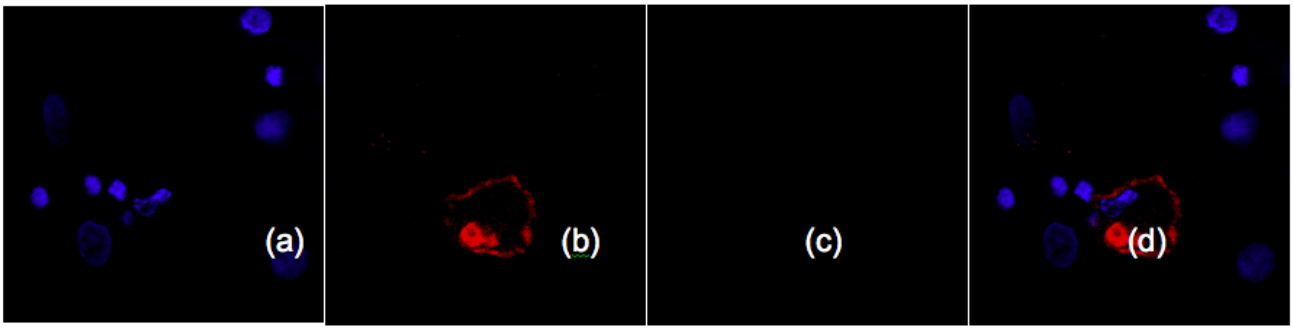


Figure 9: Immunofluorescence staining of the mammospheres that shows: (a) positive staining for DAPI, (b) positive staining for ALDH1, (c) Negative staining for CK8; (d) merge.

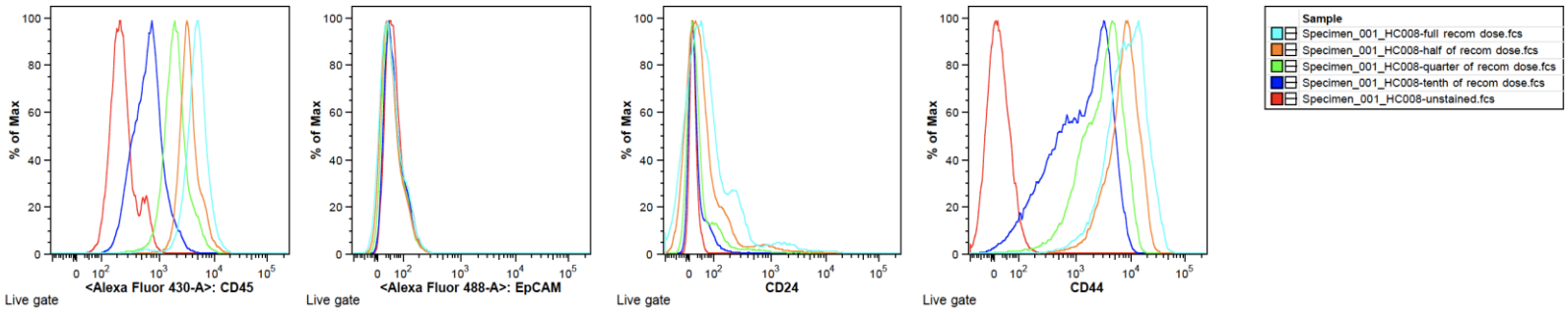


6.4 Flow Cytometry

6.4.1 Antibody titration

Antibodies obtained from BD biosciences were used for the flow cytometry testing. We first proceeded with the highest concentrations recommended by the manufacturer and proceeded with serial dilutions at half the concentration with each experiment. As seen in figure 10, most antibodies could be used with 1:10 of the maximal recommended dose with the same results. Given these results, we proceeded at these dosage concentrations in all future experiments.

Figure 10: Titration doses of FCM antibodies



6.4.2 CD45 & EpCAM

After verification of the specific antibody concentration, we proceeded with the analysis of the patients's cells with that of a healthy control with analysis done on peripheral blood . A TNBC patient (BC0027) was analyzed post-RoboSep.

FCM was used and live cells were gated from both samples. As shown in figure 11 , there was a much higher concentration and quantity of CD45(-) in the TNBC patient compared to the healthy control. It was also noted that there is a higher number of collected cells from the patient. Of note, the number of cells seen in the left lower quadrant is higher in quantity in the patient is explained due to the apheresis and large volume obtained, versus 10mL of blood analyzed from the healthy individual. Gating the CD45(-) cells, we also noted that the TNBC patient had a higher count of cells with EpCAM(+) versus the healthy control (Figure 12).However, we were not able to gate the EpCAM negative cell population given the fact that we could not ascertain whether those cells where truly EpCAM negative or dead cells.

Figure 11: CD45(-) population between a healthy volunteer and the patient

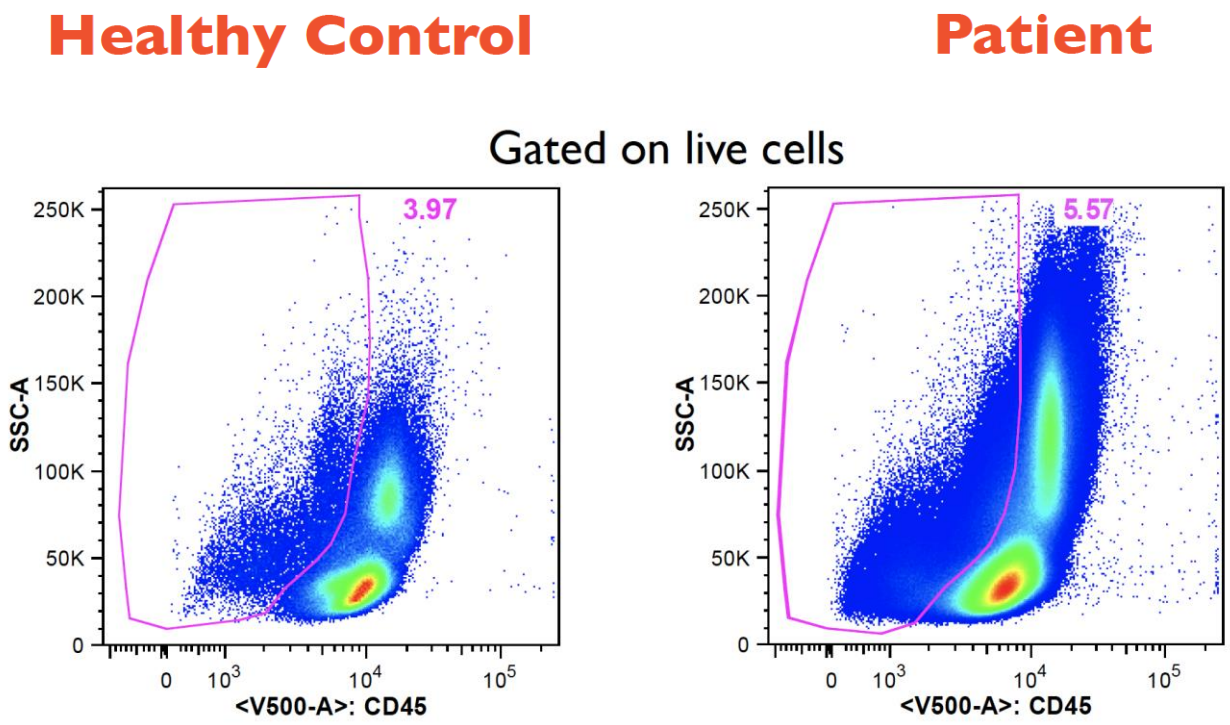
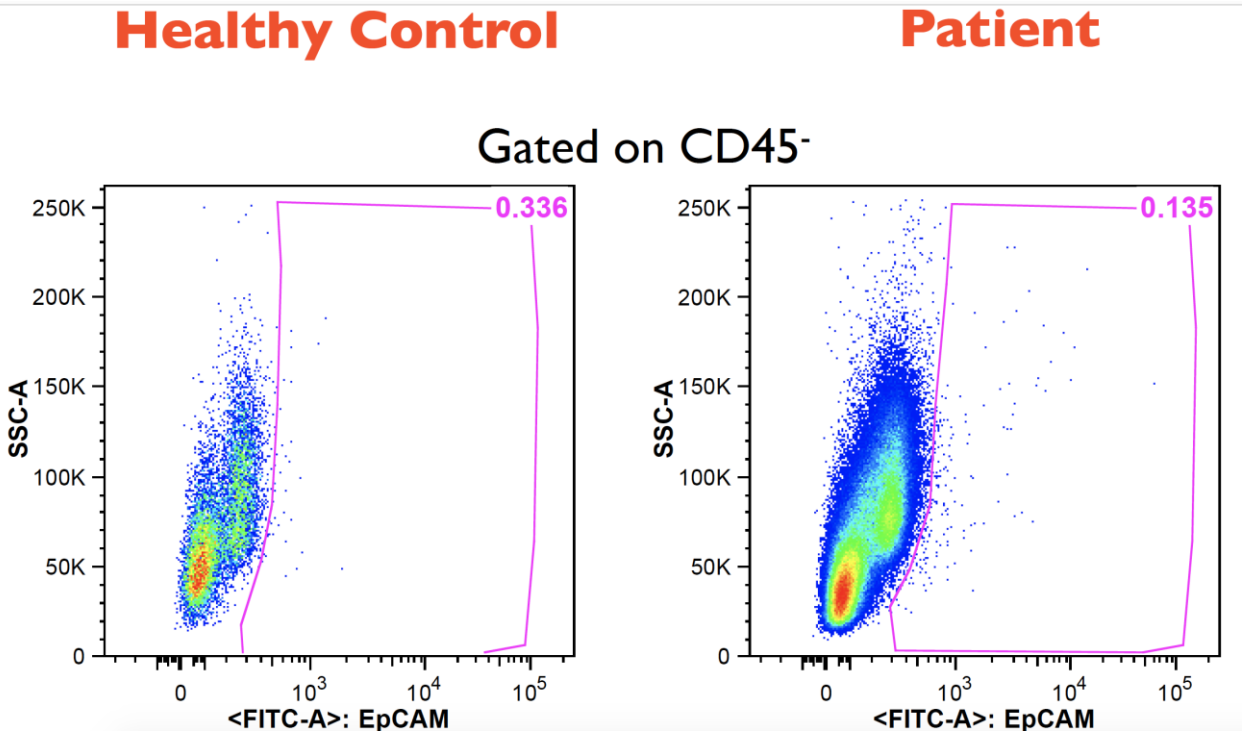


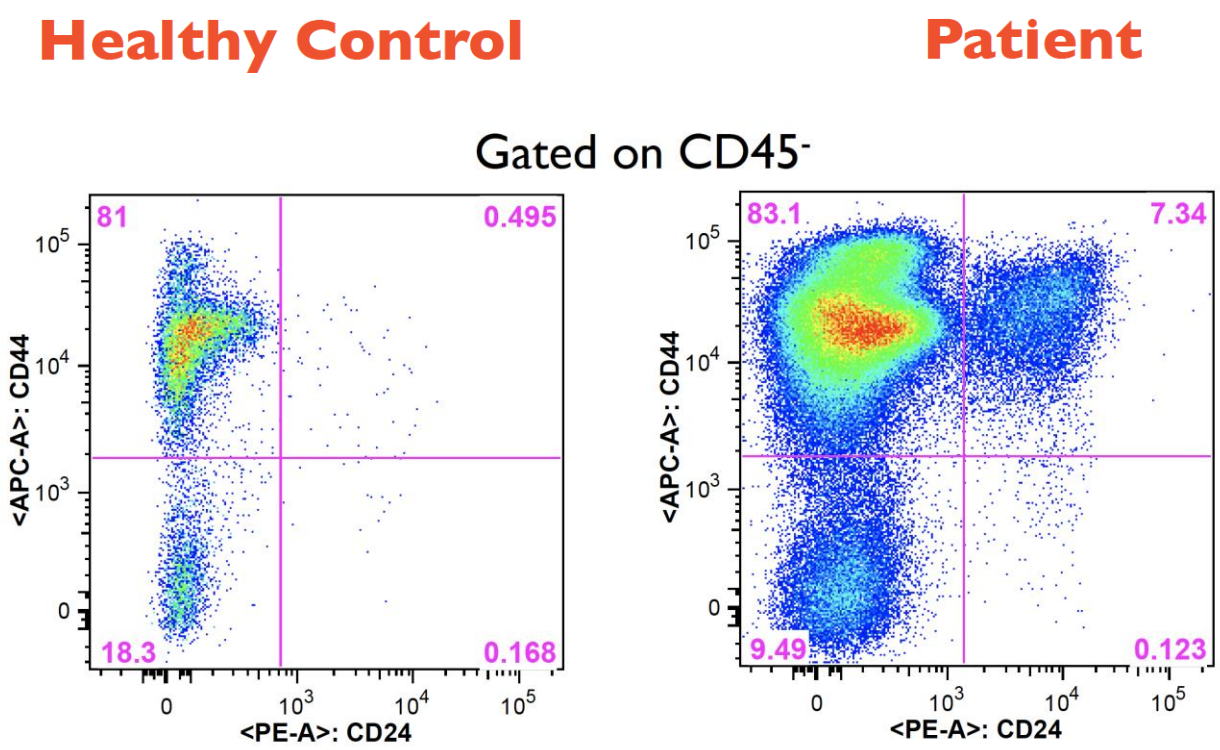
Figure 12: EpCAM positivity in gated live CD45(-) cells



6.4.3 CD45(-) and CD24/44

Using the cell phenotype definition described in figure 6, samples from a breast cancer patient were tested and compared to a healthy individual. The dyes were tested first for adherence in order to validate the results. As seen below in figure 7 , the patient was EPCAM (-) and CD24 (+), which a cell phenotype which was not present in the healthy individual. According to the literature , this would most likely represents a stem-cell like cancer cell phenotype. In addition to that, it was noted that this patient also exhibited a population not seen in the healthy individual where cells namely cells co-expressing CD24(+) and CD44(+).

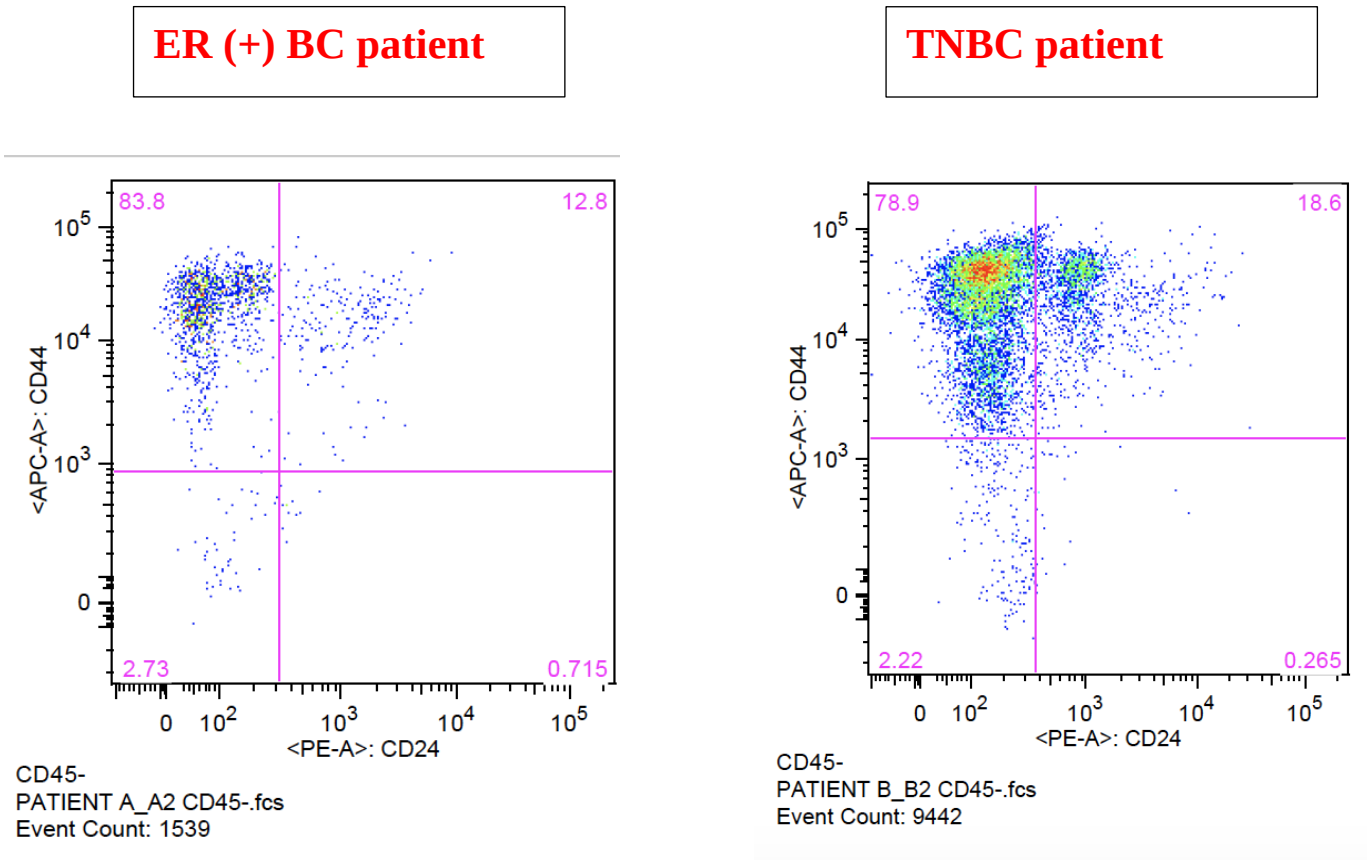
Figure 13: FCM results of CD24/44 in CD45(-) gated cells in healthy and patient samples



6.4.3 Differences in FCM in ER+ vs TNBC patients

Using the definition described in figure 6 samples from two patients were tested and compared. Patient A is a ER(+)/PR(+)/Her2/neu(-) breast cancer patient. Patient B is a TNBC patient . Both patients had successful apheresis collection and the enriched CD45 (-) cells collected were further analyzed by FCM. As seen in figure 14, the TNBC patient exhibited a higher amount of cells with a CSC-like phenotype characterized as CD 44(+) and CD 24(-).

Figure 14: CD 24 and CD44 FCM results in ER(+) BC vs TNBC



VII. DISCUSSION

There has been a growing interest in exploring the clinical potential of CTCs in precision diagnosis and treatment of breast cancer over the last decade. In this study 27 patients were successfully screened, half of the patients were luminal A (10/20), 4 were triple negative, 1 was Her2 positive and 5 were Luminal B. Of those, 20 patients underwent whole blood apheresis and cell collection was successful. The PMBCs fraction was then subjected to magnetic bead enrichment using CD45 antibodies and the CD45 negative fraction collected. An aliquot was then stained by IF to confirm CK positivity and ensure that CTCs were indeed collected. Cells were successfully cultured in Mammocult^{TM} medium to obtain mammospheres which were then characterized using specific biomarkers. Enriched fractions were also further characterized by FCM.

This study is the first of its kind using large-volume blood collection for the isolation and characterization of CTCs. This report shows that CTCs were successfully isolated, collected, and properly identified using IF and FCM. As shown by FCM , we successfully selected specific antibodies for the desired markers and created and optimized a platform for CTCs identification.

Using the results of the FCM, we were able to show that cells collected from cancer patients had a significantly higher number of CD45(-) and EpCAM(+)cells as compared to a healthy volunteer. Although given the fact that without next-generation sequencing and DNA analysis our approach cannot definitely confirm that the isolated cells are indeed CTCs, the combination of DAPI and CK8 is widely recognized as standard method to detect CTCs. Furthermore, CK8 positive cells could not be detected in healthy controls and the combination of CD44 and CD24

markers used showed a much higher proportion of cells in the breast cancer patients examined compared to healthy controls. In addition, the FCM analysis did confirm that cancer patients exhibit a much higher number of cells with CSC-like features. Furthermore, a patient with TNBC, the most aggressive type of breast cancer, was shown to exhibit predominantly stem-cell like features(CD44(+)CD24(-)) compared to an ER(+) patient.

Although CD45(-), CD24(+), CD44(+) were considered previously to be of a more benign phenotype, there is growing evidence in the literature that these cells could also behave like CSC Zhang 2011). Moreover, this CTC cell phenotype been shown to play a vital role in resistance to treatment. According to Wang et al (2017), the CD44+/CD24+ cell subset exhibits radiation resistance via decreased levels of reactive oxygen species.

Currently there is not a standardized approach for CTC detection, isolation, and characterization. Detection rates vary considerably based on which technique is employed and the type of cancer. Particularly challenging is the characterization of tumor heterogeneity and how this can influence tumor progression and therapy. In that respect examination of CTCs provides a unique opportunity to characterize changes in gene expression profiles during tumor progression and in response to therapy. The methodology described here should have sufficient sensitivity and specificity to provide a unique and promising approach for detection and characterization of CTC in real time and their heterogonous profile.

In the systematic review by Khatami et. al. (2017)the author concluded that although CTC status was a prognostic indicator , and that CTC levels during chemotherapy could be used to monitor therapeutic efficacy, simple enumeration had a low predictive value and could not predict a specific course of treatment. Hence, molecular characterization of CTCs is an important and needed step forward for personalized therapy in breast cancer, due at least in part to the high heterogeneity of CTCs.Ideally,this molecular profiling should include protein expression, phenotypic changes, and gene expression (4).

Furthermore, in the meta-analysis reported by Bidard in 2018 the authors found that CTC counts as a quantitative marker, was an indicator of poor prognosis. In the study the survival curves displayed a statistically significant survival difference starting as low as the detection of two CTCs/ per 7.5ml. Two other cutoff-points of one CTC and five CTC per 7.5ml or more were also able to distinguish a high-risk population. The authors concluded that any other trial based on CTCs detection should have to define a threshold compatible with its target population size, taking into account that one CTC/ per 7.5ml was also detected in few healthy individuals and that the low CTC count observed in non-metastatic breast cancer follows a specific distribution of rare events (31). These findings are reminiscent of the study of Yan et. al. (75) where the authors studied different cut-off points, with ≥ 5 CTCs/7.5 mL, ≥ 3 CTCs/7.5 mL being the most common.

In our study, patients who screened positive for CTCs were asked to undergo apheresis in order to isolate large numbers of CTCs. According to Fischer, even if CTC profiling holds a promising utility for diagnosis and therapy, low detection rates are delaying their routine clinical

use. Standard CTC analysis uses a blood volume of 1-10ml of peripheral blood. In their study, Fisher et. al. pointed out that such low blood volumes are insufficient and recommended screening large blood volumes for a reliable detection of CTCs, particularly in non-metastatic cancer patients (141). Hence, apheresis could be implemented as a marker-independent pre-analytic CTC enrichment step into clinical workflows(141).

VIII. CONCLUSION

Similar studies such as the one presented in this thesis have not yet been reported. The methodology presented is a novel approach for CTC detection, isolation, and characterization and our preliminary results indicate that targeting different gene markers could be useful in defining heterogeneity of CTCs.

Using this approach further and more detailed characterization of CTCs could be provided including single cell RNA sequencing. The preliminary results presented will soon be validated in the entire cohort of patients enrolled in our study.

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