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Liquid Biopsies in Metastatic Breast Cancer: a Guide to Personalized Medicine

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List of abbreviations

ACCEPT	Automated CTC Classification Enumeration and PhenoTyping (software)
AKT	A Serine/Threonine kinase 1 (gene)
A-I	Aromatase inhibitor
ASCO-CAP	American Society of Clinical Oncology/College of American Pathologists
BC	Breast cancer
BRCA1 / 2	Breast cancer 1 / 2 (genes)
CA15.3	Carcinoma antigen 15.3
CD	Cluster of differentiation
CDK4/6i	Cyclin-dependent kinases 4 and 6 inhibitor
cfDNA	Circulating free DNA
CK	Cytokeratin
CNA	Copy number alteration (acquired)
CNV	Copy number variation (germline)
CTC(s)	Circulating tumour cell(s)
ctDNA	Circulating tumour DNA
DAPI	4’,6-diamidino-2-phenlylindole
DNA	Deoxyribonucleic acid
DTC(s)	Disseminated tumour cell(s)
EBC	Early breast cancer
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor ??
EMA	European medicines agency
EMT	Epithelial to mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
ER	Estrogen receptor
ERBB2	Erythroblastic oncogene B 2 (gene, encoding HER-2/neu)
ESR1	Estrogen receptor 1 (gene, encoding ER)
ET	Endocrine therapy
FDA	Food and drug administration
FDG-PET	Fluorodeoxyglucose positron emission tomography
FISH	Fluorescence in situ hybridization
FITC	Fluorescein
FU	Follow up
HER2	Human epidermal growth factor receptor 2
HR	Hormone receptor
IF	Immunofluorescence
IHC	Immune histochemistry
LN	Lymph node
Lum	Luminal
MBC	Metastatic breast cancer
MTB	Mutational tumour burden
NSCLC	Non-small cell lung cancer
OS	Overall survival
PBMC	Peripheral blood mononuclear cell
PCR	Polyclonal chain reaction
PD-L1	Programmed death ligand 1
PE	Phycoerythrin
PFS	Progression free survival

PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase (gene)
PTEN	Phosphatase and tensin homolog (gene)
PT	Primary tumour
QC	Quality control
RECIST	Response Evaluation Criteria in Solid Tumours
RNA	Ribonucleic acid
RFU	Relative fluorescent units
RGE	Relative gene expression
SNV	Single nucleotide variation
SNP	Single nucleotide polymorphism
qRT-PCR	(quantitative) reverse transcription PCR
TNBC	Triple negative breast cancer
TP53	Tumour protein 53 (tumour suppressor gene)
WBC	White blood cell
WES	Whole exome sequencing
WGA	Whole genome amplification
WGS	Whole genome sequencing
WHO	World health organization

Summary – Samenvatting

Summary

During tumour development, cancer cells acquire various genetic and epigenetic aberrations that contribute to the ‘hallmarks of cancer’, in which unrestricted cell growth and manipulation of the tumour micro-environment are central. Due to selection and clonal expansion, multiple genetically distinct subclones emerge, that have phenotypic advantages and expand simultaneously resulting in intra-tumour heterogeneity. Over time cancer cells gradually evolve and progressively acquire a succession of the ‘hallmark’ capabilities, like: the potential for extravasation and seeding in distant tissues. Both early and late dissemination, as well as polyclonal and bidirectional seeding between different tumour sites have been described. As a consequence, different tumour sites will have their own unique evolutionary landscapes, leading to inter-metastasis heterogeneity. The efficacy of targeted therapy depends on their ability to block specific molecular aberrations. Primary tumour biopsies provide only a snapshot of cancer evolution, hence therapeutic targets for treatment of metastatic disease might be missed.

Over the last two decades, liquid biopsy has evolved to a very active field of research. It has become increasingly clear that it is of critical importance to find specific combinations of markers that can identify a cancer’s status, origin, and evolution. In this thesis I will focus on both circulating tumour cells (CTCs) and circulating tumour (ct)DNA, which are the most clinically well-developed biomarkers detected by liquid biopsy.

CTCs are shed from various tumour sites into the bloodstream or lymphatic system. CTCs are considered to represent a ‘real-time’ snapshot of the actual tumour burden. Still, they are relatively rare, representing only one in over a million blood cells. CTCs offer the potential to obtain information at the DNA, RNA, and protein level using various molecular techniques. Molecular analysis of CTCs enables researchers to detect the presence of multiple aberrations within the same cell, in order to decipher tumour heterogeneity and map clonal evolution. **Chapter 2** of this thesis is an extensive literature review focussed on the genomic and transcriptional heterogeneity found in the CTC compartment. It further discusses the technical considerations of CTC analysis and its significance for clinical decision making. In **chapter 3** we performed targeted and shallow whole genome sequencing of numerous single and sorted pools of tumour cells from three patients in order to unveil heterogeneity within the CTC and disseminated tumour cell (DTC) compartment.

HER2 is an frequently used target in the treatment of breast cancer. Multiple prospective studies investigated if anti-HER2 therapy can improve progression-free survival in patients that “gained” HER2 on CTCs at the metastatic stage. In **chapter 4** we compare different analysis methods for HER2 on CTCs (at DNA, RNA, and protein level).

Compared to CTCs, cfDNA is easier to obtain, ship and store, however it is restricted to genetic and epigenetic analysis of the DNA. Due to the very low concentration of ctDNA, highly sensitive and specific methods are needed for detection of cancer alterations like mutations or copy number changes. In **chapter 5** we discuss how ctDNA fraction can be used to predict treatment outcome and progression-free survival.

Many studies have been performed towards the use of liquid biopsies in early detection, as well as determining its prognostic and predictive value in local and advanced disease. These will be extensively reviewed in the **discussion** of this thesis.

Samenvatting

Vloeibare biopsies in gemetastaseerde borstkanker: een weg naar gepersonaliseerde behandeling.

Tijdens tumor evolutie ondergaan kankercellen verschillende genetische en epigentische veranderingen ten voordele van de zogenoemde 'hallmarks of cancer', waarbij ongeremde groei en een gunstige micro-omgeving centraal staan. Door selectie en klonale uitgroei ontstaan meerdere, genetisch verschillende, subklonen. Sommige van deze klonen hebben een gunstig fenotype waardoor ze kunnen uitgroeien, met als resultaat intra-tumor heterogeniteit. Over de tijd kunnen klonen ontstaan die in staat zijn tot extravasatie in de bloedbaan en metastatische groei in andere organen. Verschillende uitzaaiingen bestaan hierdoor uit een uniek evolutionair landschap van subklonen, waardoor er ook inter-metastatische heterogeniteit ontstaat. Een deel van de kankerbehandeling bestaat, buiten chemo- en immunotherapie, uit doelgerichte therapie, gericht tegen specifieke moleculaire afwijkingen. Met slechts één biopsie kunnen door tumorheterogeniteit belangrijke behandeldoelen gemist worden.

In de laatste twee decennia is er veel onderzoek gedaan naar een 'vloeibare biopsie', waarbij tumorale afwijkingen in het bloed worden opgespoord. In deze thesis focus ik op zowel de circulerende tumor cellen (CTCs) als op het circulerend tumor (ct)DNA.

CTCs zijn cellen die loskomen van de verschillende tumor locaties en terechtkomen in de bloedbaan of de lymfevaten, waardoor ze gezien worden als 'real-time' representatie van de aanwezige ziekte. Ze zijn vaak maar in lage concentraties aanwezig, van slecht enkele op meer dan een miljoen normale bloedcellen. Eenmaal geïsoleerd, kunnen deze cellen bestudeerd worden op afwijkingen op DNA, RNA en eiwit niveau. Door per tumorcel de afwijkingen te bestuderen, kan klonale evolutie in kaart gebracht worden. **Hoofdstuk 2** van deze thesis bevat een uitgebreid overzicht van de literatuur, die gericht is op heterogeniteit op DNA en RNA niveau binnen het CTC compartiment. Verder gaat het in op de technische aspecten van CTC onderzoek en de klinische relevantie. In hoofdstuk 3 hebben we zelf grote aantallen CTCs en DTCs (dat zijn tumorcellen in lichaamsvochten als beenmerg, hersenvocht of longvocht) nagekeken op genetische afwijkingen, om zodoende inzicht te krijgen in de heterogeniteit in het CTC en DTC compartiment.

HER2 is een vaak gebruikt doelwit in de behandeling van borstkanker. Verschillende studies hebben reeds de impact van een anti-HER2 behandeling getest in patiëntes met HER2-positieve CTCs bij initieel HER2-negatieve borstkanker. In **hoofdstuk 4** vergelijken we verschillende analyse methoden voor HER2 op CTCs (op DNA, RNA en eiwit niveau).

In vergelijking met CTCs, is cfDNA gemakkelijker te isoleren en bewerken, hoewel er alleen DNA analyse op gedaan kan worden. Door de lage concentraties van ctDNA zijn hoog sensitieve en specifieke analysemethoden nodig om veranderingen in het DNA vast te stellen. In **hoofdstuk 5** beschrijven we hoe de ctDNA fractie gebruikt kan worden voor voorspelling van behandeluitkomsten en progressie vrije overleving.

Vele klinische studies zijn uitgevoerd waarbij vloeibare biopsies gebruikt werden voor vroege kankerdetectie, alsook prognose en predictie in lokale en gevorderde borstkanker. De (pre)klinische toepassingen van vloeibare biopsies worden uitgebreid samengevat in de **discussie** van deze thesis.

1

General Introduction

Introduction into breast cancer

Cancer is a leading cause of health problems, with a worldwide estimated number of deaths of 9.56 million annually (1). Cancerous lesions develop when a cell acquires the potential for abnormal cell growth, by sustaining chronic proliferation, and is able to form a tumour. Invasive breast carcinoma generally develops from epithelial ductal or lobular cells (3). During tumour development, cancer cells acquire various genetic and epigenetic aberrations that contribute to these 'hallmarks of cancer' (4, 5). Due to selection and clonal expansion, multiple genetically distinct subclones emerge that have phenotypic advantages and expand simultaneously resulting in intra-tumour heterogeneity, as was demonstrated by single cell and multi-region sequencing of primary tumours (7, 8). Over time the cancer cells gradually evolve and progressively acquire a succession of the 'hallmark' capabilities, like: the potential for extravasation and seeding in distant tissues. Interestingly, the multistep process of tumorigenic cells to become malignant seems to be both spatially or temporally regulated. For example, tumour cells can survive in the bone marrow niche, in a dormant state, for several years, from where they recirculate and contribute to locoregional relapse or distant metastasis (10). Both early and late dissemination, as well as polyclonal and bidirectional seeding between different tumour sites have been described (11, 12, 13, 14, 15). As a consequence, different tumour sites will have their own unique evolutionary landscapes, leading to inter-metastasis heterogeneity (16) and critical genetic differences between primary tumour and metastatic sites (17). Major efforts have been undertaken to profile large quantities of breast cancer cases using next-generation sequencing, (18, 19, 20). Landmark cancer genomics datasets like The Cancer Genome Atlas (TCGA), the International Cancer Genome Consortium (ICGC), and AACR Project GENIE include tens of thousands of cases from over 30 different tumour types, including primary and metastatic breast cancers, leading to extensive and detailed catalogues of somatic changes (21, 22, 23).

Breast cancer is predominantly seen in women aged over 50 at the time of diagnosis. The lifetime risk of developing breast cancer is one in seven (European Cancer Information System, ECIS). In 2020, Belgium had the highest (age adjusted) incidence of breast cancer in the world, follow by other European countries, emphasising the excellent screening program (24). Besides age, other risk factors are implicated, such as hormone exposure: earlier age of menarche, later age or absence of pregnancy, omit breast feeding, later age of menopause, or the use of hormonal agents (25). Additionally, recent studies have indicated that about 10% of breast cancer cases are directly due to inherited "high penetrance" gene mutations, with the most well-known breast cancer associated genes being *BRCA1*, *BRCA2* and *PALB-2* (26, 27). Women that have inherited a mutated copy of either of these genes have an elevated life time risk of breast and ovarium carcinoma exceeding 80% or 30%, respectively (27). Besides the hereditary or genetic factors that increase the risk of developing mammary carcinomas, lifestyle (obesity) and environmental exposure (smoking, x-ray) may play a role (25).

The vast majority of breast carcinomas (∼75%) are described as invasive ductal carcinomas not otherwise specified (IDC-NOS) based on architectural patterns and cytological features (3). Other 'histological special types' include lobular, tubular, medullary and metaplastic carcinomas. Further histopathological analysis is important to determine tumour grade. This is an assessment of differentiation (tubule formation and nuclear pleomorphism) and proliferative activity (mitotic index), allowing tumours to be further stratified and providing key prognostic information (28). Secondly, immune histochemistry (IHC) characterisation of oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) differentiates between clinically relevant subtypes of breast cancer, extensively discussed in the St. Gallen International Consensus Guidelines

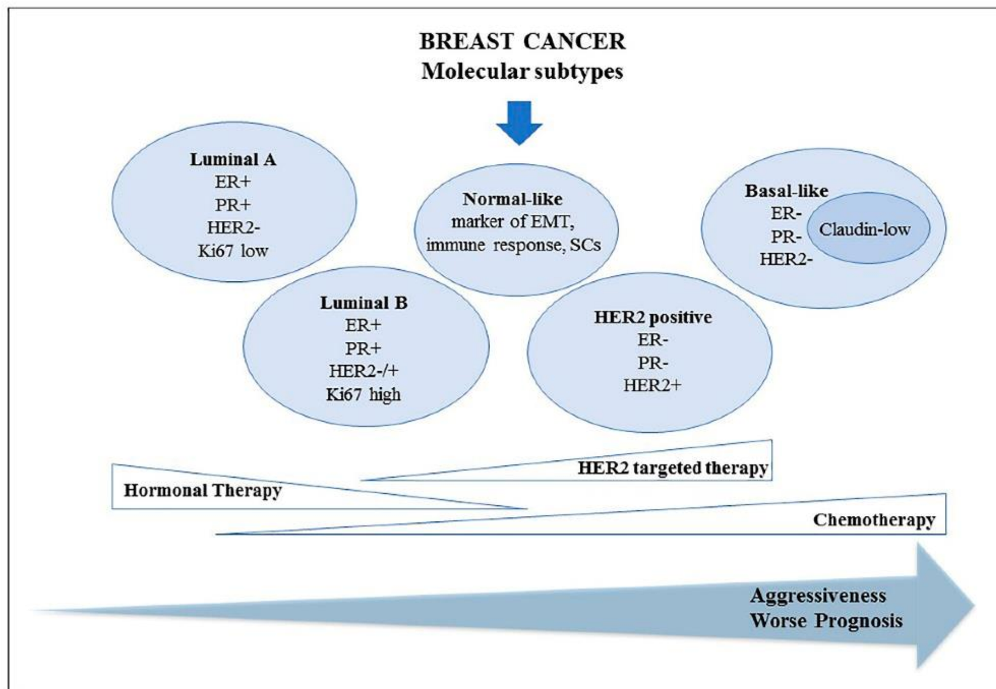

Figure 1 Breast Cancer molecular subtypes based on biomarker expression. Molecular analysis provide both prognostic and predictive information. Adapted from (6).

(29). The majority of early breast cancers are hormone receptor (ER and PR) positive (~75%), making patients eligible for hormonal-based therapies. HER2-enriched tumours account for 15-20% of breast cancers. Approximately half also express low levels of ER and PR. These patients are selected for anti-HER2 based therapies. The remaining 10–15% of breast cancers are defined by hormone receptor and HER2 negativity (i.e. triple negative cancers), which represent a key clinical entity given their lack of therapeutic options, although immunotherapy is emerging (29).

Despite above differentiations, breast cancer is a very heterogenous disease with many known molecular subtypes, that can be subclassified based upon biomarker expression (30, 31, 32, 33). The pivotal studies proposed five original subclasses based on gene expression: Luminal A, luminal B, normal-like, HER2-enriched, and basal-like (30) (**Figure 1**). These subtypes can be distinguished by the FDA-approved 50-gene molecular classifier (PAM50) (34, 35). Luminal tumours are most common (60%–70%) and characterized by the expression of hormone receptors ER and PR. They can be subdivided in Luminal A and B tumours. The first are well differentiated (lower grade), less proliferative (Ki67 low), and have strong ER/PR expression. Luminal B tumours are higher grade, have a higher expression of proliferative and cell-cycle genes, a lower expression of ER/PR, and display variable expression of the HER2/neu gene cluster. Luminal B tumours harbour an increased frequency of *TP53* mutations. They are associated with significantly shorter overall and disease-free survival, therefor requiring more aggressive treatment compared to Luminal A tumours (36, 37). There is no clear cut-off between Luminal A and B, and controversy exist over the precise thresholds for Ki67 that would justify chemotherapy or not (29). Additional multigene assays such as the 70-gene signature test (Mammaprint) and 21-gene recurrence score (OncotypeDx) help characterize the heterogeneity of ER-positive early-stage breast cancers, and serve as prognostic marker for recurrence risk (29, 38, 39). HER2-positive tumours are characterized by their very aggressive biological and clinical behaviour and have worse prognosis compared to Luminal tumours (36, 37). Patients with HER2 positive tumours are eligible for anti-HER2 based therapies like monoclonal antibodies (e.g. trastuzumab, pertuzumab), TKIs (e.g. lapatinib, tucatinib, neratinib), and antibody-drug conjugates (e.g. trastuzumab-emtansine T-

DM1, trastuzumab-deruxtecan T-DXd) (40). Basal-like tumours are negative for both ER and PR, as well as HER2, and are therefore often referred to as triple negative (TN). However, TN- and basal-like breast cancer are not synonymous, with TN breast cancer being more heterogenous and 30% of TN tumours are not basal. Basal-like tumours are frequently found to have a high mutational rate in *BRCA1* and have the worst prognosis (41). Recently, additional therapies, beyond chemotherapy, have been added to treatment of TN breast cancer, like immunotherapy and TROP directed antibody-drug conjugates in trials (e.g. Sacituzumab-govitecan (42) and Datopotamab-deruxtecan NCT05374512). A fifth subtype, known as normal-like breast cancer, closely resembles luminal A, but is triple negative. There are few studies on this subtype, but their clinical significance remains undetermined. Therefor this subtype is not included in the reference treatment guidelines for early breast cancer (29). Ongoing research revealed new breast cancer subtypes, such as Claudin-low breast cancer. It represents an aggressive molecular subtype that is comprised of mostly triple-negative tumour cells that possess stem cell-like and mesenchymal features (43).

Accurate assessment of breast cancer intrinsic subtypes is important, as they have distinct biological features. The expression status of ER, PR, and HER2 is both prognostic and predictive for therapy decisions (**Figure 1**), and further distinct prognostic signatures can be identified with gene expression profiling. Mammaprint and OncotypeDX have been approved for clinical use (29).

Somatic aberrations –caused by replication stress, mitotic errors, spindle multipolarity, and so on– contribute to carcinogenesis by inducing abnormal gene expression. These somatic alterations include single-nucleotide variants (mutations) and copy number aberrations (CNAs) (18). Although being highly influenced by mutations in oncogenes such as *ErbB2* (HER2) and tumour suppressors such as *TP53* and *BRCA1/2*, breast cancer is predominantly a copy number driven disease, as CNAs account for the greatest variability in gene expression (20, 44). Accumulation of CNA such as amplifications, deletions or rearrangements of chromosomal segments is an ongoing event in tumour evolution. Large studies revealed recurrent driver alterations and specific copy number signatures that attribute to biological phenomena like genomic duplication, aneuploidy, loss of heterozygosity (LOH), homologous recombination deficiency, and translocations (45, 46). For example, deletions at chromosome 11q and 17p, with loss of important tumour suppressor genes like *TP53* on 17p13, are known to be present in early stages of breast cancer, and are associated with relapse and worse clinical outcome (47, 48). Chromosomal LOH at 5q, containing important genes for BRCA1-dependent DNA repair, can be found in basal-like breast cancers and is associated with genomic instability (49). HER2-enriched breast cancers are characterized by the amplification of *ErbB2* located at 17q21, generally analysed by FISH (50). Integrated analysis of CNAs beyond the standard molecular subtyping (as stated in figure 1), can further subclassify breast cancers into integrative clusters that provide important biological insights into the potential molecular drivers and pathways underlying certain groups (46). These have distinct implications for the rationale development of targeted therapeutics (51).

The efficacy of targeted therapy depends on their ability to block specific molecular aberrations (**Table 1**). Many effective drugs have been developed for HER2 positive early and advanced breast cancer. Analysis of recurrent tumour biopsies only rarely identifies acquisition of *HER2* amplification among tumours originally defined as HER2-negative primary cancers (26). *HER2* mutations that activate *HER2* in the absence of gene amplification may sensitize breast cancers to the dual-kinase inhibitor neratinib (52). The most common mutations in ER+ breast cancer are activating mutations in the PI3 kinase pathway *PIK3CA* gene, arising in nearly 40% of tumours (26). These ER+ tumours respond significantly better to a combination of fulvestrant with the PI3 kinase inhibitor alpelisib (53), and therefore should always be tested. Commonly acquired mutations include those in

the ER (*ESR1*) and are associated with resistance to aromatase inhibitors in ER+ advanced breast cancers. Recent data suggest that acquisition of PIK3CA mutations may accompany resistance to fulvestrant-based endocrine therapy, suggesting that serial determination of ESR1 and PIK3CA mutation status may become clinically important (26, 54). Among patients with TNBC, mutations in the PI3 kinase pathway genes (*PIK3CA, AKT1, PTEN*) may identify sensitivity to the AKT inhibitors ipatasertib and capivasertib (26), although in the last years other treatment options have become available for TNBC. Combined chemotherapy with immunotherapy like pembrolizumab or atezolizumab is now considered for patients with TN MBC that are PD-L1 positive (55, 56). Besides,

Target	Drug & Trial	Subtype	Indication
CDK 4/6 ER/PR	ET + **Palbociclib** PALOMA-2/3 ET + **Ribociclib** MONALEESA-2/3/7 ET + **Abemaciclib** MONARCH-2/3/E	HR+ HER2-	Stage IV, from 1st line Adj. Abema+ET in high risk node-positive EBC
mTOR	Exemestane + **Everolimus** BOLERO-2	HR+ HER2-	Stage IV, progression after ET
PIK3CA	Fulvestrant + Alpelisib SOLAR-1	HR+ HER2- *PIK3CA* mt	Stage IV
PD-1 PD-L1	Chemo + **Pembrolizumab** KEYNOTE-355/522 Abraxane + **Atezolizumab** IMpassion-130	TNBC PD-L1+ (>10%) TNBC all comers TNBC PD-L1+ (>1%)	Stage IV, first line (neo)adjuvant for high risk TN EBC Stage IV, 1st line (EMA)
Trop-2	Sacituzumab-govitecan ASCENT	TNBC	Stage IV, from 3rd line
HER2	Chemo + Tras ± **Pertuzumab** NeoSphere, TRYPHAENA APHINITY, CLEOPATRA	HER2+	(neo)adjuvant for high risk EBC Stage IV, 1st line
	T-DM1 KATHERINE, EMILIA	HER2+	Adj. for nPCR in EBC Stage IV, from 2nd line
	Cape + Tras + **Tucatinib** HER2CLIMB	HER2+	Stage IV, from 3rd line
	Neratinib ExteNET NALA	 HR+ HER2+ HER2+	 Extended adj. after Tras Stage IV, from 3rd line
	T-Dxd DESTINY-breast01/03	HER2+	Stage IV, from 3rd line Pending for 2nd line
DDR (*BRCA*1/2 mt)	**Olaparib** OlympiA(D) **Talazoparib** EMBRACA	HER2-	Adj. for high risk EBC (not FDA/EMA approved) Stage IV, previous chemo in any setting or PD on ET
VEGF	Chemo + **Bevacizumab** E2100, AVADO, RIBBON-01	HER2-	Stage IV (not FDA/EMA approved)

Table 1 Targets in Breast Cancer with related FDA and EMA-approved drugs. Abbreviations: ET endocrine therapy, Tras trastuzumab, Cape Capecitabine, T-DM1 trastuzumab-emtansine, T-Dxd trastuzumab-deruxtecan, Adj. adjuvant, EBC early breast cancer, mt mutated, PD progressive disease. From ESMO handbook Sept 2022.

tumour genomic and somatic sequencing for *BRCA1* and *BRCA2* will reveal pathogenic mutations in approximately 5% of breast cancers (26). Identifying germline *BRCA1*/2 mutations selects patients with advanced breast cancer for treatment with platinum chemotherapy or PARP inhibitors (57, 58, 59).

Primary tumour biopsies provide only a snapshot of cancer evolution, hence therapeutic targets for treatment of metastatic disease might be missed (60). Importantly, from a clinical perspective, metastasis is the most critical aspect of tumourigenesis since over 90% of cancer mortality is caused by metastasis (61). In these patients, disease progression due to continuous changing biology and resistance patterns, influenced by prior therapies, seems unavoidable (62). To extend the survival of metastatic cancer patients, accurate profiling of the disease is key, as was demonstrated in the IMPACT trial (NCT00851032) (63). Investigators found that the 3-year overall survival rate was 15% for patients assigned to matched targeted therapy compared with 7% for those who did not receive precision therapy. Treatment of patients should therefore be based on ad-hoc information on the various tumour subclones present at that moment. However, repeated tumour biopsies are in many cases not feasible for technical reasons and mostly not without risk for the patient (64). Therefore, an ever-increasing effort is being made to get accurate tumour information through minimal invasive sampling of blood, called liquid biopsy.

Liquid Biopsies

Over the last two decades, liquid biopsy has evolved to a very active field of research, with over 25,000 articles listed in PubMed as of January 2000, most of them focussing on circulating tumour cells (CTCs) or circulating tumour DNA (ctDNA). Besides, circulating cell-free RNA, micro RNA, extracellular vesicles such as exosomes, tumour educated platelets, proteins, and metabolites have been explored and extensively reviewed by others (65, 66). From this body of research, it has become increasingly clear that it is of critical importance to find specific combinations of markers that can identify a cancer's status, origin, and evolution (67). However, not only circulating tumour biomarkers must be considered as with the increased use of immunotherapies as treatment options, the need to monitor the circulating immune microenvironment increases (68, 69). For this thesis I will focus on both CTCs and ctDNA, which are the most clinically well-developed biomarkers detected by liquid biopsy.

CTCs are shed from various tumour sites into the bloodstream or lymphatic system (**Figure 3**). Still, they are relatively rare, representing only one in more than a million blood cells (70). CTC count in patients with metastatic breast cancer (MBC) is a strong prognostic factor for overall survival (71, 72). The half-life of CTCs in the circulation is thought to be between 1 and 2.4 hours (73), which enables CTC analysis to be considered as a 'real-time' snapshot of disease burden. Metastasis through the bloodstream is a highly inefficient process due to a combination of physical stress, oxidative stress, anoikis (programmed cell death induced upon cell detachment from ECM), and the lack of growth factors and cytokines (74, 75). Those few CTCs that do survive either actively extravasate into the surrounding tissue or become lodged in a capillary bed (76, 77). CTCs that fully or partially undergo epithelial-to-mesenchymal transition (EMT) are more likely to survive in the bloodstream as they have an increased plasticity and motility, and are more resistant to anoikis (78, 79). Interestingly, recent evidence has emerged showing that neutrophils can escort CTCs through the blood stream and promote cell-cycle progression and subsequently increase their survival (80). Another study has implicated the recruitment of giant macrophages by platelets possibly promoting CTC survival in circulation (81). Moreover, platelets protect CTCs in the bloodstream and form aggregates that promote binding to the endothelium and subsequent extravasation into the target tissue (82). It is thought to be this sequential recruitment of platelets and granulocytes that promotes metastatic

progression of CTCs, by creating an 'early metastatic niche' (83). All these studies highlight the importance of the immune component in the circulating microenvironment (**Figure 3**). Additionally, the lymphatic environment has been demonstrated to protect circulating melanoma tumour cells from ferroptosis and increase their survival during subsequent metastasis through the blood stream (84). Overall, CTCs are considered to represent the actual tumour burden and especially mesenchymal cells are thought to be the precursors of metastasis (85). Along those lines, CTCs with an intermediate phenotype (partial EMT) might have the highest plasticity to adapt to both the bloodstream and distant organ sites (78). More recently, the focus has shifted towards CTCs that circulate as clusters of cells. These clusters are very rare but have up to a 50 times increased metastatic potential (86), and are associated with poor prognosis (87, 88). CTC clusters are even capable of passing capillaries by unfolding into single-file chains, and then rapidly reorganise (89). As mentioned above, platelets can co-aggregate with these CTC clusters, within these newly formed microenvironments they produce high levels of TGF-β that increase EMT of the CTCs (82). This acquisition of EMT could also augment chemoresistance within these clusters (90). In all, EMT, as well as stromal-derived factors or CTC-immune cell interactions may provide survival signals that increase CTC survival and enhance their metastatic potential. Dissecting the contributions of these various mechanisms requires the ability to isolate CTCs from the bloodstream.

Many devices have been developed to enrich and detect CTCs. Enrichment, in order to increase the concentration of CTCs, has been performed by use of biological properties (positive or negative selection based on protein expression), physical properties (i.e. density, size, deformability, or electric charges), or combinations of these techniques (91). Although, the only FDA approved device for enrichment and enumeration of CTCs is the CellSearch® system, that uses positive selection with magnetic bead-coupled anti-EpCam antibodies (71). Besides enumeration using immunologic assays, CTCs can be used in molecular or functional assays (92). CTCs offer the potential to obtain information at the DNA, RNA, and protein level (**Figure 2**) using various molecular techniques (93). Furthermore,

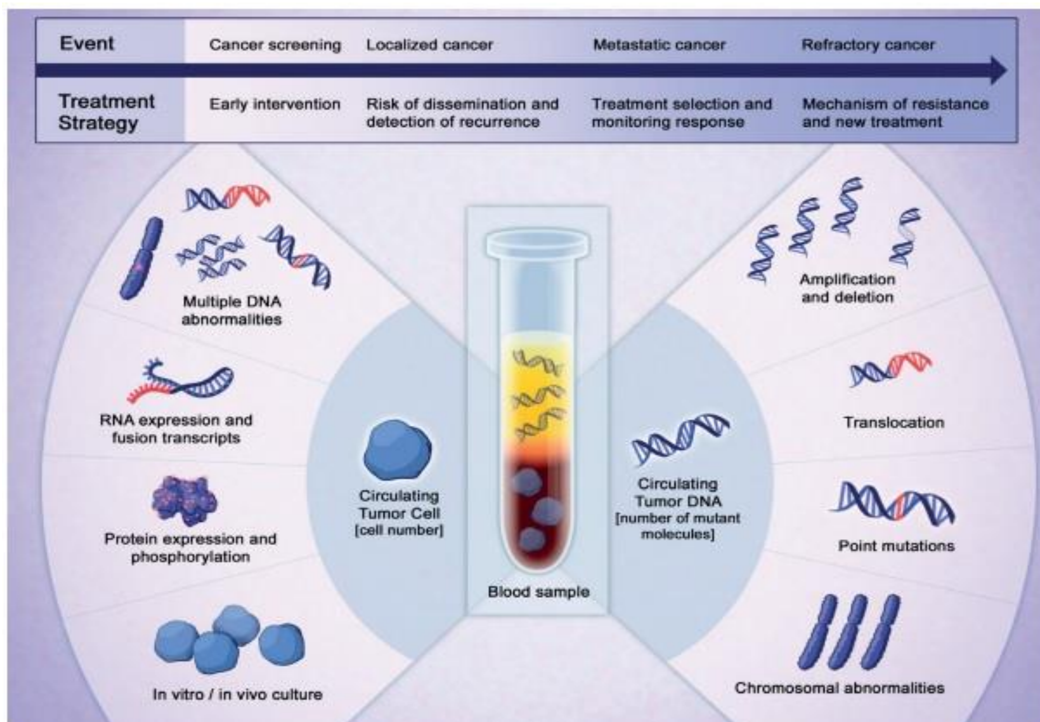

Figure 2. Clinical applications of CTC and ctDNA analyses in cancer care. Illustration of molecular analyses that are enabled by the isolation of CTCs and ctDNA from blood specimens. These may be applied to guide different treatment strategies from the initial diagnosis until advanced disease. Figure adapted from (2).

CTCs are used in *in vitro* (i.e. establishment of cell lines) and *in vivo* studies (i.e. xenograft models), to facilitate functional studies like drug testing (94, 95, 96, 97). Although, establishing cultures or xenografts requires many CTCs per patient, and is therefore only possible for patients with advanced disease.

Molecular analysis of CTCs enables researchers to detect the presence of multiple aberrations within the same cell, in order to decipher tumour heterogeneity and map clonal evolution (98), while conventional molecular analysis of whole tumours provides genotype or phenotype information of only the dominant clones or cumulative information of all clones. **Chapter 2** of this thesis is an extensive literature review focussed on the genomic and transcriptional heterogeneity found in the CTC compartment (99). It further discusses the technical considerations of CTC analysis and its significance for clinical decision making. In **Chapter 3** we performed targeted and shallow whole genome sequencing of 136 samples of singles and pools (varying 5-150 cells per pool) of tumour cells from three patients in order to unveil heterogeneity within the CTC and disseminated tumour cell (DTC) compartment. Interestingly, we observed only a limited heterogeneity of the CTC compartment in advanced stages of disease.

Circulating tumour (ct)DNA is the fraction of circulating cell free (cf)DNA that originates from tumour cells. ctDNA is released by apoptotic or necrotic cancer cells from the primary tumour, metastases, or CTCs, or might even be actively secreted after digestion by immune cells (100). It can be highly diluted, especially during cancer therapy, when normal cells are as well damaged by surgery, chemotherapy, or radiotherapy. cfDNA has a typically fragment length of 85-230 base pairs (101). ctDNA can be differentiated from normal cfDNA as it carries tumour-related genetic and epigenetic alterations that might be relevant to cancer development, progression and resistance to therapy (102). The half-life of cfDNA in the circulation is between 16 minutes and 2.5 hours (102, 103, 104, 105), which is comparable to CTCs. It is cleared from the circulation via direct nuclease action or via renal excretion (105, 106, 107). It is even described that ctDNA can be taken up by host cells inducing cell transformation, which fosters tumour progression (100, 108).

cfDNA is easier to obtain, ship and store compared to CTCs, however it is restricted to genetic and epigenetic analysis of the DNA (**Figure 2**) (109). Straightforward DNA extraction will deliver fragments within a small size range that does not need cutting or shearing for downstream analysis. This makes ctDNA research far less laborious and expensive compared to CTC studies. However, due to its very low concentration, highly sensitive and specific methods are needed for detection of cancer alterations like mutations or copy number changes.

Clinical use of liquid biopsies

Liquid biopsies have the potential to provide a more comprehensive profile of both primary tumours and metastases, compared to a sample harvested directly from the solid tumour. This is especially true considering the spatial heterogeneity of primary tumours. Blood sampling can be focussed on various stages of the disease being early detection or screening, prognostication in primary cancer, detection of minimal residual disease (MRD) to select patients at risk for recurrence or metastatic disease, or at time of advanced disease for monitoring therapy response or even therapy selection (110, 111). On the downside, compelling evidence that CTC guided treatment is improving patient survival is lacking and has so far only be studied in few trails. One on these, known as STIC-CTC, based treatment of advanced breast cancer patients on CellSearch results and suggest that patient with high CTC counts should be treated aggressively and have better outcome when receiving chemotherapy as front-line treatment (72, 112). Multiple prospective studies (CirCe01, LAP105594, NCT01048099) investigated if

treatment of patients with various anti-HER2 drugs can improve progression-free survival (PFS) in patients that “gained” HER2 in CTCs at the metastatic stage (113), though no major break throughs have been made. In **chapter 4** we compare different analysis methods for HER2 on CTCs (at DNA, RNA, and protein level) and discuss the why above trials were not able to demonstrate clinical significance. In all, these data show that more clinical testing is needed to propel this technology as a tool for guiding treatment. In recent years, ctDNA research has overtaken CTCs as studied analyte in liquid biopsies. ctDNA quantity, measured by CNA or mutation burden, is a strong prognostic factor for overall survival in MBC (114, 115). In **chapter 5** we discuss how ctDNA fraction can be used to predict treatment outcome and progression-free survival. ctDNA is rapidly entering clinical use. In NSCLC, real-time PCR for the qualitative detection of *EGFR* exon 19-21 deletions and mutations is used determine whether patients are eligible for treatment with EGFR inhibitors (116). Also in advanced breast cancer cfDNA presents an alternative way to conduct genomic testing in patients (117). It is particularly useful to identify mutations in the cancer that are acquired through treatment and may not be readily detectable before treatment or that might evolve over time. The most prevalent acquired mutation in breast cancer is mutation in the *ESR1* gene in advanced ER+ breast cancer. These are selected in the cancer by prior aromatase inhibitor (A-I) therapy. Identification of *ESR1* mutations in ctDNA predicted resistance to subsequent A-I and showed improved progression-free and overall survival with fulvestrant (118, 119). Many studies have been performed towards the use of liquid biopsies in early detection, as well as determining its prognostic and predictive value in local and advanced disease. These will be extensively reviewed in the **discussion** of this thesis.

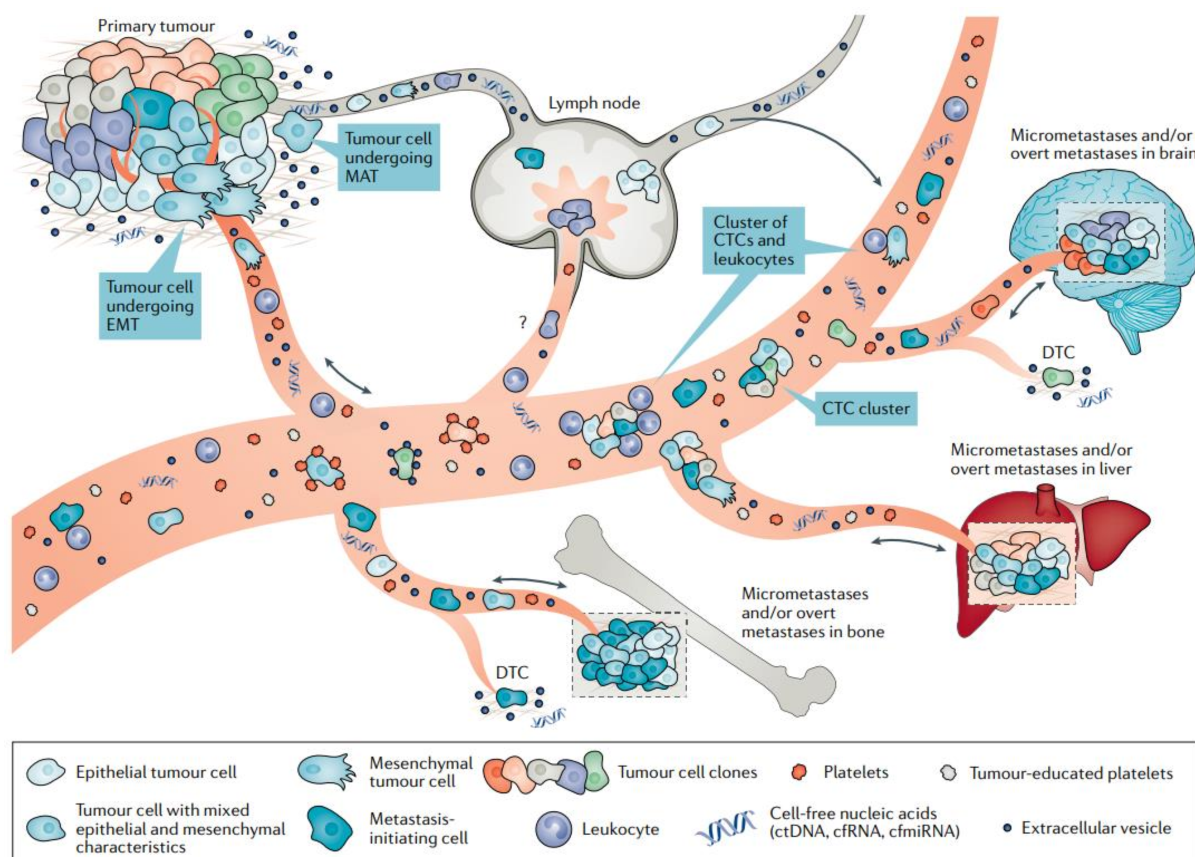

Figure 3. Biology of tumour blood dissemination: stepwise progression from CTC release to overt metastasis formation. The diversity of molecular mechanisms leading to tumour dissemination are mirrored by a substantial heterogeneity in the pool of CTCs present in the blood. There are multiple entry sites for cells into the blood (primary tumour, lymph node, metastasis) and molecular mechanisms implicated in invasion (EMT, mesenchymal-amoeboid transition (MAT), single or collective migration). Figure adapted from (9).

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Evaluation and Consequences of Heterogeneity in the Circulating Tumor Cell Compartment

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Evaluation and Consequences of Heterogeneity in the Circulating Tumor Cell Compartment

ABSTRACT

A growing understanding of the molecular biology of cancer and the identification of specific aberrations driving cancer evolution have led to the development of various targeted agents. Therapeutic decisions concerning these drugs are often guided by single biopsies of the primary tumor. Yet, it is well known that tumors can exhibit significant heterogeneity and change over time as a result of selective pressure. Circulating tumor cells (CTCs) are shed from various tumor sites and are thought to represent the molecular landscape of a patient's overall tumor burden. Moreover, a minimal-invasive liquid biopsy facilitates monitoring of clonal evolution during therapy pressure and disease progression in real-time. While more information becomes available regarding heterogeneity among CTCs, comparison between these studies is needed. In this review, we focus on the genomic and transcriptional heterogeneity found in the CTC compartment, and its significance for clinical decision making.

INTRODUCTION

Metastatic disease is responsible for over 90% of cancer-related deaths (1). Due to a growing insight in the molecular mechanisms driving cancer evolution and identification of specific molecular aberrations involved, an increasing number of patients is now considered candidate for treatment with so called targeted agents (2, 3). However, when it comes to therapy decision making, intra-patient heterogeneity should be taken into account. Here we discuss the molecular heterogeneity within the circulating tumor cell (CTC) compartment in various tumor types. Furthermore, we review the causes and consequences of this heterogeneity and the clinical perspective.

Intra-tumor heterogeneity

Advances in DNA sequencing techniques and comparison of tumor samples obtained from different sites and at different time points, have revealed an extensive view on clonal evolution and intra tumor heterogeneity (ITH). During tumor development, cancer cells acquire various aberrations, including both passenger (neutral) and driver (advantageous) mutations. Due to selection and clonal expansion, multiple genetically distinct subclones can emerge that often evolve following a pattern of branched evolution, which has been described for various solid tumor types (4-15). This branched evolution comprises multiple subclones that have a phenotypic advantage within a particular environment and evolve simultaneously resulting in ITH, whereas a linear evolutionary pattern describes a random genetic drift where fitter clones outgrow ancestral clones, resulting in a relatively homogeneous tumor at any given moment (16). Exome sequencing of multiple tumor foci from clear-cell renal carcinomas revealed that only one-third of the identified driver aberrations were present in every region analyzed from an individual tumor, suggesting these to be early founder aberrations. In contrast, 71% of driver mutations were heterogeneous between tumor regions, although appearing clonally dominant within individual regions, showing branched evolution with spatially separated dominant subclones (6).

During the development of metastatic disease, tumor cells shed from the primary tumor are able to travel to distant organ sites to seed metastatic tumors (17). Moreover, in breast, prostate, and pancreatic cancers, it has been shown that these cells disseminate long before metastatic colonization

becomes clinical evident (18, 19). Both early and late dissemination, as well as polyclonal and bidirectional seeding between different tumor sites, and parallel evolution have been described (20, 21). Hence, different tumor sites will consist of unique evolutionary landscapes, leading to inter-metastasis heterogeneity (12, 21-23).

Although clonal diversity can be resolved by spatial sampling (7) in combination with deep-sequencing of tumor tissue to determine (sub)clonality of certain mutations (9-11, 24, 25), the field is shifting towards single cell sequencing (SCS) studies to shed light on this heterogeneity. SCS allows to study rare tumor cell populations and clonal expansion, and is already widely used in hematopoietic cancers, including Acute Myeloid Leukemia (26, 27). However, in solid tumors, patients often exhibit multiple lesions composed of genetically diverse subclones that evolve in parallel over time (28, 29), hampering the evaluation of targetable aberrations in a patient's metastatic disease (22, 30, 31). Hence, single tumor biopsies fail to represent the clonal landscape of the overall tumor burden. Moreover, changing biology and resistance patterns, influenced by prior therapies, stresses the need for repeated sampling of a patients tumor burden, to expose the molecular landscape at various moments in time (23, 32).

Circulating tumor cells

CTCs are shed into the peripheral blood from various tumor deposits and represent the actual tumor mass as was demonstrated by comparative analysis of CTCs, primary tumors, and metastases in various tumor types (33-37). CTC capturing systems have revealed that aggressive tumors release thousands of cancer cells into the circulation each day (38-41), although most CTCs only persist for a short time in the circulation, with an estimated half-life between 1 and 24 hours (38, 42, 43). It is assumed, however, that CTCs with an intermediate phenotype between epithelial and mesenchymal have the highest plasticity and can survive in the circulation (44-46). Although CTCs are a frequent phenomenon in cancer, only a small fraction (<0.01%) eventually succeed in forming metastasis (47, 48). This was further demonstrated with the identification of specific subsets of CTCs with tumor-initiating capacity (39, 40, 49, 50).

In general, CTCs are relatively rare, representing only one in more than a million blood cells (40). Still, CTC count of patients with metastatic cancer is a strong prognostic factor for overall survival in several tumor types (51-60). Moreover, changes in CTC counts during treatment are used as a marker for therapy response (42, 55, 61-64). Genotyping of circulating tumor (ct)DNA, derived from tumor deposits and lysed CTCs, also has the potential to serve as a marker for tumor burden, therapy response, and even therapy resistance patterns, when followed longitudinally (32, 65-68). Moreover, mutation levels in plasma can reflect the multifocal clonal hierarchy of tissue biopsies from a patient with metastatic breast cancer during therapy (23). Compared to CTCs, ctDNA is easier and less laborious to obtain. Nonetheless, CTCs represent pure and intact tumor cells. Molecular analysis on DNA, RNA, and protein level (33, 69), as well as functional cellular characteristics can only be interrogated in CTCs (39). In addition, molecular analysis of CTCs enables researchers to detect the presence of multiple mutations within the same cell, in order to decipher tumor heterogeneity and map clonal evolution. When combining genomic and transcriptomic evaluation of CTCs, a potential linkage between mutational status and pathway activation can be observed (70).

CTCs can be analyzed both as pure cells as well as enriched fractions. Mutation detection of DNA extracted from CTC-enriched samples demonstrated activating mutations in the *EGFR*, *KRAS*, and *AR* genes in patients suffering from lung cancer, colorectal cancer (CRC), and castration-resistant prostate cancer (CRPC) respectively (65, 71, 72). Additionally, RNA analysis of enriched CTC fractions have been

performed using reverse transcription PCR (RT-PCR) amplification of tumor-specific transcripts, such as AR splice variant 7 in CRPC, and translocations like *EML4–ALK* in lung cancer and *TMPRSS2–ERG* in prostate cancer (42, 73-75). However, sequencing of enriched fractions is complicated by low levels of tumor-specific templates and contamination by abundant leukocyte-derived sequences, limiting the sensitivity and specificity (76, 77). Advances in next generation sequencing (NGS) strategies and computational analyses help resolve this challenge. Nevertheless, single CTC sequencing strategies can provide a direct insight into CTC heterogeneity by identifying co-existing mutations within a cell. Heitzer and colleagues, profiled individual CTCs isolated from patients with metastatic CRC, using array-Comparative Genomic Hybridization (CGH) and targeted panel sequencing of 68 genes. Various genomic aberrations in CTCs were found, indicative for their subclonal origin from specific areas of the original tumor (33).

Overall, cancer presents a problem of continuous spatial and temporal complexity, particularly due to selection pressures such as anti-cancer drugs, that may promote dominance of previously minor or dormant lineages (78). It is important to note that subclonal diversity is viewed as a snapshot, and only serial analysis of CTCs can clarify the much needed dynamic view of tumor genomes, as pointed out in **figure 1**. Both in metastasis research, as well as in clinical practice, it is important to know whether a minor subclone is emerging or has been outcompeted by the dominant subclone (16). Longitudinal CTC studies have been performed to investigate the clonal changes in both phenotypical and molecular profiles associated with disease evolution and therapy resistance (79-81). Hence, CTCs might reflect the characteristics of the current status of the biologically and clinically relevant subclones irrespective of a detailed anatomical distribution, and should ideally be suited to provide dynamic assessments of tumor characteristics in patients with metastatic disease. Even more since repeated sampling of multiple metastatic lesions is an invasive procedure and often not feasible.

Although increasingly sophisticated technologies have become available to detect and isolate CTCs, as is already extensively reviewed (82-88), further progress in CTC research is needed to envision heterogeneity and clonal evolution within the CTC compartment. Major questions in CTC research implicate the clonal relationship between CTCs and the number of CTCs that have to be analyzed in order to capture the overall profile of the dominant disease driving (sub)clones in a patient suffering from widespread metastatic disease. In this review, we will focus on the genomic and transcriptional heterogeneity found in the CTC compartment, and its significance for clinical decision making.

GENOTYPIC CTC HETEROGENEITY

A growing number of research articles have been published demonstrating genotypic heterogeneity in the circulating compartment, emphasizing the need for studies analyzing multiple purified CTC samples. This can be performed focusing on several types of aberrations such as gene rearrangements, mutations, and CNA profiles. Here we compare the results regarding genomic variation in CTCs of various tumor types (summarized in **table 1**). We found that in many patients rearrangements as well as specific and global mutation profiles were highly heterogeneous. Concerning CNA profiles, homogeneity in overall profiles was reported frequently, although in both breast and prostate cancer intra-patient variation was observed. Furthermore, changes in CNA profiles over time were documented and in depth analysis of copy number profiles of specific genes in various tumor types demonstrated extensive heterogeneity.

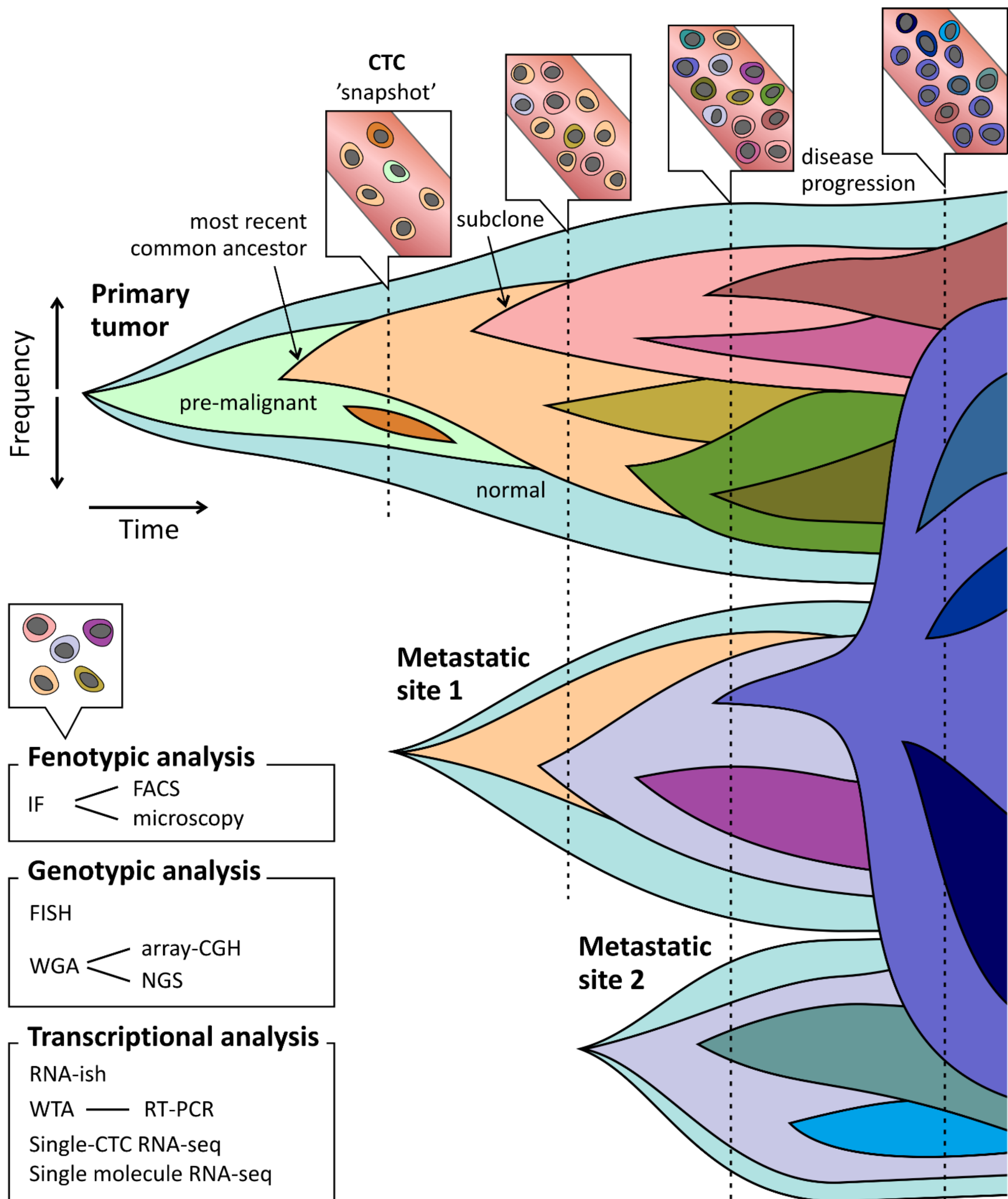

Figure 1: CTCs as snapshot of the evolving tumor landscape. Clonal evolution depicted as emergence of clones after acquisition of driver mutations. New (sub) clones derive from ancestral clones following linear and branched evolution. Outgrowth and repression (therapeutic or outcompeting) of these subclones can lead to emergence and disappearance of driver mutations respectively. Seeding and re-seeding of tumor cells causes development of changing tumor landscapes at multiple sites. Selective therapy pressure can lead to outgrowth of resistant clones at time of disease progression. CTCs sampling can function as a snapshot of the overall tumor bulk (primary tumor and metastases). When profiling CTCs at multiple time points emerging and decreasing subclones can be unveiled. Techniques to profile CTCs include phenotypical and molecular analyses.

Rearrangements

Several research groups studied rearrangements of the *ALK* gene in CTCs using Fluorescence *in situ* hybridization (FISH) (89-91). In non-small cell lung cancer (NSCLC), *EML4-ALK* fusion is present in approximately 3-7% of cancers, and these patients are eligible for targeted treatment with crizotinib and ceritinib (92-94). Pailler and colleagues demonstrated that percentages of *ALK*-rearranged CTCs ranged between 28% and 100% in patients with ALK-positive tumors, and varied within these patients during crizotinib therapy. This suggests that the *ALK*-rearranged CTC population might be a consequence of clonal selection from a specific subpopulation of primary tumor cells, and that outgrowth of this subpopulation can be an indication for therapy resistance (89). Percentages of *ALK*-rearranged CTCs were confirmed by two other studies. In a first report, one-fourth of the total 177 CTCs of one patient harbored *ALK* rearrangements (90) and in the other, 100% of the CTCs of 5 patients were *ALK*-rearranged (91), whereas in the primary tumor tissue this was around 50% in both studies. Furthermore, *ROS1* rearrangements were found in CTCs of four patients with lung cancer (95). FISH has also been used to analyze *ERG* rearrangement in prostate cancer CTCs (96, 97). *TMPRSS2-ERG* gene fusion was either homogeneously present in all CTCs of one patient or absent (96). Although presence of this *ERG* rearrangement demonstrates a significant association with PSA response to abiraterone in this study, TMPRSS2-ERG status could not predict a decline in PSA or other clinical outcomes in response to abiraterone therapy in a clinical trial evaluating enriched CTC populations (97).

Hotspot mutations

In breast cancer, *PIK3CA* is mutated in up to 25% of patients, with mutation frequencies rising to 40% in the hormone receptor-positive subgroups (98, 99). Analyzing the *PIK3CA* genotype has clinical relevance with respect to drug resistance, e.g. against HER2-targeted therapy. Hence, various studies are performed investigating the *PIK3CA* mutational status in CTCs. In a first study, two single CTCs per patient were analyzed (100). In two patients *PIK3CA* mutations were found in all CTCs of these patients (resp. 1 and 2 CTCs). In a similar, but much larger study, *PIK3CA* mutations were detected in 16 patients, two of whom harboring a heterogeneous mutational status in their single and pooled CTCs (101). De Laere and colleagues profiled CTCs of 26 hormone receptor positive patients, ranging between 4 and 311 CTCs per patient. In 19 cases (73%) *PIK3CA* mutations were detected. Of these, six cases were found almost homogeneously mutant for one specific mutation, whereas another six patients were extensively heterogeneous with subclones harboring one or multiple *PIK3CA* mutations (102). In contrast, another study detected *PIK3CA* mutations in only one out of 17 patients, which might be due to different patient selection (103). Single CTCs of 24 samples (containing 2-50 CTCs) of 12 patients were examined for presence of *PIK3CA* mutations. In one patient an exon 9 mutation was detected in two out of nine serial samples, both at a heterogeneous level (103). Pestrin *et al*. identified *PIK3CA* mutations in CTCs in 6 out of 18 patients (104). In three cases with multiple CTCs analyzed, all CTCs were homogeneously mutant. One patient had a heterogeneous mutational status, with 3 out of 16 single CTCs harboring three different *PIK3CA* mutations (104). When combining aforementioned studies, from a total of 47 *PIK3CA* mutated patients, 15 had a heterogeneous circulating compartment with mutated CTCs present at a subclonal level. Also in a study on CRC, *PIK3CA* mutations were present at a subclonal level in four patients; one of whom harbored two different *PIK3CA* mutations in separate CTCs (105).

Since *PIK3CA*, *BRAF*, *KRAS*, and *PTEN* are relevant genes in predicting resistance to anti-EGFR therapy (106), mutations in these genes are frequently studied using CTCs. A recent study isolated 37 single CTCs from six patients with metastatic CRC for sequencing of a 68 CRC-associated gene panel to determine mutational landscapes in CTCs and the corresponding primary tumors and metastases (33).

Point mutations in *APC, KRAS, PIK3CA*, and *TP53* in the primary tumors were also present in the single CTCs. However, 20 'branch' mutations were found exclusively in CTCs, although targeted ultra-deep sequencing revealed the presence of 17 of these mutations at subclonal level in either the primary tumor or metastases (33). Two more studies performed targeted sequencing of *BRAF*, *KRAS*, and *TP53* of respectively 741 and 126 single CTCs (105, 107). The first study detected the presence of *KRAS* mutations in one-third of CTCs of one patient (105), while in the other, 6 out of 18 patients demonstrated a heterogeneous CTC compartment regarding these genes (107). Moreover, two studies examined heterogeneity of *KRAS* mutations in pools of CTCs (108, 109). Fabbri *et al*. reported one patient harboring three pools of CTCs with different mutational statuses. Two specific *KRAS* mutations were detected in the first pool, and another *KRAS* mutations was found in a second pool of pure CTCs (108). Also, temporal heterogeneity was shown as enriched CTC fractions exhibiting different mutational status of *KRAS* during treatment (109). However, one can argue on the sensitivity of mutation detection in enriched samples containing low CTC-counts, as often seen during therapy. Furthermore, mutational analysis was performed on multiple single CTCs collected from two patients with stage-IV melanoma. All CTCs were consistently *BRAF*^{V600E} mutated analogous to the primary tumor (110).

In a study towards *TP53* mutations, single and pooled CTCs of two patients with metastatic triple-negative inflammatory breast cancer, known for harboring a *TP53* mutation in their primary tumor, were recovered for molecular analysis (111). In the first patient, 2 of 6 single CTC harbored two different *TP53* mutations, one of these was also found in the pool of 14 CTCs. In the second patient, 3 of 5 single and 5 of 6 clusters of CTCs had a *TP53*^{R110delC} mutation. In contrast, *TP53* and *RB1* were homogeneous in all CTCs of lung cancer patients (112).

Temporal heterogeneity was demonstrated in pools of pure CTCs from patients with NSCLC receiving tyrosine kinase inhibitors. Serial analysis showed emergence of activating mutations in the gene encoding the EGFR conferring a mechanism of acquired resistance to therapy (65). *EGFR* mutation detection was also performed on enriched CTC samples. In 4 out 31 cases, multiple *EGFR* mutations were documented, suggesting possible CTC heterogeneity (113). However, the actual mutational landscape and subclonality can only be detected in single CTC samples or multiple pools of pure CTCs.

Global mutational profile

A recent study applied whole exome sequencing (WES) of 19 single CTCs from a patient with metastatic prostate cancer (34). Although non-uniform coverage, a heterogeneous mutation profile was detected in single CTCs. To compensate for the low coverage and random polymerase errors that did occur in individual CTCs, single-CTC data was pooled. Half of the somatic SNV in CTCs could be detected in the primary and metastatic sites, whereas the rest were CTC-specific mutations (34). Moreover, Ni *et al*. determined single nucleotide variation landscapes in CTCs of four patients with lung cancer by single-cell exome sequencing (35). The exome data showed extensive variation from cell to cell and presence of 'private' CTC mutations, not detected in tissue samples. The authors raise the question of false discovery due to interfering technical errors compatible with the MALBAC method used (35).

Copy number alterations

Methods used to study genome-wide CNA include array-CGH and whole genome or exome sequencing. In prostate cancer, a wide range of CNA in pools of pure CTCs were detected in nine patients, using array-CGH. But more specifically, CTCs showed uniform copy number gains in both the *AR* and *CCND1* locus (114). In one study where two single breast cancer CTCs per patient were analyzed for CNA, all CTCs displayed a typical breast cancer related copy number profile (100), with six patients harboring *CCND1*

amplification in both CTCs. Yet, differences in CNA between CTC couples were to a greater or lesser extent visible in all cases. Furthermore, multiple CTCs of 16 patients with breast cancer were analyzed using array-CGH. Ten of these patients showed molecular heterogeneity based on CNA. Although, in seven cases were *ERBB2* amplification was detected, it was homogeneous in all CTCs (101).

However, in multiple studies in various tumor types, homogeneity in the copy number profile was demonstrated. WES was applied to lung cancer CTCs in two studies (35, 112). Five out of six patients had highly homogeneous copy number profiles, although one patient harbored substantial CNA heterogeneity (112). In another study, the copy number profiles of the single CTCs were highly similar and shared most of the same CNAs as the primary and metastatic tumor cells. Furthermore, CNA patterns were indicative for specific lung cancer subtypes (35). A recent study isolated 37 single CTCs from six patients with metastatic CRC for copy number profiling with array-CGH (33). In general, many of the CTCs shared a number of gains and losses with the primary and metastatic lesions. However, they also observed private copy number changes in CTCs as well as heterogeneity between CTCs (33). To define CNA in melanoma, the genomes of 15 individually isolated CTCs from seven patients were analyzed by single-cell CGH (115). All of the analyzed CTCs displayed multiple chromosomal changes and carried aberrations typical for melanoma. In five of six cases with multiple CTCs isolated, hierarchical clustering of the CTCs showed a clonal relationship (115).

Sampling at multiple time-points to evaluate genetic evolution based on CNA profiles was performed in three studies (36, 80, 116). Dago and colleagues thoroughly analyzed CNA of multiple single CTCs of one patient with prostate cancer by WGS at various time points. Three different clonal lineages were found. One specific clone was present at subclonal level at the first blood draw, but demonstrated outgrowth at time of the third blood draw. A third clone only emerged at the fourth time point (80). Both array-CGH and WGS were applied for copy number analyses in one patient with breast cancer harboring extensive numbers of CTCs (36). CNA demonstrated high similarities between the 31 single and 21 pools of CTCs ranging between 5 and 100 CTCs. Furthermore, a high degree of analogy was also found with CNA in primary and metastatic tissue samples (36). In a large breast cancer cohort, array-CGH of CTCs revealed a wide range of CNA, including those known for breast cancer (116). In one patient, where multiple sampling was performed, CTCs of the second blood draw revealed numerous additional CNA beyond the baseline profile, while the third sample, divided in two pools, was comparable with itself and the second. Interestingly, the patient initially responded to her cancer treatment, but subsequently developed disease progression. In two other cases temporal homogeneity was documented between first and second blood draw. Furthermore, CTCs and the primary tumor were moderately and highly correlated, respectively (116).

Then, various studies have thoroughly analyzed CNA of specific target genes using FISH. In 4 patients with lung cancer, *ROS1* copy numbers were heterogeneous between CTCs (95). In prostate cancer, FISH was applied to study CNA of *AR*, *BRCA1*, *MYC*, and *PTEN* (96, 117, 118). Leversha and colleagues report a considerable variability in CTCs of individual patients. In one patient, a subset of CTCs showed *AR* amplification, whereas all CTCs had high copy number gain for *MYC* (117). A similar heterogeneity in *AR* amplifications and loss of the tumor suppressor gene *PTEN* was detected by Attard *et al*. when profiling 49 patients suffering CRPC (96). FISH analysis further revealed *BRCA1* losses appearing in minute fractions of CTCs in four of seven patients (118). In breast cancer, fluorescent cell sorting was combined with FISH to analyze *EGFR* amplification in CTCs (50). 11% and 6% of CTCs from ALDH1 positive and negative populations respectively, harbored *EGFR* amplification (50). Furthermore, *EFGR* copy number gain was found in 37% of CTCs of three patients with CRC, based on array-CGH data (105).

Table 1: Genomic heterogeneity in CTCs

#CTC	#Pts	Isolation	Analysis	Targets	Heterogeneity	Ref.
Lung cancer						
n.s.	32	MF (ISET)	FA-FISH	*ALK* rearrangements	18 ALK+ patients exhibited between 7 and 24 CTCs/ml, mean percentage of *ALK*-rearranged CTCs was 63% (range 28-100%). All ALK- patients had <4 rearranged CTCs.	89
n.s.	5	MF (ISET)	FA-FISH	*ALK* rearrangements	5 patients showed *ALK*-gene in all CTCs (100%), while in the primary tumor only half of the tumor cells show *ALK*-gene rearrangements.	91
177	1	microfluidics + cytospin	FISH	*ALK* rearrangements	25% of the total 177 CTC of 1 patient harbored *ALK*-gene rearrangements, and 54% of the 200 primary tumor cells did.	90
n.s.	8	MF (ISET)	FA-FISH	*ROS1* rearrangements	*ROS1* rearrangements were detected in the CTCs of all 4 ROS+ patients. *ROS1* copy number was heterogeneous within these CTCs. CN was increased at time of disease progression.	95
8	1	CS + MM	WES	CNA; mutations; indels	CNA show inter-CTC homogeneity, and represent metastatic tumor. SCLC and NSCLC can be differentiated based on CN-profile. Mutations and indels were highly heterogeneous in all CTCs.	35
8 + pools	2	CS + DEPArray	WGS; TAS	CNA; *TP53, RB1* mutations	CNA strongly correlated, but 1 of 6 CTC harbored substantial CNA differences. *TP53* and *RB1* mutations were homogeneous.	112
1 pool	4	microfluidics	Allele-specific PCR	*EGFR* mutations and CNA	Temporal heterogeneity in *EGFR* mutations. Genotypes of enriched CTC fractions evolved during therapy, with consistent presence of the primary *EGFR* activating mutation and the emergence of a drug-resistant mutation.	65
Colorectal cancer						
37	6	CS + MM	aCGH; Panel	CNA; 68 CRC-related gene panel	Multiple CRC related CNA and mutations were found in CTC and tissue samples. Various CTC-specific mutations, but most were retraced at subclonal level by ultra-deep sequencing of the tissue samples. Inter-CTC heterogeneity, with some private mutations.	33
741	33	CS + MM	qPCR; TAS	*EGFR* CNA; *PIK3CA, KRAS*, and *BRAF* mutations	CN-gain of *EGFR* was found in 27% of CTCs of 3 patients, 1 patient had *KRAS* mutations in 33% of CTCs, 39% of CTCs of 4 patients harbored *PIK3CA* mutations.	105
126	31	CS + MM	TAS	*TP53, KRAS* and *BRAF* mutations	CTCs were analyzed of 18 patients. 6 patients harbored heterogeneous CTC populations.	107
pools	21	DGC + DEPArray	TAS; PyroSeq	*KRAS* mutations	In 1 patient, 3 pools of CTCs had different mutational statuses, two mutations were found in the first pool and another mutation in a second pool of isolated CTCs.	108
pools	2	CS enriched	qPCR	*KRAS* mutations	Temporal heterogeneity: enriched CTC fractions exhibited different mutational status of KRAS during treatment.	109
Prostate cancer						
n.s.	49	CS	On-chip FISH	*ERG* rearrange-ments; *PTEN* and *AR* CNA	FISH on CTCs reveals homogenous *ERG* rearrangements but heterogeneous *AR* amplifications and *PTEN* deletions.	96
n.s.	77	CS + cytospin	FISH	*AR* and *MYC* CNA	There was considerable variability in the morphology of CTCs in individual patients. 1 patient showed heterogeneity of FISH patterns, with *AR* amplification in a subset of CTCs, but all with high copy number gain for *MYC*.	117
n.s.	7	DGC + cytospin	FISH	*BRCA1* CNA	In 4 of 7 patients, *BRCA1* losses appeared in a fraction of CTCs.	118

pools	9	IE/FACS	aCGH	CNA	CTCs from all patients revealed a wide range of CNA. Replicate CTC isolates where comparable showing gains in the *CCND1* and *AR* locus.	114
41	1	HD-CTC + MM	WGS	CNA	Three different clonal lineages were found. Clone B was present subclonally at first blood draw, but demonstrated outgrowth in the third blood draw. A third clone emerged at fourth blood draw.	80
19 + 10	2	MagSweeper + MM	WES	Somatic SNV	Although non-uniform coverage, a heterogeneous mutation profile was detected in single CTCs. When pooling the CTC data, found SNVs were comparable to the primary tumor.	34
Breast cancer						
261 + pools	42	CS + DEPArray	aCGH; qPCR; TAS	CNA; *ERBB2* CNA; *PIK3CA* mutations	2 patients had heterogeneous *PIK3CA* mutational status in their single and pooled CTCs. 10 of 16 patients harboring PIK3CA mutations showed molecular heterogeneity based on CNA. *ERBB2* amplification was uniformly detected in all CTCs of 7 patients.	101
26	12	CS + flow sorting (MoFlo XDP)	aCGH; qPCR; TAS	CNA; *CCND1* CNA; *PIK3CA* mutations	CNA were found breast cancer related in all CTCs, but differences in CNA between related CTCs were visible in all cases. 1 patient harbored a mutation in exon 20 of the *PIK3CA* gene in both CTCs and 1 patient harbored another *PIK3CA* mutation in 1 of 1 CTCs.	100
147 + pools	26	CS + DEPArray	TAS	*PIK3CA* hotspots	11 of 26 patients were found to harbor a heterogeneous *PIK3CA* mutational status in their CTC compartment.	102
115 + pools	18	CS + DEPArray	TAS	*PIK3CA* hotspots	3 patients were homogeneously mutated in all CTCs. 1 patient was found to have three different *PIK3CA* mutations.	104
185	17	MagSweeper + MM	TAS	*PIK3CA* hotspots	1 patient showed to harbor a heterogeneous CTC compartment based on *PIK3CA* status.	103
11 + pools	2	CS + DEPArray	TAS	*TP53* mutations	In one patient, 2 of 6 single CTC harbored two different *TP53* mutations. In the second patient, 3 of 5 single and 5 of 6 clusters of CTCs showed a $TP53^{R110delC}$ mutation.	111
402	3	DGC + cytospin	IF/FISH (BioView)	*EGFR* CNA	10 of 91 ALDH1+/HPSE+ cells showed *EGFR* amplification. This was 19 of 311 in the ALDH1-/HPSE+ population.	50
31 + pools	1	CS ór DGC + MM	WGS; aCGH	CNA	CNA show homogeneity within all isolated CTCs.	36
n.s.	3	IE/FACS	aCGH	CNA	Temporal heterogeneity: Serial testing of enriched CTC populations revealed numerous additional CNA beyond the baseline profile.	116
Melanoma						
24 + 18	2	Microfluidic + LCM	TAS	*BRAF* mutations	Consistency in the $BRAF^{V600E}$ mutation, and analogous to the mutation found in the primary tumor.	110
15	7	IM + MM	CGH	CNA	In 5 of 6 patients with ≥1 isolated CTC, hierarchical clustering showed a clonal origin.	115
Multiple cancers						
n.s.	20	IM + cytospin	FISH	CNA	6 patients had a homogeneous pattern of aneusomy in all CTCs. In 10 patients a heterogeneous pattern was observed, including 6 cases with two distinct clones.	49

Abbreviations: aCGH, array comparative genomic hybridization; CNA, copy number alterations; CS, CellSearch enrichment; CTC, circulating tumor cell; DGC, density gradient centrifugation; FA-FISH, filter adapted fluorescent *in situ* hybridization; HD-CTC, high-definition CTC assay; IE/FACS, immunomagnetic enrichment and fluorescence-activated cell sorting; IF, immunofluorescence; IM, immunomagnetic enrichment; LCM, laser capture microscopy; MF, microfiltration; MM, micromanipulation; TAS, targeted amplicon sequencing; qPCR, quantitative polyclonal chain reaction; WES, whole exome sequencing; WGS, whole genome sequencing; n.s., not specified.

TRANSCRIPTIONAL CTC HETEROGENEITY

While in diploid cells chromosomal DNA molecules are present with only two copies, a single cell harbors thousands of copies of each mRNA transcript, which facilitates single-cell RNA approaches (119). Yet, single cell RNA studies are affected by transcriptional bursting or pulsing (120, 121). This phenomenon can account for the high variability in gene expression between cells in isogenic populations, and therefore transcriptional heterogeneity should be evaluated with caution. On the other hand, variability in gene expression may also contribute to resistance of sub-populations of cancer cells to chemotherapy (122). Gene-expression studies in single CTCs may be essential for determining the nature and extent of tumor heterogeneity, linking phenotypic differences with genetic and epigenetic aberrations. However, preserving RNA is more difficult than DNA and concerns have been raised about the impact of sample processing on CTC expression profiles (123). Hence, several devices have been developed for direct and fast isolation of CTCs using a microfluidic approach (37, 75, 81, 124-126).

Single cell expression profiling is performed using RNA-*in situ* hybridization (ish), RT-PCR, and RNA-sequencing (seq). While RNA-ish has the advantage of direct analysis of the RNA without whole transcriptome amplification, expression of far more genes can be evaluated using RT-PCR or RNA-seq. Differentiating the changes in gene expression that are biologically relevant from those caused by technical and biological noise remains a significant hurdle for single-cell transcriptome studies. Hence, single cell mRNA-seq protocols are being developed with improved transcriptome coverage, high reproducibility, and low technical variation (127, 128).

Hereafter, we review various publications on transcriptional heterogeneity in CTCs. Often, patient-specific global expression profiles were observed. However, when looking in detail, significant heterogeneity between CTCs is found regarding specific transcripts, which is often linked to therapy selection or response. **Table 2** gives an overview of the experimental details of these studies.

Metastasis-associated gene expression

In prostate cancer RT-PCR of 84 EMT-related genes was applied to analyze multiple single CTCs of 8 patients (129). Heterogeneous upregulation of EMT-associated gene expression was found, especially in CRPC. RT-PCR was also used to target vimentin, EpCAM, and stem cell gene NANOG mRNA for EMT evaluation in approximately 400 breast CTCs (130). Temporal heterogeneity was shown as expression patterns changed after surgery, with emerging of a sub-population of EpCAM positive CTC expressing NANOG and/or vimentin. Yu *et al.* applied RNA-ish for scoring the relative abundance of epithelial versus mesenchymal transcripts within individual breast cancer CTCs of 15 patients, both during therapy or at time of progression (81). Clear heterogeneity was shown, with various proportions of CTCs that were mesenchymal. Moreover, relative changes during treatment in the expression of epithelial and mesenchymal markers in CTCs correlated with response and prognosis. For one patient, single CTCs were analyzed with RNA-ish over 7 time points and two different treatment regimens. An increased number of mesenchymal CTCs was repeatedly detected in the samples taken at time of disease progression (81). Additionally, single molecule RNA-seq was applied on CTCs to identify signaling pathways that contribute to EMT, and 45 enriched genes were identified (81). In metastatic pancreatic cancer, RNA-ish was used for detection of CTC-specific transcripts of Wnt2, which is known for its role in tumor sphere formation and metastasis initiation (37). Wnt2 transcripts were identified in 23 out of 66 (35%) cytokeratin-positive CTCs from 2 out of 8 patients. Heterogeneity was also shown in the primary tumors. The small number of Wnt2-positive cells was consistent with RNA-seq analysis, which showed rare *Wnt2* RNA reads in both enriched CTCs and primary tumors (37). This demonstrates Wnt2-positive CTCs are present at subclonal

level and represent a rare subset of the primary tumor population. Ting and colleagues isolated 7, 29, and 77 single CTCs from patients with pancreas, breast, and prostate cancer respectively (125). In more than 15% of all CTC samples, CTCs exhibit a very high expression of stromal-derived extracellular matrix (ECM) genes, which have an important role in metastatic spread. One specific ECM glycoprotein gene was expressed at high levels in 100% of pancreatic CTCs compared to 31% of breast and 9% of prostate CTCs (125).

Global gene expression profiling

Recently, genome-wide expression profiling of single cells using NGS has been achieved (127, 128). In a study regarding patients with metastatic breast cancer, a homogeneous global expression pattern was shown, with all CTCs clustering together patient wise, except for two patients (124). Furthermore, in advanced melanoma, some highly expressed transcripts in single CTCs were detected (128). Although slight differences in gene expression, CTCs show a uniform and high upregulation of cell-cycle and melanoma specific markers, as well as uniform up- or downregulation of certain plasma membrane proteins (128). The same single cell mRNA-seq protocol was used for CTCs isolated from patients with metastatic prostate cancer (131). High rates of RNA degradation consistent with apoptosis amongst CTCs was noted, although prostate-specific and cancer-specific transcripts could still be elucidated. 181 genes were overexpressed in the CTCs compared to normal prostate tissue (131). Unsupervised clustering revealed that all CTCs, except two, cluster in a patient specific manner. Specific transcripts, e.g. related to CRPC or *ERG*-fusion, were detected homogeneously within the same patients (131). In another RNA-seq study on prostate cancer, hierarchical clustering analysis also demonstrated patient-specific CTCs clustering, separated from cancer cell lines. However, single CTCs from nine individual patients with at least 3 CTCs analyzed, showed considerably higher heterogeneity in their transcriptional profiles compared to single cells from prostate cancer cell lines (126). Moreover, RT-PCR of a panel of 87 cancer genes demonstrated heterogeneity among individual breast cancer CTCs, separating them into two major subgroups based on 31 highly expressed genes (132). This was in contrast to several breast cancer cell lines tested.

Prostate cancer specific gene expression

Isolated single CTCs were tested for expression level of the PSA gene *KLK3* (34). The expression profile of *KLK3* was heterogeneous between the 26 out 48 selected individual CTCs, for which sufficient part of the transcriptome was covered (34). Besides, expression patterns of AR splice variants have been studied at a single cell level using either RT-PCR or RNA-seq (73, 126). One study demonstrated that more than half of all patients had multiple AR splice variants present within different CTCs and that a subpopulation of single CTCs had simultaneous expression of several AR splice variants (126). These results are in line with other data showing that acquisition of AR-independent alterations conferring resistance to antiandrogen therapies is very heterogeneous in patients with CRPC (79). Temporal heterogeneity between multiple enriched CTC samples from 21 patients with prostate cancer was shown by emerging of AR-V7 in one out nine patients treated with taxane chemotherapy. In contrast, seven out of twelve patients who were AR-V7 positive at baseline, only harbored full length AR at time of progression (73). Relations between therapy response and presence of variants are increasingly studied (74, 97), although usually not at multiple time points or with multiple CTC samples, which is needed to study tumor evolution.

Table 2: Transcriptional heterogeneity in CTCs

#CTC	#Pts	Isolation	Analysis	Targets	Heterogeneity	Ref.
Breast cancer						
n.s.	17	HBCTC-Chip	RNA-ish; RNA-seq-DGE	EMT markers	Heterogeneous fractions of Epithelial (E), Mesenchymal (M), and EM-CTCs; In TNBC more homogeneous pool of M-CTCs. Temporal heterogeneity was examined in 10 patients. At progressive disease, patients harbored emerging numbers of M-CTCs.	81
105	35	MagSweeper + MM	qRT-PCR	87 cancer-associated genes	Two major subgroups of CTCs, i.e. high expression of EMT genes and high metastasis-associated genes. Heterogeneity based on CTCs not clustering by patient-ID and 8 patients had CTCs in both clusters.	132
15 pools + 14 clusters	10	negCTC-iChip + MM	RNA-Seq	Whole transcriptome expression level	At global gene expression level, all isolated CTCs clustered closely by patient of origin. Based on *JUP* and 31 cluster-associated genes, CTC-clusters could be differentiated from pooled single CTCs.	124
~400	20	IM (Maintrac) + AP	PCR + gelelectro-phoresis	HER2, EpCAM, Vimentin, and NANOG	Expression patterns changes after surgery, with emerging of a sub-population of EpCAM positive CTC expressing NANOG and/or vimentin.	130
Prostate cancer						
77	13	negCTC-iChip + MM	RNA-seq	Whole transcriptome expression level	Single CTCs from nine individual patient with at least 3 CTCs analyzed, showed considerably greater intra-patient heterogeneity in their transcriptional profiles compared to single cells from prostate cancer cell lines.	126
20	4	MagSweeper + MM	RNA-seq	Whole transcriptome expression level	All CTCs, except two, cluster in a patient specific manner. 181 cancer-specific genes were overexpressed in the CTCs, compared to normal tissue. Specific transcripts, e.g. related to CRPC or *ERG*-fusion, were detected homogeneously within the same patients.	131
48	2	MagSweeper + Nanowell	RNA-seq	*KLK3* (PSA) mRNA	*KLK3* expression was variable between the 26 individual CTCs, for which a sufficient number of genes including *KLK3* were detected.	34
38	8	MF + MM	qRT-PCR	84 EMT-related genes	Heterogeneous upregulation of EMT-associated gene expression, especially in CRPC.	129
pools	21	IM (AdnaTest)	qRT-PCR	AR full length + AR-V7	Temporal heterogeneity: 1 out 9 patients converted to AR-V7 positive, at progression on Taxane. While 7 out 12 patient who were at baseline AR-V7 positive became negative at progression.	73
Pancreatic cancer						
265	15	HBCTC-Chip	RNA-ish; RNA-Seq-DGE	*WNT2*	RNA-ish showed heterogeneity of *WNT2* expression in CTCs and the primary tumor. This was confirmed by RNA-seq with DGE, showing rare *WNT2* RNA reads in the enriched CTC sample and the primary.	37
Melanoma						
6	1	MagSweeper + MM	RNA-seq	Whole transcriptome	CTCs show a uniform upregulation of melanoma markers, including melanoma associated tumor antigens (MAGE), as well as uniform up- or downregulation of certain plasma membrane proteins.	128
Multiple cancers						
7, 29, 77	n.s.	negCTC-iChip + MM	RNA-seq	Whole transcriptome	High expression of stromal-derived ECM proteins in >15% of CTC samples. One glycoprotein was expressed in 100% of pancreatic CTCs compared to 31% of breast and 9% of prostate CTCs.	125

Abbreviations: CTC, circulating tumor cell; DGE, digital gene extraction; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; IM, immunomagnetic enrichment; MF, microfiltration; MM, micromanipulation; qRT-PCR, quantitative reverse transcription polyclonal chain reaction; RNA-ish, RNA *in situ* hybridization; RNA-seq, RNA sequencing; n.s., not specified.

DISCUSSION

Technical considerations

Studies across multiple tumor types have demonstrated the feasibility of analyzing molecular profiles of single CTCs. Although technical improvements are needed, it becomes clear that CTC profiling contributes to our understanding of tumor heterogeneity, disease evolution (through serial sampling), and clinical management. To maximize the potential of CTC profiling, key issues in CTC research must be addressed regarding both technical and biological challenges.

Evolution in multiple-marker and marker-independent CTC enrichment has already increased yield and diversity of CTCs (50, 81, 133), although it is not as extensively validated as EpCAM enrichment strategies. Furthermore, efforts have been made to improve both amplification methods (134-136) and sequencing techniques (34, 127, 128) as well as subsequent data interpretation and bioinformatics (10, 137, 138), reviewed in more detail by Van Loo and Voet (24). This all contributes to more reliable detection of aberrations and evaluation of heterogeneity in CTC research.

A major question in CTC research remains how many CTCs should be profiled to account for heterogeneity. Often, the molecular characteristics of only a few CTCs out of the entire pool of CTCs from a patient have been adequately analyzed (34, 100). As a consequence their diversity remains largely unknown. In primary breast cancer for example, single-molecule sequencing indicated that many of the diverse mutations occur at low frequencies (<10%) in the tumor mass (139). Navin demonstrated, using a power analysis, that detection of a 10% subclone would require sequencing at least 20 single cells to achieve a 0.87 detection power (140). Besides, subclonality can be evaluated using multiple small pools of pure CTCs (**Figure 2**) and determining the variant allele frequencies. Herewith technical errors typical for single cell research (24) can be reduced, although more CTCs need to be available and isolated. Furthermore, in depth comparative research towards CTCs and multiple metastases (31) should clarify whether the whole tumor burden contributes equally to the CTC pool or if some subclones might be underrepresented or absent.

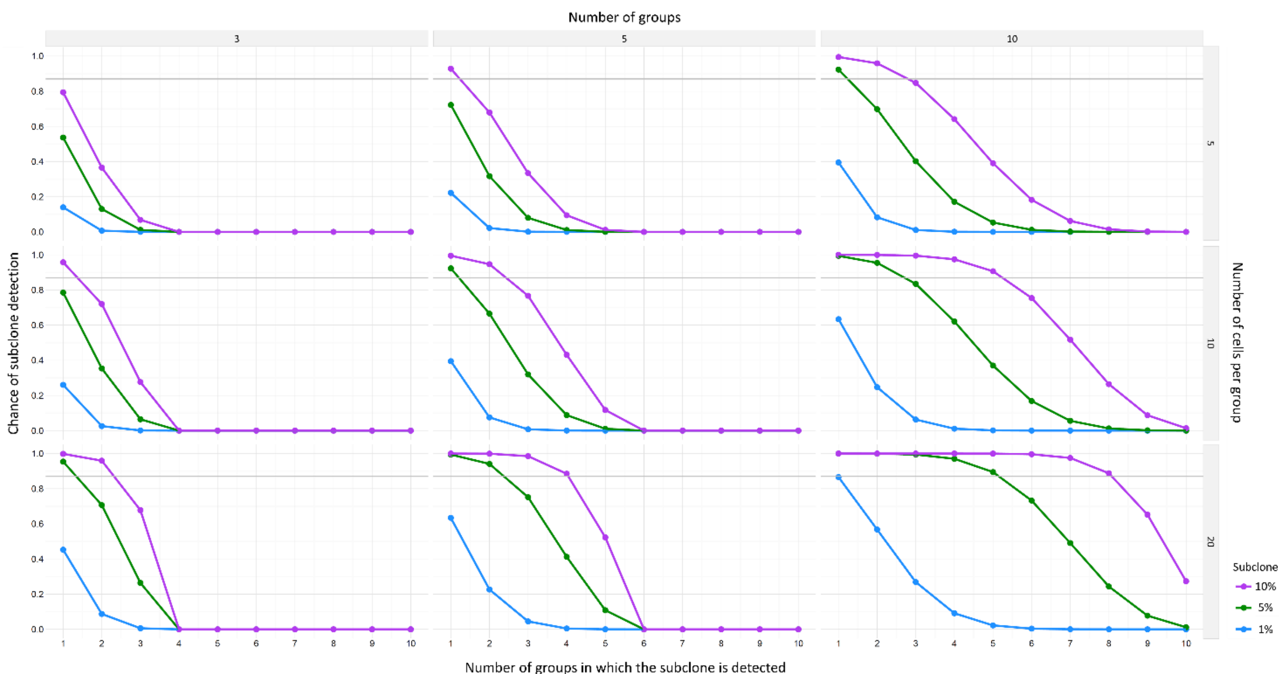

Figure 2: Power analysis for detection of minor subclones in pools of CTC. Chances of detection of minor subclones (i.e. 1%, 5%, or 10%), calculated with a power of 0.87, for three different number of groups (i.e. 3, 5, or 10 groups) and three different number of cells per group (i.e. 5, 10, or 20 cells). As depicted in the lower right graph (10 groups of 20 cells), there is a 90% change of detecting a 1% subclone in 1 out of 10 groups, or detecting a 5% subclone in 5 out of 10 groups, or detecting a 10% subclone in 8 out of 10 groups.

Clinical implications and future perspectives

Currently, biomarkers predicting therapy response are frequently assessed using primary tumor biopsies, reflecting only parts of a patient's disease at a specific moment in time (141). It is well-known that targetable molecules can change during the course of the disease. CTCs have shown to be useful in understanding and predicting acquired resistance to therapies, and might in the future be used to circumvent this. In lung cancer, serial analysis identified emergence of activating mutations in the *EFGR* gene in some patients receiving EGFR-targeting therapy, conferring a mechanism of acquired resistance to therapy (65). Moreover, clonal selection of *ALK*-rearranged CTCs during crizotinib therapy was detected in patients with lung cancer (89). Serial RNA analysis of prostate CTCs demonstrated emergence of AR-V7 during taxane chemotherapy (73), and TMPRSS2-ERG status in CTCs is a predictive biomarker of abiraterone acetate sensitivity in CRPC (97). Hence, repeated CTC sampling may have the potential to guide optimal therapy regimens depending on the evolving molecular profile of the tumor burden within an individual patient. However, CTC characterization is currently only performed in clinical trials (142). Therefore, efforts to increase clinical utility, have to be made. A comprehensive analysis of multiple patient samples, including CTCs, cfDNA, and tissue samples, on both RNA and DNA level can provide a holistic view of a patient's (sub)clonal landscape. The development of multi-compartment molecular databases of large patient cohorts will enable the creation of algorithms able to predict outcome at a more individual patient level (3, 143, 144).

A key issue remains to what extent heterogeneity in the circulating compartment affects therapy outcome and whether one should take a minor subclone into account if it comes to treatment selection. The analysis of subclonal heterogeneity may help clinicians understand why patients do not respond homogeneously to targeted drugs. Furthermore, longitudinal molecular analysis of individual CTCs can uncover clonal evolution caused by therapy pressure (32, 78, 145). In a patient with CRPC, sequentially progressive on chemo and targeted therapy, comparable CTC clones were observed before the start and during standard chemotherapy. However, subsequent clinical response to targeted therapy was associated with the drastic depletion of the fist clone and emergence of a second clone, while a third tumor lineage was detected at time of disease progression (80). As acquired drug resistance and disease relapse is common, drugs may only ablate specific subpopulations of tumor cells, allowing resistant cells to grow, evolve and seed new tumor foci that may not respond to cytotoxic or targeted therapies (32, 78, 145). Hence, a tremendous potential of CTCs lies in profiling them over the entire clinical course to study the evolutionary history of tumors and to optimize clinical trial design. In the TRACERx trial (NCT01888601), primary tumors of 842 NSCLC patients will be sequenced, as well as cfDNA and CTCs, obtained at multiple time points during therapy. To evaluate the effect of clonal heterogeneity and selection pressure on clinical outcomes, and to identify targetable driver events, repeated tumor sampling will be performed at time of disease recurrence. These patients will be eligible for the DARWIN trial (NCT02183883). This trial aims at evaluating whether targeting driver events, detected by the TRACERx trial, has a different clinical outcome in patients harboring the driver dominantly compared to subclonally.

In conclusion, molecular characterization of CTCs provides the opportunity to repeatedly assess the biological features of cancer during the evolution of the disease. Therefore, CTCs may facilitate the development of new therapeutic strategies and enable clinicians to tailor therapy to an individual patient in a longitudinal fashion. The relevance of CTC heterogeneity as a cause or consequence of resistance to targeted therapy is yet to be unveiled. Hence, a tremendous potential of CTCs lies in single-cell profiling techniques that will contribute to understanding the predictive value of driver molecular aberrations in subclones of CTCs and emergence of resistant populations on targeted therapy.

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Conflicts of interest

The authors declare no conflicts of interest regarding the publication of this paper.

Abbreviations

General: CGH, comparative genomic hybridization; CNA, copy number alteration; CRC, colorectal cancer; CRPC, castration resistant prostate cancer; CTC, circulating tumor cell; DGE, digital gene extraction; EMT, epithelial-to-mesenchymal transition; FISH, fluorescent *in situ* hybridization; ITH, intra tumor heterogeneity; NCSLC, non-small cell lung cancer; NGS, next generation sequencing; SCS, single cell sequencing; SNV, single nucleotide variation; WGA, whole genome amplification; WGS, whole genome sequencing; WTA, whole transcriptome amplification; ***Genes:*** *ALK*, anaplastic lymphoma kinase; *APC*, adenomatous polyposis coli; *BRAF*, v-Raf murine sarcoma viral oncogene homolog B; *BRCA1*, breast cancer 1; *CCND1*, cyclin-D1 ; *EGFR*, epidermal growth factor receptor; *EML4*, echinoderm microtubule-associated protein-like 4; *ERBB2*, avian erythroblastosis oncogene B 2; *ERG*, ETS-related gene; *KLK3*, kallikrein 3; *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *MYC*, V-myc avian myelocytomatosis viral oncogene homolog; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase; *PTEN*, phosphatase and tensin homolog; *RB1*, retinoblastoma 1; *ROS1*, ROS Proto-Oncogene 1; *TMPRSS2*, transmembrane protease serine 2; *TP53*, tumor protein 53; ***Markers:*** ALDH, aldehyde dehydrogenase; AR, androgen receptor; ; EpCAM, epithelial cell adhesion molecule; HER2, receptor tyrosine-protein kinase erbB-2; PSA, prostate-specific antigen.

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Mutational and copy number analysis of numerous sorted circulating and disseminated tumour cells to interrogate subclonal evolution in advanced breast cancer.

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Mutational and copy number analysis of numerous sorted CTC and DTC to interrogate subclonal evolution in advanced breast cancer.

ABSTRACT

Background. Liquid biopsies have been studied as an accessible method to capture spatial tumour heterogeneity and repeatedly evaluate targetable aberrations in cancer. Here, we analysed pure single and pools of circulating and disseminated tumour cells (CTC and DTC) to study genetic heterogeneity. **Methods.** From, three patients with metastatic breast cancer, CTC and DTC (bone marrow, pleural fluid, and cerebrospinal fluid) were CellSearch® enriched and DEPArray® sorted into 136 samples. Sorted cells, primary tumour, circulating free (cf)DNA, and enriched CTC/DTC fractions were analysed for mutations and copy number alterations (CNA). **Results.** Two patients harboured a driver *PIK3CA* mutation in all samples. Variant allele frequencies matched the ploidy status calculated from copy number data. The majority of CNA were homogeneously present in most samples of each patient, including breast cancer subtype specific CNA. One patient who rapidly progressed harboured additional CNA in the CTC/DTC compartment, reflecting the aggressive course and possibly subtype switch. These clones emerged at distinct timepoints in the different compartments. Furthermore, various mutations were present in unique samples. **Conclusion.** Repeated tumour analysis with liquid biopsies unveils changing molecular characteristics over time, and even a difference between simultaneous primary and metastatic disease. We show that all major subclones can be captured by combined sequencing of enriched CTC and cfDNA at disease progression.

INTRODUCTION

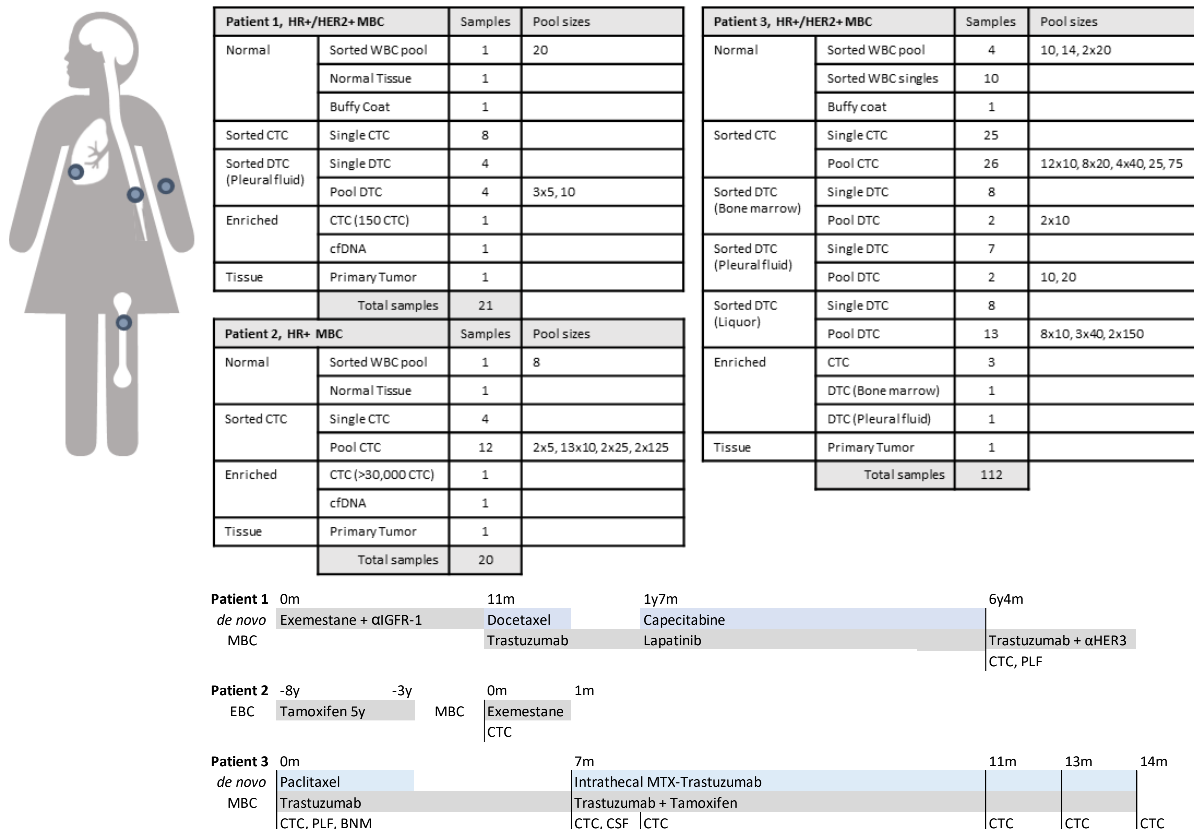
A growing understanding of the molecular biology of cancer and the identification of specific aberrations driving cancer evolution have led to the development of various targeted agents. Therapeutic decisions are generally guided by unique tumour biopsies. Yet, tumours can exhibit significant heterogeneity and change over time, e.g. as a result of selective therapeutic pressure (1, 2). Patients often present with multiple metastatic lesions, each composed of numerous genetically diverse subclones (3, 4, 5). Circulating Tumour Cells (CTCs), shedded directly from the one or multiple tumour sites (6, 7, 8, 9), hold considerable promise to provide a convenient and safe alternative for real-time and repeated tumour biopsies. The enumeration of CTCs bears prognostic significance in patients with MBC (10, 11) and other malignancies (12, 13). Furthermore, phenotypical and molecular characterization of CTCs are informative with regards to targetable aberrations present during the course of the disease (14, 15, 16, 17).

Although mutation detection in DNA extracted from CTC-enriched samples demonstrated activating mutations in for example EGFR, KRAS, and AR genes in patients suffering from lung, colorectal, or castration-resistant prostate cancer respectively (17, 18, 19), sequencing of enriched fractions is complicated by low levels of tumour-specific templates and contamination by abundant leukocyte-derived sequences, limiting the sensitivity and specificity (20, 21). Molecular analysis of pure isolated CTCs enables the detection of multiple mutations within the same cell, in order to decipher tumour heterogeneity and map clonal evolution (22, 23, 24). Here, we aim to unveil clonality within the CTC and disseminated tumour cell (DTC) compartment at a mutational and copy number level, using both multiple single and pools of pure isolated tumour cells.

MATERIALS AND METHODS

Patients and samples

Patients were recruited after giving written informed consent at the Oncology Centre of GZA Hospitals Sint-Augustinus (Antwerp, Belgium). A detailed description of the patients oncological history is written in the supplementary methods. Patient 1 was diagnosed with *de novo* ductal MBC, hormone receptor (HR)+ and HER2+, with bone and visceral metastasis (liver and pleura). Shortly after, she developed brain metastasis. Whole brain radiation in combination with systemic chemo and HER2 directed therapy led to 5.5 years of stable disease. Subsequently, she developed progressive liver, pleura, and bone metastasis and CTCs and DTCs from pleural fluid were collected. Patient 2 was enrolled when she developed metastatic disease eight years after the diagnosis of HR+ HER2- early breast cancer. PET-CT showed bone, lymph node, and pleura metastasis. At the first blood draw, >30.000 CTCs per 7.5 ml blood were enriched. Patient 3 presented with *de novo* MBC with extensive local disease and diffuse bone and visceral metastasis (PET images and pictures of local disease are depicted in **Figure S3**). Shortly after, also leptomeningeal metastasis was detected. Primary tumour biopsy showed HR+ HER2+ lobular breast cancer. Multiple samples were taken at different time points (**Figure 1 and 4B**). She received two lines of chemo-trastuzumab, after which CTC counts dropped for only several months.



Patient 1, HR+/HER2+ MBC		Samples	Pool sizes
Normal	Sorted WBC pool	1	20
	Normal Tissue	1	
	Buffy Coat	1	
Sorted CTC	Single CTC	8	
Sorted DTC (Pleural fluid)	Single DTC	4	
	Pool DTC	4	3x5, 10
Enriched	CTC (150 CTC)	1	
	cfDNA	1	
Tissue	Primary Tumor	1	
	Total samples	21	

Patient 2, HR+ MBC		Samples	Pool sizes
Normal	Sorted WBC pool	1	8
	Normal Tissue	1	
Sorted CTC	Single CTC	4	
	Pool CTC	12	2x5, 13x10, 2x25, 2x125
Enriched	CTC (>30,000 CTC)	1	
	cfDNA	1	
Tissue	Primary Tumor	1	
	Total samples	20	

Patient 3, HR+/HER2+ MBC		Samples	Pool sizes
Normal	Sorted WBC pool	4	10, 14, 2x20
	Sorted WBC singles	10	
	Buffy coat	1	
Sorted CTC	Single CTC	25	
	Pool CTC	26	12x10, 8x20, 4x40, 25, 75
Sorted DTC (Bone marrow)	Single DTC	8	
	Pool DTC	2	2x10
Sorted DTC (Pleural fluid)	Single DTC	7	
	Pool DTC	2	10, 20
Sorted DTC (Liquor)	Single DTC	8	
	Pool DTC	13	8x10, 3x40, 2x150
Enriched	CTC	3	
	DTC (Bone marrow)	1	
	DTC (Pleural fluid)	1	
Tissue	Primary Tumor	1	
	Total samples	112	

Figure 1. Patient samples. Detailed description of collected samples per patient. Patient 1 and 3 were diagnosed with de novo hormone receptor (HR) and HER2 positive metastatic breast cancer (MBC). Patient 2 was included at time of progression to MBC. Archival primary tumour tissue (HR+) was used. Samples include sorted white blood cells (WBC), circulating and disseminated tumour cells (CTC and DTC) into single cell samples or pools of varying size (between 5 and 150 cells per pool). Mixed samples include Cellsearch enriched CTC/DTC and cell free (cf)DNA from plasma. Abbreviations: BNM bone marrow, CSF cerebrospinal fluid, PLF pleural fluid, MTX Methotrexate.

CTCs and DTCs were enriched from 7.5 ml blood or effusion using the CellSearch® CTC enumeration kit and subsequently further purified using the DEPArray® system, as previously described (25). Individual CTCs, DTCs and white blood cells (WBC) as well as small pools were successfully recovered for each of the three MBC patients. Samples per patients are depicted in **Figure 1**. Fresh frozen tissue from solid metastases and the primary tumour, as well as bulk CTC and DTC samples (CellSearch® Profile) were sequenced as comparators for mutation and copy number profiles. DNA of buffy coat was sequenced to enable germline variant detection.

Targeted AmpliSeq sequencing and shallow WGS

A detailed description of the materials and methods can be found in the supplementary methods.
After DNA extraction, *Ampli*1™ whole genome amplification (WGA), and library preparation samples were subjected to Ion Torrent deep amplicon sequencing using the Ion AmpliSeq™ Cancer Hotspot Panel v2 covering 50 oncogenes and tumour suppressor genes (see supplementary methods). State-of-the-art bio-informatics are described in the supplementary methods. Annotated variants where further selected based on coverage >20x and variant allele frequency (VAF) >10% for pools and bulk, VAF >30% for single cells, and VAF 0% for reference sample (buffy coat or normal tissue) (**Figure S1**).
Shallow WGS was performed to determine the copy number profile. *Ampli*1™ WGA products were used for library preparation and sequencing on a Illumina HiSeq 4000, as described in the supplementary methods. After read trimming, mapping, merging, and sorting, BED files were subjected to the Ginkgo pipeline (26), especially designed for copy-number alteration (CNA) analysis of single cells, although also very capable of analysing bulk data. Single-cell CNAs were identified with Ginkgo using variable-length bins of around 500 kb. Bin counts were normalized using a buffy coat reference and corrected for GC bias (supplementary methods). Gains and losses within single cells are assigned to regions with copy numbers ≥3 and ≤1 respectively (as cancer cells can be triploid or more), for pools the cut-off is ≥2.3 and ≤1.7 respectively (which can result from a mix of cells with altered and normal CN). Amplifications and deletions have copy numbers ≥4 or ≤0 respectively.

RESULTS

Quality control

A high average read depth and width was obtained (**Figure S2A**) with Ion AmpliSeq™ panel sequencing. The allelic dropout (ADO) rate across individual CTCs and WBCs was 30.85% (SEM 17.73%) and for sorted pools 9.98% (SEM 5.62%) (**Table S1A**). The mean false positive (FP) rate was 0.63% (SEM 0.73%) (**Table S1B**). This is all within the range reported previously (24, 27). In total we found 33, 21, and 93 exon variants over all samples of patients 1 to 3, respectively (**Figure S1**). Similar to previously reported data by Rothé and colleagues (28), the number of variants found in sorted WBC samples are significantly lower compared to sorted CTC or DTC samples.
Illumina shallow WGS raw data demonstrated coverages of 2-30 (median 5) million reads per samples, phred scores of 37.7-38.8 and high concordance between duplicate lanes (**Figure S2B**). The data was analysed in the GINKGO pipeline (26). Binned read counts were used to measure further data quality metrics, i.e. GC bias and coverage dispersion. We found a GC bias within the range (typically 0.4-0.6) for uniform or non-preferential amplification (26). Coverage dispersion was evaluated by the Median Absolute Deviation (MAD) of neighbouring bins. MAD-scores were consistently horizontal across all samples (**Figure S2C**). This was comparable with single cell studies using DOP-PCR (26). The coverage uniformity was further confirmed by Lorenz curves (**Figure S2D**).

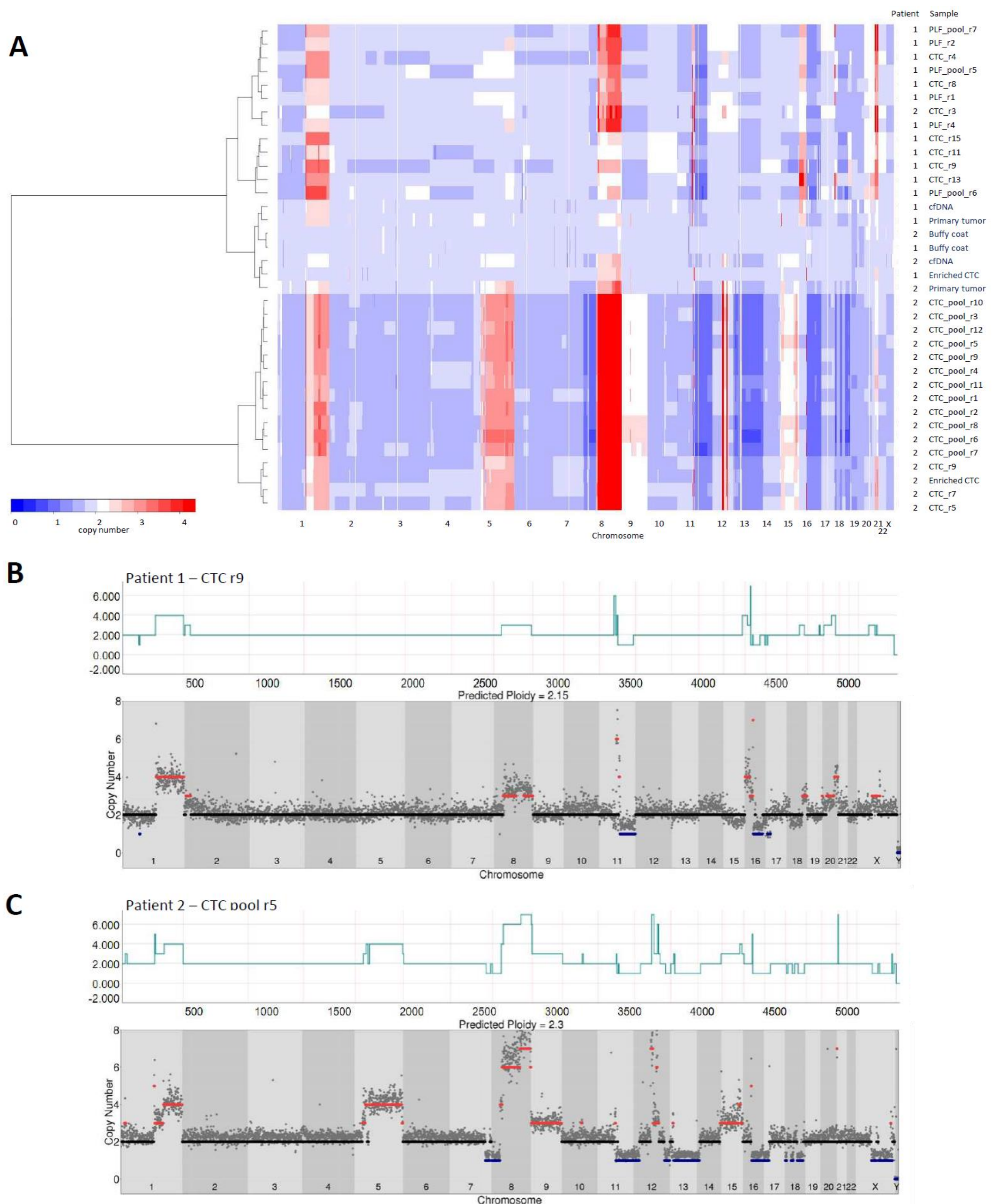
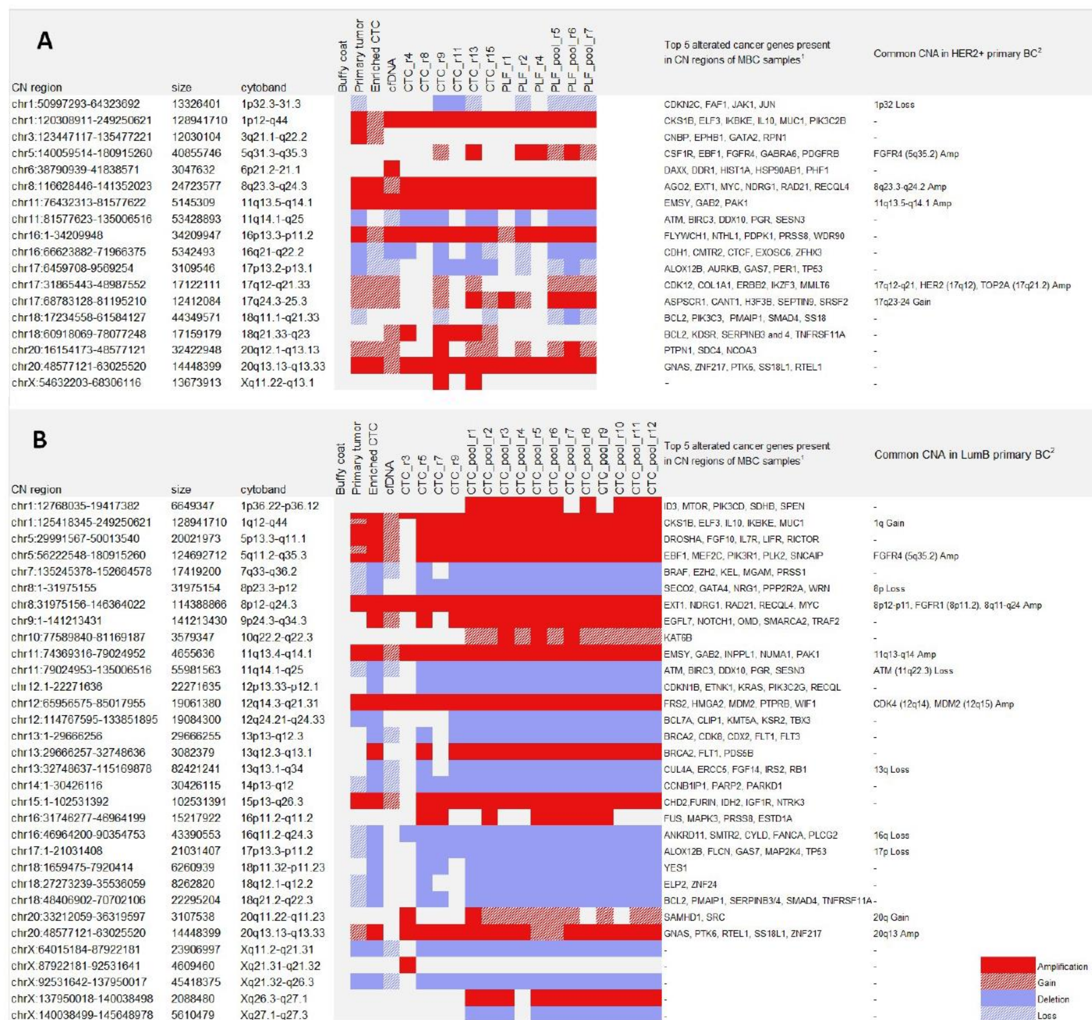


Figure 2A. Heatmap of normalized read counts across segment breakpoints using Euclidian distance. Cluster analysis shows clear separation between samples of the two patients. Buffy coats and mixed samples, like primary tumours and cfDNA, cluster together (written in blue), including the enriched CTC fraction of patient 1, confirming the relative low quantity of CTCs present in that sample (150 versus >30,000 in the enriched sample of patient 2). Clear aneuploidy was seen in 1q, 8q 11q and 16q, which reflects metastatic breast cancer in general. Further aneuploidy and CNA were patient specific. **B+C. Copy number profile.** Representative examples of detailed CN profile of a single CTC from patient 1 (**A**) and small pool of CTCs from patient 2 (**B**). Upper parts show the calculated copy number per bin. Lower graphs show CN status per chromosome. There is a general diploidy with patient and sample specific deletions (CN -1) and amplifications (CN ranging 3-7).

CNA landscapes and subclonality

Several As breast cancer is a copy number (CN) driven disease, we explored heterogeneity within the CTC and DTC compartment based on the CNA profiles generated after shallow WGS. Hierarchical clustering of CTC, DTC, and other samples demonstrates clear separation between patient 1 and 2 (**Figure 2**). Both buffy coats have a CN neutral profile and cluster together with the enriched CTC fraction of patient 1, confirming the relative low quantity of CTCs present in that samples (i.e. 150/7.5ml versus >30,000/7.5ml in the enriched CTC sample of patient 2). cfDNA and enriched CTC samples have lower tumour purity with reduced signal on CN level compared to pure CTC and DTC, therefor clustering with non-tumour samples. In patient 1, the buffy coat, primary tumour, enriched CTC fraction and one single CTC cluster separate from all other CTC and pleural fluid DTC samples. In both patients distinct aneuploidy of chromosome arms 1q and 8q, as are deletions in 11q and 16q are present (**Figure 2B and C, Table 1**), all frequently observed in metastatic breast cancer (29). For example, most samples of patient 1 (ER+HER2+) contain increased copies of the *ERBB2* gene mainly as a result of 17q arm aneuploidy, apart from a single and pooled PLF sample harbouring a local amplification.



A

CN region	size	cytoband	Top 5 altered cancer genes present in CN regions of MBC samples[1]	Common CNA in HER2+ primary BC[2]
chr1:50997293-64323692	13326401	1p32.3-31.3	CDKN2C, FAF1, JAK1, JUN	1p32 Loss
chr1:120308911-249250621	128941710	1p12-q44	CKS1B, ELF3, IKBKE, IL10, MUC1, PIK3C2B	-
chr3:123447117-135477221	12030104	3q21.1-q22.2	CNBP, EPHB1, GATA2, RPN1	-
chr5:140059514-180915260	40855746	5q31.3-q35.3	CSF1R, EBF1, FGFR4, GABRA6, PDGFRB	FGFR4 (5q35.2) Amp
chr6:38790939-41838571	3047632	6p21.2-21.1	DAXX, DDR1, HIST1A, HSP90AB1, PHF1	-
chr8:116628446-141352023	24723577	8q23.3-q24.3	AGO2, EXT1, MYC, NDRG1, RAD21, RECQL4	8q23.3-q24.2 Amp
chr11:76432313-81577622	5145309	11q13.5-q14.1	EMSY, GAB2, PAK1	11q13.5-q14.1 Amp
chr11:81577623-135006516	53428893	11q14.1-q25	ATM, BIRC3, DDX10, PGR, SESN3	-
chr16:1-34209948	34209947	16p13.3-p11.2	FLYWCH1, NTHL1, PDPK1, PRSS8, WDR90	-
chr16:66623882-71966375	5342493	16q21-q22.2	CDH1, CMTR2, CTCF, EXOSC6, ZFHX3	-
chr17:6459708-9569254	3109546	17p13.2-p13.1	ALOX12B, AURKB, GAS7, PER1, TP53	-
chr17:31865443-48987552	17122111	17q12-q21.33	CDK12, COL1A1, ERBB2, IKZF3, MMLT6	17q12-q21, HER2 (17q12), TOP2A (17q21.2) Amp
chr17:68783128-81195210	12412084	17q24.3-25.3	ASPSCR1, CANT1, H3F3B, SEPTIN9, SRSF2	17q23-24 Gain
chr18:17234558-61584127	44349571	18q11.1-q21.33	BCL2, PIK3C3, PMAIP1, SMAD4, SS18	-
chr18:60918069-78077248	17159179	18q21.33-q23	BCL2, KDSR, SERPINB3 and 4, TNFRSF11A	-
chr20:16154173-48577121	32422948	20q12.1-q13.13	PTPN1, SDC4, NCOA3	-
chr20:48577121-63025520	14448399	20q13.13-q13.33	GNAS, ZNF217, PTK6, SS18L1, RTEL1	-
chrX:54632203-68306116	13673913	Xq11.22-q13.1	-	-

B

CN region	size	cytoband	Top 5 altered cancer genes present in CN regions of MBC samples[1]	Common CNA in LumB primary BC[2]
chr1:12768035-19417382	6649347	1p36.22-p36.12	ID3, MTOR, PIK3CD, SDHB, SPEN	-
chr1:125418345-249250621	128941710	1q12-q44	CKS1B, ELF3, IL10, IKBKE, MUC1	1q Gain
chr5:29991567-50013540	20021973	5p13.3-q11.1	DROSHA, FGF10, IL7R, LIFR, RICTOR	-
chr5:56222548-180915260	124692712	5q11.2-q35.3	EBF1, MEF2C, PIK3R1, PLK2, SNCAIP	FGFR4 (5q35.2) Amp
chr7:135245378-152664578	17419200	7q33-q36.2	BRAF, EZH2, KEL, MGAM, PRSS1	-
chr8:1-31975155	31975154	8p23.3-p12	SECO2, GATA4, NRG1, PPP2R2A, WRN	8p Loss
chr8:31975156-146364022	114388866	8p12-q24.3	EXT1, NDRG1, RAD21, RECQL4, MYC	8p12-p11, FGFR1 (8p11.2), 8q11-q24 Amp
chr9:1-141213431	141213430	9p24.3-q34.3	EGFL7, NOTCH1, OMD, SMARCA2, TRAF2	-
chr10:77589840-81169187	3579347	10q22.2-q22.3	KAT6B	-
chr11:74369316-79024952	4655636	11q13.4-q14.1	EMSY, GAB2, INPPL1, NUMA1, PAK1	11q13-q14 Amp
chr11:79024953-135006516	55981563	11q14.1-q25	ATM, BIRC3, DDX10, PGR, SESN3	ATM (11q22.3) Loss
chr12:1-22271636	22271635	12p13.33-p12.1	CDKN1B, ETNK1, KRAS, PIK3C2G, RECQL	-
chr12:65956575-85017955	19061380	12q14.3-q21.31	FRS2, HMGA2, MDM2, PTPRB, WIF1	CDK4 (12q14), MDM2 (12q15) Amp
chr12:114767595-133851895	19084300	12q24.21-q24.33	BCL7A, CLIP1, KMT5A, KSR2, TBX3	-
chr13:1-29666256	29666255	13p13-q12.3	BRCA2, CDK8, CDX2, FLT1, FLT3	-
chr13:29666257-32748636	3082379	13q12.3-q13.1	BRCA2, FLT1, PDS5B	-
chr13:32748637-115169878	82421241	13q13.1-q34	CUL4A, ERCC5, FGF14, IRS2, RB1	13q Loss
chr14:1-30426116	30426115	14p13-q12	CCNB1IP1, PARP2, PARKD1	-
chr15:1-102531392	102531391	15p13-q26.3	CHD2,FURIN, IDH2, IGF1R, NTRK3	-
chr16:31746277-46964199	15217922	16p11.2-q11.2	FUS, MAPK3, PRSS8, ESTD1A	
chr16:46964200-90354753	43390553	16q11.2-q24.3	ANKRD11, SMTR2, CYLD, FANCA, PLCG2	16q Loss
chr17:1-21031408	21031407	17p13.3-p11.2	ALOX12B, FLCN, GAS7, MAP2K4, TP53	17p Loss
chr18:1659475-7920414	6260939	18p11.32-p11.23	YES1	-
chr18:27273239-35536059	8262820	18q12.1-q12.2	ELP2, ZNF24	-
chr18:48406902-70702106	22295204	18q21.2-q22.3	BCL2, PMAIP1, SERPINB3/4, SMAD4, TNFRSF11A	-
chr20:33212059-36319597	3107538	20q11.22-q11.23	SAMHD1, SRC	20q Gain
chr20:48577121-63025520	14448399	20q13.13-q13.33	GNAS, PTK6, RTEL1, SS18L1, ZNF217	20q13 Amp
chrX:64015184-87922181	23906997	Xq11.2-q21.31	-	-
chrX:87922181-92531641	4609460	Xq21.31-q21.32	-	-
chrX:92531642-137950017	45418375	Xq21.32-q26.3	-	-
chrX:137950018-140038498	2088480	Xq26.3-q27.1	-	-
chrX:140038499-145648978	5610479	Xq27.1-q27.3	-	-

Table 1. Regions with altered copy number per sample. Samples of patient 1 (**A**) and patient 2 (**B**) include buffy coat, primary tumour, enriched CTC, cfDNA, sorted CTC (with recovery number), and DTC from pleural fluid (only patient 1). Amplifications (red) and deletions (blue) per chromosome region with size (number of base pares) and corresponding cytoband position. [1] Data adapted from (29). [2] Data adapted from (30), table 1.

Approximately half of the CNAs found in patient 1 are homogeneously present in most of the samples (**Table 1A**). These include amplifications (or gains in pooled samples) in 1p, 8q, 11q, 16p, 17q, 20q, and deletions in 11q, 16q, and 17p. In contrast, few alterations that are present in the -time matched- primary tumour (amplification within 3q, and deletions in parts of 1p and 18q), are only subclonal in CTCs or DTCs. Others are new compared to the primary tumour, i.e. 6p21 (in cfDNA), 5q31-35 (mainly in PLF), and 18q21-23 (only in CTCs). Most of the alterations found in patient 2 are homogeneous (**Table 1B**). Still some newly detected CNAs are seen 8 years after the primary tumour was sampled. CNAs within 1p, 9p, 12p, various in chr 13, 18p, and Xq are present at a clonal level. Only a subpopulation of CTCs in pure pools, harbour 10q22, 16p11, 20q11 amplifications, which were not detectable in the enriched CTC fraction or cfDNA (31).

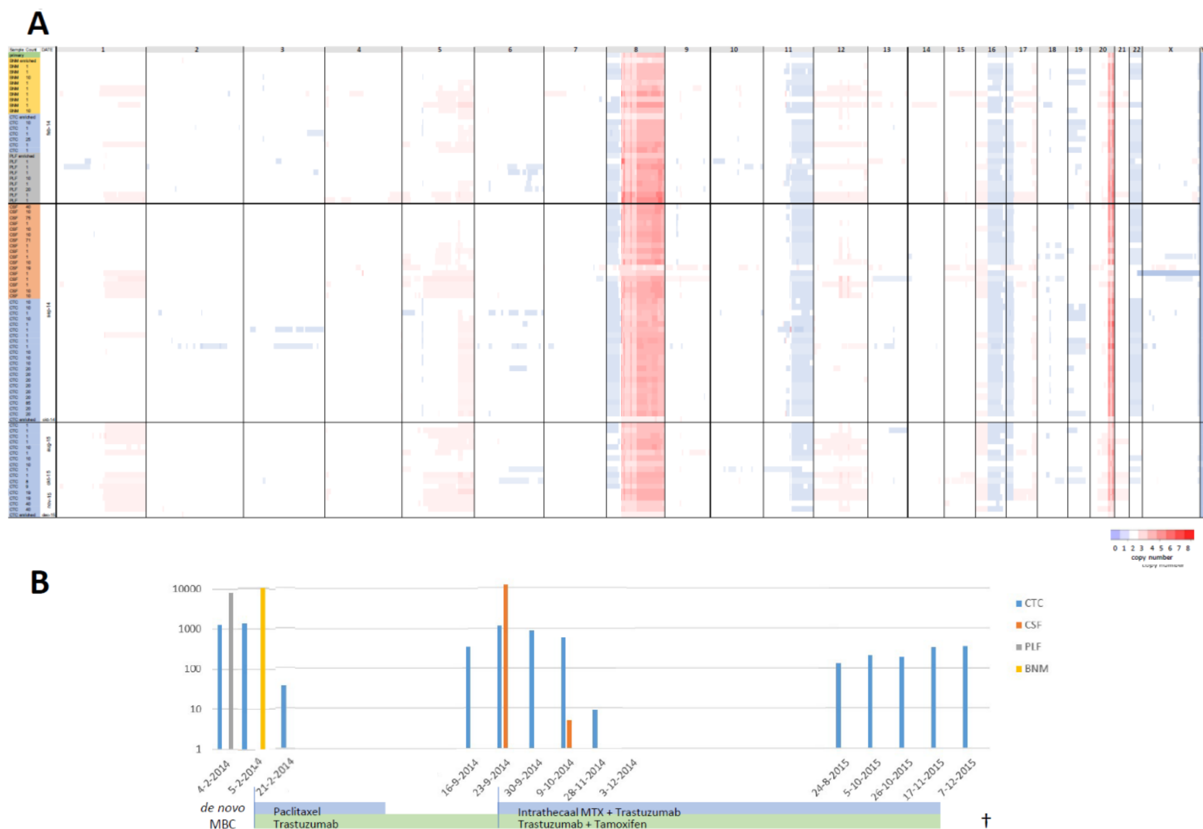


Figure 3. Heatmap of normalized read counts. Copy number (CN) profile per sample: primary tumour, CTC or DTC from bone marrow (BNM), pleural fluid (PLF), or cerebrospinal fluid (CSF) of patient 3 (**A**), taken at three phases of disease (separated by horizontal black line), i.e. at diagnosis (February 2014), first progression (September 2014) and after second progression (from August 2015). CN profiles are correct for ploidy status as calculated by Ginkgo. In general, there is a very homogeneous appearance of the copy number profile, with amplifications in chromosome 5, 8, and 20; and significant losses in 8, 11, 16, 17, and 22. Amplification with CN of ≥4 was only seen chromosomes 8 and 20. Co-occurring alterations of chromosome arms 1q, 5q, parts of 12q, and 16p were initially predominantly present in the bone marrow, then emerging in the cerebrospinal fluid, to only become dominant in the CTC compartment after second progression. **B**: Details on CTC count per 7,5ml (3ml for CSF) and therapy choice per timepoint (MTX: methotrexate).

Timeline studies

From patient 3 we were able to extensively collect samples from multiple anatomical sites and at various time points (**Figure 3B**). This patient presented with *de novo* metastatic disease, that was initially thought to be ER+HER2+, though with current ASCO/CAP guidelines, nowadays this patient would be classified as group 2 HER2 negative. At baseline we collected tumour cells from the blood, pleural fluid and bone marrow. After the first administration of paclitaxel and trastuzumab the CTC count dropped from approximately 1000 to 50 cells per 7.5 ml of blood. Subsequent samples were taken at time of progression under this treatment, and showed again high CTC count. At that point, leptomeningeal metastasis was diagnosed, and high DCT numbers up to 10,000 tumour cells were isolated from 3 ml of cerebrospinal fluid (CSF, or liquor). Systemic therapy was switched to trastuzumab plus tamoxifen (due to general condition and age) and intrathecal therapy with methotrexate and trastuzumab was started as well. Within two weeks the numbers of CTC and DTC from CSF dropped below ten cells/3 ml, and subsequently we were not able to capture any DTC again. Final CTC sampling occurred at time of progression. **Figure 3A** demonstrates the heatmap of CN profiles from CTC and DTC of patient 3, separated in the three timepoints of diagnose and respectively the first and second episode of progressive disease. In general there is a very homogeneous appearance of the copy number profile. Alterations seen in the primary tumour are present in all liquid biopsy samples, with prominent aneuploidy of chromosome arm 8q (copy number counts of 4 to 10 in all samples but WBC) and parts of 20q, as well as deletions in 8p, 11q, 16q, 17p, and 22. Additional amplifications with CN of 3-4 are seen in 1q, 5q, 12, and 16p, in part of the CTC and DTC samples. This specific signature, present in half of the CSF DTCs, is predominantly found in the BNM compartment a few months before, and only becomes dominant in the CTC compartment at second progression (**Figure 3A**).

Mutational profiling by targeted AmpliSeq

In all three patients many variants were detected, the most being unique variants present in only one sample (**Figure S1)**. After filtering and selection of variants present in multiple samples, a *PIK3CA* mutation was found in both patient 1 and 2 (**Figure 4A**). No clonal pathogenic mutations according to the OncoKB^{TM}(32) were detected in samples of patient 3, though two samples contain a probably damaging *TP53* G361W mutation. Patient 1 harboured a *PIK3CA* p.H1047R mutation in the primary tumour, which is homogeneously present in 13/14 (93%) of CTC and DTC samples in various variant allele frequencies (VAF). The *PIK3CA* p.E542K variant found in patient 2 was heterozygously present in almost all CTC samples, but not in the enriched bone marrow fraction. In total we found 33, 21, and 93 unique exon variants over all samples of patients 1 to 3, respectively (**Figure S1B**). After further selection for presence COSMIC and/or annotation in PolyPhen(33), remaining unique variants are depicted in **Figure 4B**. Variant allele frequencies (VAF) are mostly ~50% (heterozygous), or lower in pooled samples, though some variants have VAFs of 100%, almost solely present in single cells, at least partly due to ADO (as ADO rate was 30% in single cells, **Table S1**).

When combining the copy number and mutational data we found heterogeneity in ploidy status in patient 1 (**Figure 5**). The copy number of the *PIK3CA* gene was reflected in variant allele frequency (VAF) of the *PIK3CA* H1047R mutation. This mutation was found to be heterozygous level in six CTC and DTC samples, varying between VAF of 66-75% (i.e. 2 mutant copies while triploid, and 3 mutant copies while tetraploid). In five other samples, the H1047R mutation was found to be homozygous, mostly with a copy number of two, although one single CTC harboured 2 normal and 2 mutated copies. Two single cells had VAF of 0 and 100, which can caused by either loss of heterozygosity or allelic drop out. The VAF of the *PIK3CA* H1047R mutation was 30% in the primary tumour, which had a tumour cell

A

PATIENT 1	Hotspot PIK3CA
Normal tissue	0
WBC 20	0
Primary tumor	30
CTC 1	47
CTC 1	100
CTC 1	0
CTC 1	76
CTC 1	57
CTC 1	54
CTC 1	59
CTC 1	50
CTC 1	21
CTC 1	77
CTC 10	61
CTC 10	64
CTC 5	76
CTC 10	46
aa effect	H1047R
polyphen	poss.dam
annotation	rs121913279, COSM775, COSM94986

PATIENT 2	Hotspot PIK3CA	Novel RET	Novel RET
Normal tissue	0	0	3
WBC 8	0	0	
Enriched CTC	30	0	0
Enriched BNM	0	100	
CTC 1	0	40	0
CTC 1	47		
CTC 1	42		
CTC 1	79		
CTC 10	52	0	0
CTC 10	57	0	0
CTC 10	37		29
CTC 10	46		
CTC 10	51	0	0
CTC 10	55		
CTC 10	59		
CTC 10	56		0
CTC 10	52	0	13
CTC 10	53		
CTC 10	32		
CTC 10	47		
aa effect	E542K	L769L	L870H
polyphen	prob.dam	-	prob.dam
annotation	rs121913273, COSM760, COSM125369	rs1800861	-

PATIENT 3	Novel TP53
WBC singels (x9)	0
WBC pools (x4)	0
Buffy coat	
Primary tumor	
CTC singles (x3)	
CTC singles (x21)	0
CTC pools (x23)	0
CTC 10	18
Enriched CTC	
BNM singles (x8)	
BNM pools (x2)	
Enriched BNM	
PLF singles (x7)	
PLF pools (x2)	
Enriched PLF	
CSF singles (x7)	0
CSF pools (x12)	0
CSF 10	15
aa effect	G361W
polyphen	prob.dam
annotation	-

Covered<20X

WT 0% VAF 100%

B

PATIENT 1	Hotspot FLT3	Hotspot TP53	Hotspot TP53	Hotspot TP53	Novel EGFR	Novel KDR	Novel KIT	Novel PIK3CA	Novel TP53
Normal tissue	0	0	0	0	0	0	0	0	0
WBC 20	0	0	0	0	0	0	0	0	0
Primary tumor	0	0	0	0	0	0	0	0	0
CTC 1	0	0	0	100	0	0	15	0	0
CTC 1	0	100	0		0	0	0	0	0
CTC 1		0	0			0	0	50	0
CTC 10		0	0		19	0		0	0
CTC 10	0	0	0		0	10	43	0	0
CTC 5	0	0	0		0	0		0	0
CTC 10	10	0	15		0	0	0	0	26
aa effect	D835Y	Q192*	V216A	R342*	A839T	A1168S	Y343C	Y343C	P219H
polyphen	prob.dam	-	prob.dam	-	prob.dam	prob.dam	benign	benign	prob.dam
annotation	FLT3_g.53109G>T, COSM783	TP53_g.12643C>T, COSM10733, COSM117946, COSM117949, COSM3370929, COSM117947, COSM1649390, COSM117948	TP53_g.12716T>C, COSM44567	TP53_g.16915C>T, COSM11073, COSM99721	COSM13430	COSM321154	COSM21436	COSM21436	TP53_g.12725C>A, COSM46215

PATIENT 2	Novel RET	Hotspot CDKN2A	Hotspot PDGFRA	Novel CDKN2A
Normal tissue	0	0	0	0
WBC 8	37	0	0	0
Profile CTC	0	0	0	0
Profile BNM	0	0	48	0
CTC 10		60	0	13
aa effect	N763K	V82M	V824V	E69G
polyphen	prob.dam	prob.dam	-	poss.dam
annotation	COSM1505474	COSM12469, COSM3906642, COSM3906644, COSM3906643	rs2228230, COSM22413	rs372670098

PATIENT 3	Hotspot CTNNB1	Hotspot PDGFRA	Hotspot TP53	Hotspot TP53	Novel EGFR	Novel ERBB2	Novel FGFR2	Novel IDH1	Novel KIT	Novel KIT	Novel KIT	Novel PDGFRA	Novel PTEN	Novel PTPN11	Novel TP53	Novel TP53	Novel TP53	Novel TP53	Novel TP53	Novel VHL
WBC 1	0	0	0	0		0	0		0			0		0		40	0	0	0	
WBC 1	0		0						33	3	0	0				0	0	0	0	
WBC 10		0						0	5				0	0	22			0		0
WBC 14	0	0	0	0	0	0	0		0	13	0	0		0		0	0	0	0	
Buffy coat		0						0	0				0	0	0			0		0
Primary tumor		0						0	0				0	0	0			0		0
CTC 1		35						0	0					0	0			0		0
CTC 1		0						0	0				0	30						0
CTC 1		0						0	0				0	0	0			69		
CTC 1		0				0	0		0	0	0			11						
CTC 1	0	0		0	0	0	0		0	0	74	0		0				0	0	
CTC 1	0	0	0	0	0	100	0		0	0	0	0		0		0	0	0	3	
CTC 1	0	0	0	0	0		98		0	0	0	0		0		0	100		0	
CTC 10		0						17	0				0	0	0			0		83
CTC 10	0	0	0	0	0	0	0		0	0	0	0		0		0	0	0	12	
CTC 10	15	0	29	0	81	0	0		0	0	0	0		0		0	0	0	0	
BNM 1		0							0				100	0	0			0		
CSF 1	0	0	0	31	0	0	0		0	0	0	0		0		0	0			
CSF 40	0	0		0		0	0		0	0	0	14		0					0	
aa effect	A13T	V824V	S166*	E294*	Q787Q	R840W	P263L	R119W	G856G	H697H	A698S	Y676H	K6K	R498L	P72X	P151S	P222P	R110S	A364T	G93V
polyphen	benign	-	-	-	-	prob.dam	poss.dam	poss.dam	-	-	benign	poss.dam	-	prob.dam	-	poss.dam	-	prob.dam	benign	prob.dam
annotation	rs121913394, COSM5747	rs2228230, COSM22413	TP53_g12485C>A, COSM11508	TP53_g13860G>T, COSM10856, COSM126981, COSM3388169, COSM2744501	rs1050171, COSM1451600	rs369828131	rs779326224	COSM1015578	COSM1056111	COSM1430169	rs756394678	rs747598419	COSM1349460	rs397507542	COSM18569, COSM1268331, COSM1268332	rs28934874, TP53_g12439C>T, COSM10905, COSM984959, COSM984961, COSM984963, COSM3378358, COSM1640859,	rs72661118, TP53_g.12735G>A, COSM46134	TP53_g.11559C>A, COSM45604	TP53_g.16981G>A, COSM46361	COSM3364920

Covered<20X

WT 0% VAF 100%

Figure 4 A. Mutation analysis. Pathogenic mutations with variant allele frequency (VAF), detected per sample: tissue, white blood cell (WBC), circulating or disseminated tumour cells (CTC or DTC) from pleural fluid (PLF). Both patients harbour a different PIK3CA mutation generally present in all samples,

but 1 single CTC. **B**. Unique variants per sample. After filtering as stated in the method section and further selection of exon variants described previously in COSMIC or as reference single nucleotide polymorphism (SNP) with rsID. Mostly variant allele frequencies (VAF) are heterozygous or lower in pooled samples, though some. None are present in normal tissue or sorted WBC, nor in the primary tumour. Abbreviations: BNM, bone marrow; CSF, cerebrospinal fluid; PLF, pleural fluid; aa, amino acid; prob.dam, probably damaging; poss.dam, possibly damaging.

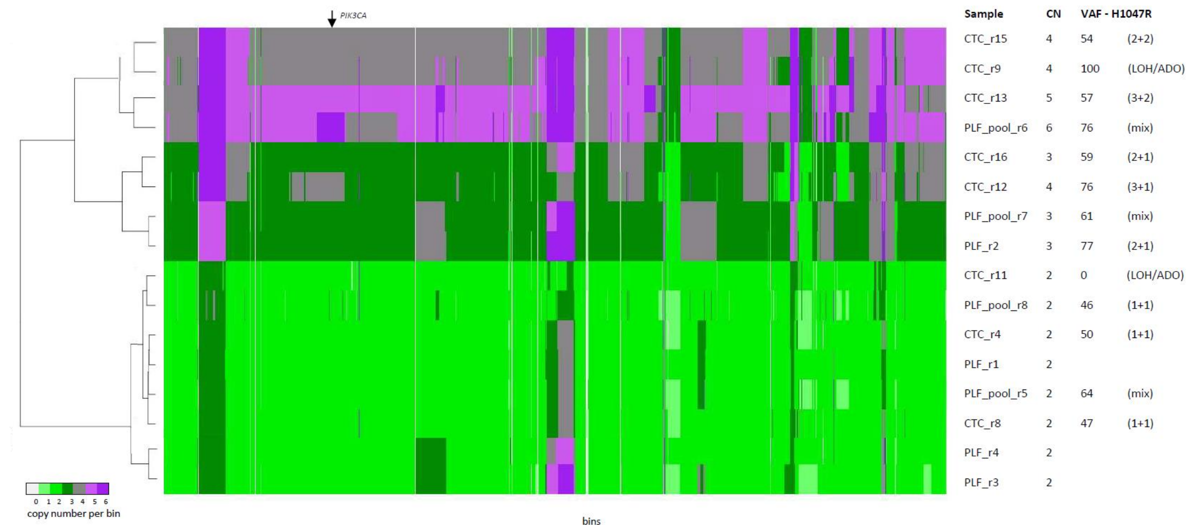


Figure 5. Heatmap of copy number values across all segment breakpoints using Euclidian distance metric. Correlation between average ploidy status based on CN profile (light green:2, dark green: 3, shades of purple: 4-6) and variant allele frequency (VAF) of the PIK3CA H1047R mutation, with suspected number of mutant or wild type alleles (example: 3+1). LOH: Loss of heterozygosity, ADO: Allelic drop out.

content of approximately 70%. Similar results were found for patient 2 with regards to the *PIK3CA* E542K mutation (**Figure S4**). In this patient the majority of samples were pure pools with VAFs of the mutation mostly around 50% and a mean ploidy status that was diploid or triploid. In the single CTCs the E542K VAF matches the ploidy status that was diploid (47%) or tetraploid (42% and 79% resp.) (**Figure 4A, Figure S4**). Balanced amplification of both alleles as well as preferential amplification of the mutant allele have been described for *PIK3CA* (34).

DISCUSSION

CTCs are frequently studied as part of the metastatic cascade in the early stages of breast cancer, trying to identify those cells that are able to intravasate into the blood stream and subsequently extravasate to form micro- and macrometastasis. These studies focus on phenotypic changes like epithelial-to-mesenchymal transition and CTC clusters (35). Contrarily, CTCs can be studied in overt metastatic disease, where shedding of these cell potentially represent all different tumour sites, justifying the use of EpCAM and cytokeratin for selection of CTCs (36). With this, there is a risk of finding tumour DNA in sorted WBC, as hybrid cells (CTC-macrophage) have been described (37, 38). In this study we aimed to extensively look into the CTC and DTC profile of three patients with a high CTC count, to investigate spatiotemporal heterogeneity, using both copy number and mutational profiles.

We found an overall intra-patient homogeneity in the CNA profile, involving copy number gain of the long arm of chromosome 8 in all three patient samples, as well as amplifications in 1q and deletions in chromosome arms 11q, 16q and 17p. Amplification of 8q, especially 8q21 and 8q24, is the most common CNA in breast cancer, and is associated with tumour progression and chemoresistance (39, 40), and is often present with high copy numbers (41). Besides activation of oncogenes, inactivation of tumour suppressor genes is key in cancer development. The latter can occur when one allele is mutated in combination with loss of heterozygosity (LOH) of a chromosomal segment containing the wild-type allele (42, 43), or with loss of both wild-type alleles (homozygous deletion). Deletions at chromosome 11q and 17p, with loss of important tumour suppressor genes like *TP53* on 17p13, are known to be present in early stages of breast cancer, and are associated with relapse and worse clinical outcome (44, 45). Also similar to our data, co-occurrence of 1q-gain and 16q-loss is a frequent cytogenetic abnormality in breast cancer. Transcriptome and functional pathway analysis suggested cooperation of overexpressed 1q genes and underexpressed 16q genes in the genesis of both ductal and lobular carcinomas (46). These data support our findings of 1q, 8q, 11q, 16q and 17p alterations to be present in all samples including the primary tumour.

This general homogeneity is coherent with time of sampling, i.e. end stage metastatic disease. Compared to genetic heterogeneity in DTCs in early breast cancer (47), CTCs in the metastatic setting are often homogeneous, as fit and aggressive subclones become dominant either primarily or due to selective pressure from therapeutic interventions (35, 48, 49, 50). A continuous escape from treatment is expected and evolution can continue from reservoirs of minor subclones (51). Following subclones during the entire course of disease therefor seems sensible.

CNA in liquid biopsies can be used to identify breast cancer subtypes, as ER+ tumours are known to harbour 1q gain and 11q and 16q losses, compared to ER- HER2+ and TN subtypes. Moreover, in patient 1 (ER+ HER2+) we detected 17q, including *ERBB2*, and 20q amplification typical for HER2+ tumours, and not arm 16q deletion as this alteration matches HER2- subtypes (52). The opposite was homogeneously found in the CN profile of patient 2 (ER+ HER2-). Interestingly, in patient 3 (initially diagnosed as ER+ HER2+) we were not able to detect amplification of the 20q, 17q, or even the locus containing *ERBB2*, while we found a general 16q loss in all samples, including the primary tumour. Archival immune histochemistry (IHC) results were 2+ or equivocal, showing only weak to moderate HER2 expression. Initially the in situ hybridisation (ISH) result was reported as being positive with a HER2/CEN17 ratio of 2.4. Though with the changed ASCO/CAP guidelines, nowadays this patient would be classified as group 2 HER2 negative (3.85 HER2 signals/nucleus) (53, 54). We have shown previously that the majority of CTCs from this patient express HER2 at low or intermediate levels, while only few CTCs were actually HER2+ (14). This patient progressed within 7 months on trastuzumab, emphasising the need for regular re-evaluating tumour characteristics during the course of the disease, as is shown possible with liquid biopsies. Moreover, various additional amplifications were seen in 1q, 5q, 12, and 16p, in part of the CTC and DTC samples of patient 3, stressing the aggressive nature, possibly even subtype switch, of the metastatic disease in this patient that was ER+ on primary tumour biopsy (52), as chromosome 1q and 12p amplifications are associated with TNBC/basal-like breast cancer and poor survival (55, 56). These co-occurring alterations, absent in the primary tumour, were initially predominantly present in the bone marrow, then emerging in the cerebrospinal fluid, to only become dominant in the CTC compartment after second progression. Studies have shown early bone marrow involvement functioning as a reservoir, before overt metastatic disease (47, 57). New CNAs may occur as a consequence of clonal evolution, or were present as minor subclones not detected in the primary tumour due to insufficient sequencing depth or spatial heterogeneity (58).

Since two decades large scale sequencing studies have revealed the landscapes of somatic mutations in tumours. For example, when sequencing over 13 thousand genes of 11 breast tumours, 519 harboured a mutation (59). Notwithstanding only a few mutational events affect driver genes that play important roles in tumorigenesis. Large tumour sequencing studies such as The Cancer Genome Atlas (TCGA)(60) and the International Cancer Genome Consortium (ICGC)(61) contributed to defining a list of driver genes (62, 63). Ongoing efforts are now revealing the landscape of driver alterations in metastatic disease (64, 65, 66). In a comprehensive study, 568 cancer driver genes were identified through a large-scale transcriptome analysis (66). They showed an extensive heterogeneity in mutation burden within the 2711 breast cancer samples. From these, 99 driver genes were extracted. Most of them not annotated for breast cancer before, stressing the importance including patients in trials during the full treatment trajectory. We detected a known *PIK3CA* mutation in the primary tumour and all other samples of both patient 1 and 2, common in ER+ breast cancer (67, 68). However, no clonal drivers were found in samples of patient 3. Minor subclones within the expected homogeneous liquid biopsy landscape during overt metastatic disease, could be detected by sequencing many pure pools of CTC and DTC. Power analysis showed that a 1% subclone can only be detected when sequencing 10 groups of 20 CTCs, although there is a 90% chance of detecting a 5% subclone in only 5 pools of 10 CTC (69). The *TP53* gene was frequently mutated in all three patients at a subclonal level (**Figure 4B**), as were genes encoding for growth factor receptors within unique samples, like *FGFR2* in a single CTC of patient 3.

This subclonal evolution was also noted based on CNA profiles. Besides the general homogeneity as described above, there were few newly acquired CNA, mostly amplifications, in the liquid biopsy samples compared to the primary tumours. For patient 1, gain in region 5q31-35 was only seen in two CTC samples and almost all DTC from pleural fluid, though not detected in the enriched CTC or cfDNA fractions. This region includes a *FGFR4* amplification which is associated with endocrine therapy and CDK4/6 inhibitor resistance (70). Currently over 20 trials are registered on clinicaltrials.gov using FGFR inhibitors alone or in combination with endocrine therapy. In patient 2, we noticed new 10q22 gain and 16p11 amplification (31) present in CTC samples. Furthermore, co-occurring alterations in chromosome arms 1q, 5q, 12q, and 16p in patient 3 developed over time.

Multiple spatial biopsies of the primary tumour helps capturing heterogeneity in CNA and mutations, though some variation is only captured in the single-cell data from primary breast tumours (71). Still, advanced disease has shown differences compared to local breast cancer, even in *de novo* metastatic breast cancer. Repeated liquid biopsies, preferably a combination of both enriched CTC fractions and cfDNA can trace these outgrowing and changing clones, to help therapy decision making.

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Author contributions
A.B. and L.D. conceived, designed and supervised the study. A.B., B.D.L. contributed to develop the methodology. A.B., B.D.L., PJ.V.D. and L.D. contributed to sample acquisition and provided technical support. A.B., B.D.L, S.V.L, and L.D. analyzed and interpreted the data. A.B. wrote the first draft of the paper which was edited and approved by all authors.

Data availability
The dataset is available on request by emailing the corresponding author.

Ethics approval and consent to participate
The ethical committee of the Antwerp University Hospital (UZA) and University of Antwerp (UA) approved this non-interventional study (UA A11-18). Written informed consent was obtained from all patients. The study was performed in accordance with the Declaration of Helsinki.

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Competing interests
The authors do not have any competing financial and/or non-financial interests in relation to the work described.

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TABLE S1

A

Patient 1 (11 SNPs)		Patient 2 (12 SNPs)		Patient 3 (12 SNPs)									
WBC pool	0,00%	WBC pool	8,33%	CTC1	27,27%	BNM1	10,00%	CTC pool1	10,00%	BNMpool1	0,00%	WBC1	25,00%
Primary tumor	0,00%	Profile CTC	0,00%	CTC2	30,00%	BNM2	37,50%	CTC pool2	9,09%	BNMpool2	10,00%	WBC2	55,56%
		Profile BNM	25,00%	CTC3	36,36%	BNM3	20,00%	CTC pool3	9,09%	PLF pool1	10,00%	WBC3	25,00%
CTC1	10,00%			CTC4	40,00%	BNM4	33,33%	CTC pool4	9,09%	PLF pool2	10,00%	WBC4	27,27%
CTC2	30,00%	CTC1	63,64%	CTC5	30,00%	BNM5	14,29%	CTC pool5	18,18%	CSF pool1	9,09%	WBC5	60,00%
CTC3	18,18%	CTC2	44,44%	CTC6	10,00%	BNM6	42,86%	CTC pool6	9,09%	CSF pool2	9,09%	WBC6	45,45%
CTC4	40,00%	CTC3	50,00%	CTC7	36,36%	BNM7	0,00%	CTC pool7	18,18%	CSF pool3	9,09%	WBC7	10,00%
CTC5	10,00%	CTC4	71,43%	CTC8	20,00%	BNM8	NA	CTC pool8	0,00%	CSF pool4	9,09%	WBC8	18,18%
CTC6	18,18%	**Average**	**57,38%**	CTC9	45,45%	PLF1	14,29%	CTC pool9	9,09%	CSF pool5	9,09%	WBC9	27,27%
CTC7	18,18%			CTC10	9,09%	PLF2	66,67%	CTC pool10	9,09%	CSF pool6	9,09%	**Average**	**32,64%**
CTC8	36,36%	CTC pool1	16,67%	CTC11	50,00%	PLF3	22,22%	CTC pool11	9,09%	CSF pool7	9,09%		
CTC9	27,27%	CTC pool2	16,67%	CTC12	33,33%	PLF4	25,00%	CTC pool12	18,18%	CSF pool8	9,09%	WBC pool1	0,00%
CTC10	55,56%	CTC pool3	8,33%	CTC13	9,09%	PLF5	66,67%	CTC pool13	9,09%	CSF pool9	20,00%	WBC pool2	9,09%
Average	**26,37%**	CTC pool4	16,67%	CTC14	77,78%	PLF6	36,36%	CTC pool14	9,09%	CSF pool10	NA	WBC pool3	0,00%
		CTC pool5	8,33%	CTC15	33,33%	PLF7	NA	CTC pool15	9,09%	CSF pool11	NA	WBC pool4	0,00%
CTC pool1	20,00%	CTC pool6	16,67%	CTC16	36,36%	CSF1	18,18%	CTC pool16	9,09%	CSF pool12	9,09%	**Average**	**2,27%**
CTC pool2	18,18%	CTC pool7	16,67%	CTC17	18,18%	CSF2	40,00%	CTC pool17	8,33%	CSF pool13	9,09%		
CTC pool3	18,18%	CTC pool8	16,67%	CTC18	10,00%	CSF3	20,00%	CTC pool18	9,09%	**Average**	**9,39%**	Primary tumor	8,33%
CTC pool4	18,18%	CTC pool9	16,67%	CTC19	18,18%	CSF4	33,33%	CTC pool19	0,00%			Profile CTC	0,00%
Average	**18,64%**	CTC pool10	8,33%	CTC20	18,18%	CSF5	20,00%	CTC pool20	9,09%			Profile BNM	0,00%
		CTC pool11	8,33%	CTC21	33,33%	CSF6	9,09%	CTC pool21	9,09%			Profile PLF	0,00%
		CTC pool12	16,67%	CTC22	33,33%	CSF7	50,00%	CTC pool22	9,09%				
		Average	**13,89%**	CTC23	9,09%	**Average**	**28,99%**	CTC pool23	0,00%				
				CTC24	9,09%			CTC pool24	0,00%				
				Average	**28,08%**			**Average**	**8,72%**				

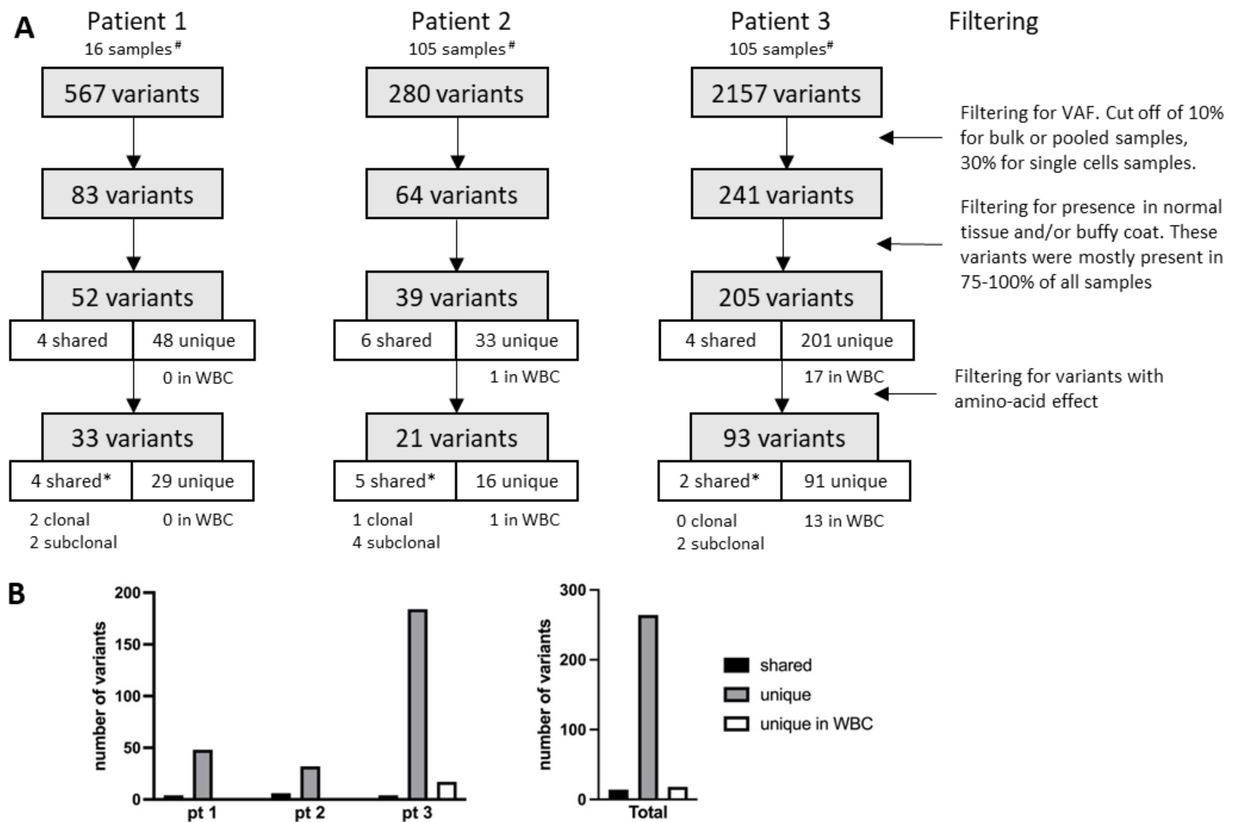
Single Cells		Sorted pools	
ADO	30,85%	ADO	9,98%
SEM	17,73%	SEM	5,62%

B

Patient 1 (501 events)		Patient 2 (250 events)		Patient 3 (862 events)									
WBC pool	0,00%	WBC pool	1,20%	CTC1	2,09%	BNM1	0,58%	CTC pool1	1,04%	BNMpool1	0,23%	WBC1	0,12%
Primary tumor	0,40%	Profile CTC	0,80%	CTC2	1,74%	BNM2	1,51%	CTC pool2	0,81%	BNMpool2	0,23%	WBC2	0,58%
		Profile BNM	1,60%	CTC3	1,86%	BNM3	0,70%	CTC pool3	0,23%	PLF pool1	0,23%	WBC3	0,23%
CTC1	0,60%			CTC4	2,67%	BNM4	0,70%	CTC pool4	0,12%	PLF pool2	0,00%	WBC4	0,23%
CTC2	0,80%	CTC1	2,80%	CTC5	2,55%	BNM5	0,81%	CTC pool5	0,23%	CSF pool1	0,12%	WBC5	0,35%
CTC3	0,00%	CTC2	2,40%	CTC6	1,74%	BNM6	1,04%	CTC pool6	0,23%	CSF pool2	0,23%	WBC6	0,23%
CTC4	0,40%	CTC3	2,80%	CTC7	1,74%	BNM7	0,81%	CTC pool7	0,23%	CSF pool3	0,12%	WBC7	0,23%
CTC5	0,40%	CTC4	2,00%	CTC8	1,51%	BNM8	0,58%	CTC pool8	0,23%	CSF pool4	0,00%	WBC8	0,23%
CTC6	0,40%	**Average**	**2,50%**	CTC9	0,23%	PLF1	0,35%	CTC pool9	0,12%	CSF pool5	0,00%	WBC9	0,23%
CTC7	0,80%			CTC10	0,23%	PLF2	0,23%	CTC pool10	0,00%	CSF pool6	0,23%	**Average**	**0,27%**
CTC8	0,40%	CTC pool1	1,60%	CTC11	0,35%	PLF3	1,04%	CTC pool11	0,12%	CSF pool7	0,23%		
CTC9	0,20%	CTC pool2	0,80%	CTC12	0,35%	PLF4	0,35%	CTC pool12	0,12%	CSF pool8	0,12%	WBC pool1	0,58%
CTC10	0,80%	CTC pool3	2,00%	CTC13	0,35%	PLF5	0,35%	CTC pool13	0,23%	CSF pool9	0,23%	WBC pool2	0,35%
Average	**0,48%**	CTC pool4	1,60%	CTC14	0,46%	PLF6	0,23%	CTC pool14	0,23%	CSF pool10	0,23%	WBC pool3	0,23%
		CTC pool5	1,20%	CTC15	0,12%	PLF7	0,46%	CTC pool15	0,12%	CSF pool11	0,00%	WBC pool4	0,23%
CTC pool1	2,20%	CTC pool6	0,80%	CTC16	0,23%	CSF1	0,35%	CTC pool16	0,23%	CSF pool12	0,00%	**Average**	**0,35%**
CTC pool2	2,20%	CTC pool7	2,80%	CTC17	0,12%	CSF2	0,23%	CTC pool17	0,12%	CSF pool13	0,00%		
CTC pool3	2,00%	CTC pool8	1,60%	CTC18	0,35%	CSF3	0,23%	CTC pool18	0,23%	**Average**	**0,13%**	Primary tumor	0,00%
CTC pool4	2,00%	CTC pool9	2,00%	CTC19	0,12%	CSF4	0,12%	CTC pool19	0,00%			Profile CTC	0,12%
Average	**2,10%**	CTC pool10	0,80%	CTC20	0,23%	CSF5	0,23%	CTC pool20	0,12%			Profile BNM	0,00%
		CTC pool11	0,80%	CTC21	0,12%	CSF6	0,23%	CTC pool21	0,12%			Profile PLF	0,12%
		CTC pool12	2,00%	CTC22	0,23%	CSF7	0,23%	CTC pool22	0,23%				
		Average	**1,50%**	CTC23	0,12%	**Average**	**0,52%**	CTC pool23	0,00%				
				CTC24	0,00%			CTC pool24	0,23%				
				Average	**0,81%**			**Average**	**0,22%**			TOTAL FPR	0,63%
												SEM	0,73%

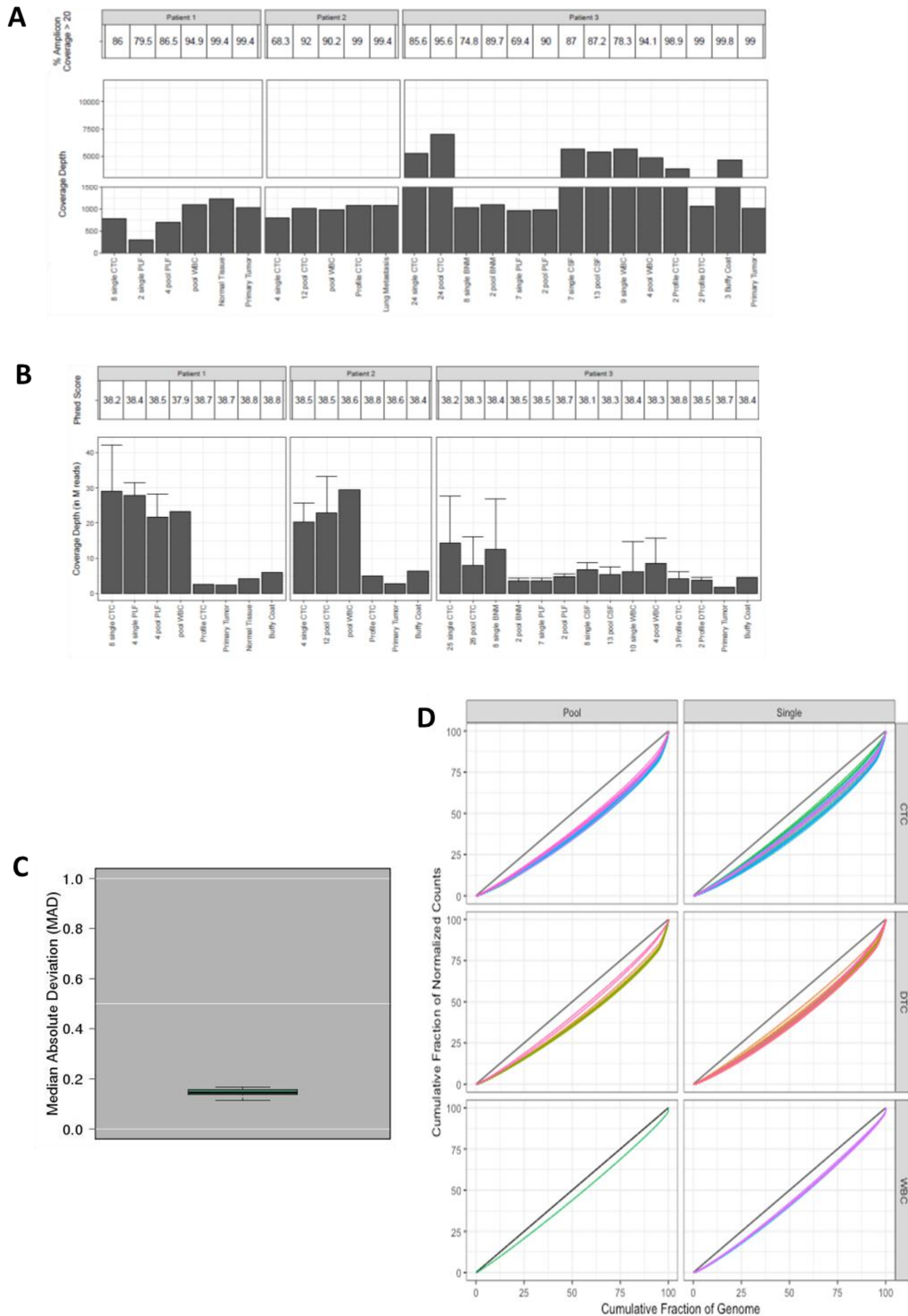
Supplementary table 1. Quality control of Ion Torrent targeted sequencing. A. Allelic dropout (ADO) per sample, per patient. The ADO across individual CTCs and WBCs was 30.85% (SEM 17.73%) and for sorted pools 9.98% (SEM 5.62%). **B.** False positive rate (FPR) per sample, per patient. The mean FPR was 0.63% (SEM 0.73%).

FIGURE S1



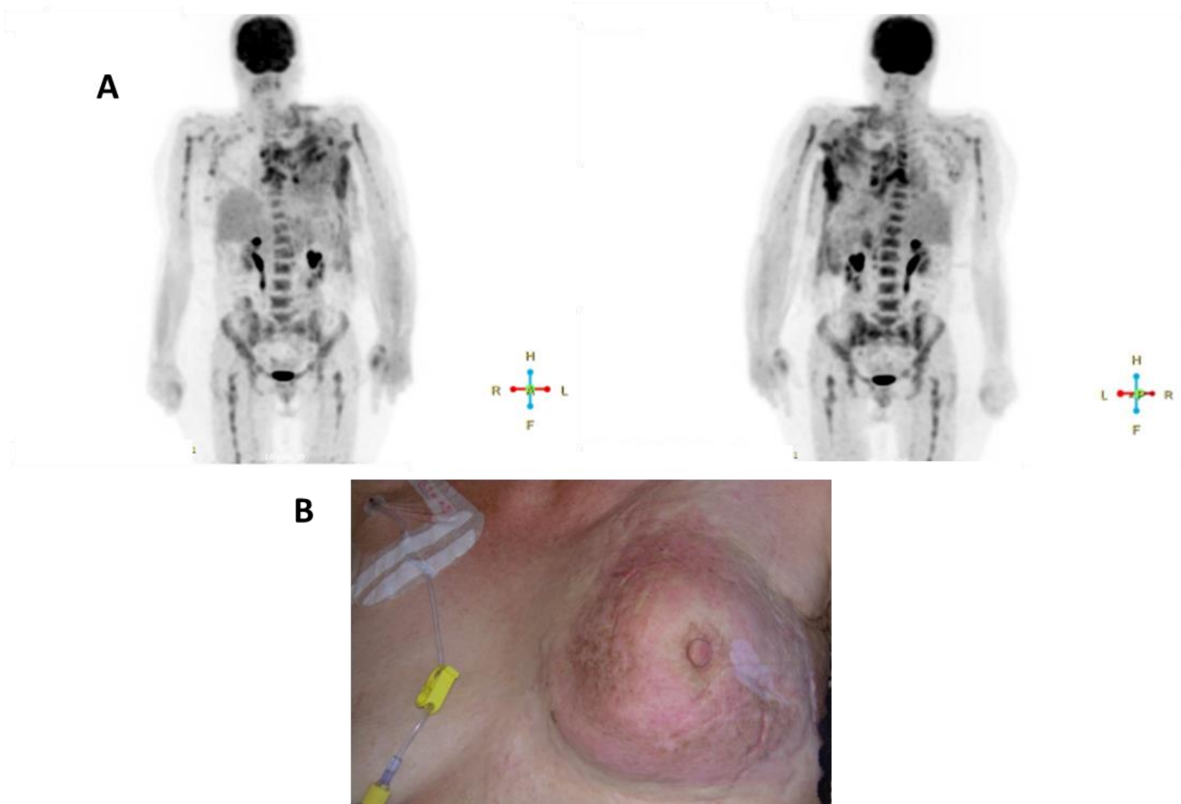
Supplementary figure 1. Filtering of single nucleotide variant (SNV) after prior quality control. **A** Number of variants found after processing as described in method section. Further filtering based on variant allele frequencies (VAF), subsequent filtering for false positive variants present in normal bulk samples (tissue or buffy coats), and finally filtering for variants that possibly can have an effect on the amino-acid. Resulting in 33 variants (4 shared*) for patient 1, 21 variants (5 shared*) for patient 2 and 93 variants (2 shared*) for patient 3. # Samples excluding bulk normals * Variants present in multiple samples, none were present in WBC samples. **B** Number of variants per patient present in multiple samples (black), unique samples (grey) or unique sorted WBC samples (white), after filtering for presence in normal tissue and/or buffy coat.

FIGURE S2



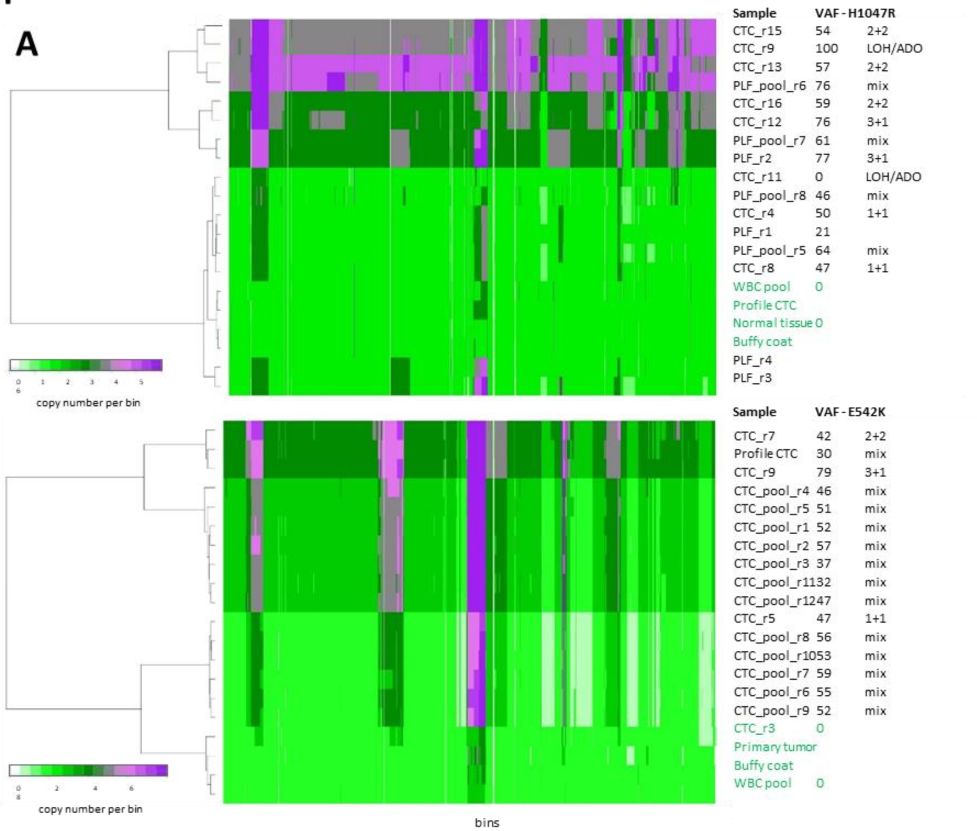
Supplementary figure 2. Quality control data of Ion Torrent targeted sequencing data (A) and Illumina shallow WGS (B-D)**. A.** Average read depth and width per sample type, per patient. **B.** Average coverage depth and Phred scores per sample type, per patient **C.** Overall Median Absolute Deviation (MAD) score of neighbouring bins **D.** Lorenz curves displaying coverage uniformity per sample type.

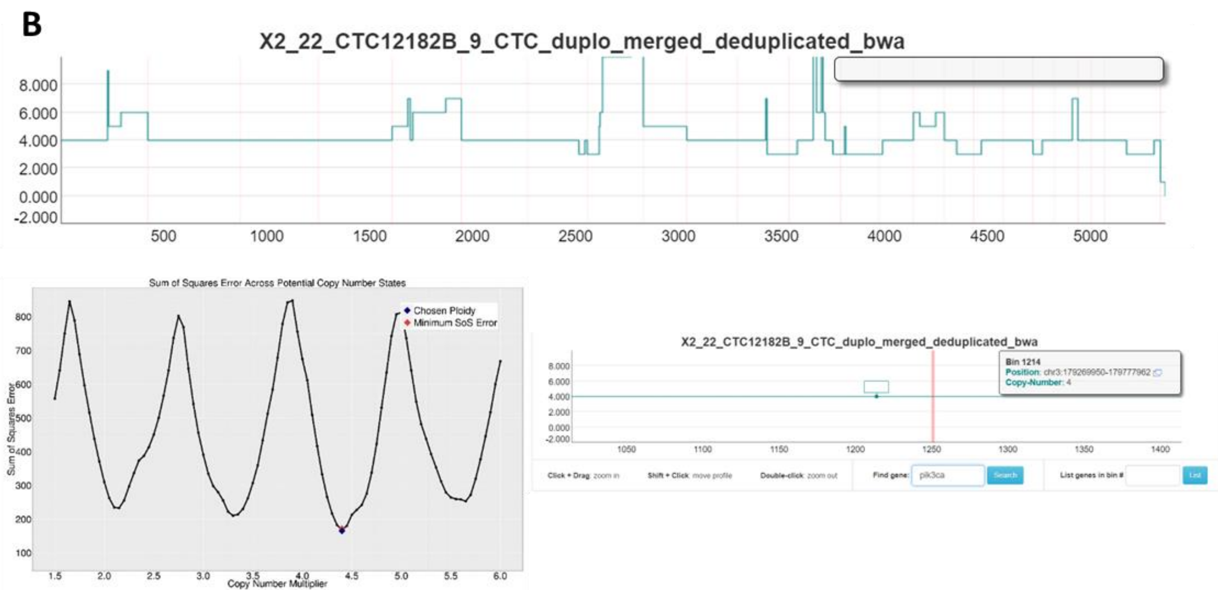
FIGURE S3



Supplementary Figure 3. Images of patient 3 at time of diagnosis. A. PET images of anterior (left) and posterior (right). Conclusion based on PET and CT: diffuse bone and probably also bone marrow metastasis. Multiple mediastinal, hilar, and bilateral (L more than R) axillary pathological lymph nodes. Extensive tumour infiltration in the left breast with central ulceration and skin invasion. Diffuse infiltration at the base of the left breast with extension towards the left lateral thorax and invasion of the pectoralis muscle. Left sided pleural fluid with subsequent compression atelectasis of the complete left lower lobe and part of het left upper lobe. No obvious liver involvement. **B.** Picture taken from the left breast showing mastitis carcinomatosa due to extensive tumour infiltration.

FIGURE S4





Supplementary figure 4A. Heatmap of copy number values across all segment breakpoints using Euclidian distance metric. Correlation between average ploidy status, based on CN profile, and variant allele frequency (VAF) of the PIK3CA mutation. In both patients some samples have a mean ploidy of 3 or 4 (darker greens and purple). Variant allele frequencies of the PIK3CA mutations point towards similar ploidy status as CNA data. **B.** Copy number profile and ploidy calculation of single CTC recovery 9 from patient 2, showing a mean ploidy of four.

SUPPLEMENTARY METHODS

Patients and samples

Patients were recruited after giving written informed consent at the Oncology Centre of GZA Hospitals Sint-Augustinus (Antwerp, Belgium). Patient 1 was diagnosed with *de novo* ductal MBC, hormone receptor (HR)+ and HER2+, with bone and visceral metastasis (i.e. liver and pleural metastasis), for which she was treated with Exemestane and an IGFR-1 inhibitor. Second line treatment with Docetaxel-Trastuzumab was started after 11 months due to development of brain metastasis, although there was stable disease in the bone and liver. After 8 months there was general progression and the patient was switched to Capecitabine-Lapatinib and whole brain radiotherapy. Five years and 9 months later she developed progressive liver, pleura, and bone metastasis and a study with Trastuzumab and an anti-HER-3 antibody was started in fourth line. At this time, CTCs and DTCs from pleural fluid were collected. Patient 2 was enrolled when she developed metastatic disease eight years after the diagnosis of HR+ HER2- early breast cancer. PET-CT showed bone, lymph node and pleura metastasis. At the first blood draw, >30.000 CTCs per 7.5 ml blood were enriched. Patient 3 presented with *de novo* MBC with extensive local disease and diffuse bone and visceral metastasis. PET images and pictures of local disease are depicted in **Figure S8**. They demonstrate diffuse bone and probably also bone marrow metastasis. Multiple mediastinal, hilar, and bilateral axillary pathological lymph nodes. Extensive tumour infiltration in the left breast with central ulceration and skin invasion. Diffuse infiltration at the base of the left breast with extension towards the left lateral thorax and invasion of the pectoralis muscle. Left sided pleural fluid with subsequent compression atelectasis of the complete left lower lobe and part of het left upper lobe. No obvious liver involvement. Shortly after, also leptomeningeal metastasis was detected. Primary tumour biopsy showed HR+ HER2+ (IHC 2+, SISH+) lobular breast cancer. Multiple samples were taken at different time points (**Figure 1 and 4B**). She received two lines of chemo-trastuzumab (**Figure 4B**), after which CTC counts dropped for only several months.

CTCs and DTCs were enriched from 7.5 ml blood or effusion using the CellSearch® CTC enumeration kit (Menarini Silicon Biosystems Inc, Huntingdon Valley, PA, USA) and subsequently further purified using the DEPArray® system (Menarini Silicon Biosystems Inc), as previously described (1). Individual CTCs, DTCs and white blood cells (WBC) as well as small pools were successfully recovered for each of the three MBC patients. In total 37 and 27 single and 38 and 21 pools of 10-150 EpCAM-positive CTC and DTC respectively, and 10 single and 6 pools of leukocytes acting as germline and quality controls, were isolated with DEPArray, and passed QC checks (genomic integrity index ≥3) for sequencing (2). Samples per patients are depicted in **Figure 1**. DNA was isolated and amplified using the *Ampli*1™ kit and double stranded using the *Ampli*1™ ReAmp/ds kit (Menarini Silicon Biosystems Inc) (3). Whole genome amplified (WGA) samples were subjected to Illumina whole genome sequencing (WGS) and Ion Torrent AmpliSeq panel sequencing. Fresh frozen tissue from solid metastases and the primary tumour, as well as bulk CTC and DTC samples (CellSearch® Profile, Menarini Silicon Biosystems Inc) were sequenced as comparators for mutation and copy number profiles. DNA of buffy coat was sequenced to enable germline variant detection.

Targeted AmpliSeq sequencing

The *Ampli*1™ cancer hotspot panel (CHP) custom panel β (Menarini Silicon Biosystems Inc), covering 2265 Hotspot COSMIC variants in 315 amplicons of 50 oncogenes, is compatible with the *Ampli*1™ WGA product (**Figure 1**). Libraries were prepared using a single-tube ultrahigh-multiplex PCR according

COPY NUMBER	HOTSPOTS				
MET	*ABL1*	*DDR2*	*GNA11*	*KIT*	*PTEN*
FGFR3	*AKT1*	*EGFR*	*GNAQ*	*KRAS*	*PTPN11*
EGFR	*ALK*	*ERBB2*	*GNAS*	*MEK1*	*RB1*
ALK	*APC*	*ERBB4*	*HNF1A*	*MET*	*RET*
AR	*ATM*	*EZH2*	*HRAS*	*MLH*	*SMAD4*
MYC	*BRAF*	*FBXW7*	*IDH1*	*NOTCH1*	*SMARCB1*
PTEN	*CDH1*	*FGFR1*	*IDH2*	*NPM1*	*SMO*
	CDKN2A	*FGFR2*	*JAK2*	*NRAS*	*SRC*
	CSF1R	*FGFR3*	*JAK3*	*PDGFRA*	*STK11*
	CTNNB1	*FLT3*	*KDR*	*PIK3CA*	*TP53* *VHL*

Table 1. AmpliSeq™ Cancer hotspot panel (CHP) Version 2

to the Ion AmpliSeq™ DNA Library Preparation User Guide (MAN0006735, Life Technologies). Quality control of the amplicon libraries were evaluated by Bioanalyzer 2100 (Agilent Technologies) using Agilent High Sensitivity DNA kit and subsequently sequenced on the Ion PGM (Personal Genome Machine) system. Alignment and variant calling was performed using the TorrentSuite v4.2. A Burrows-Wheeler algorithm (TMAP) is included in the TorrentSuite, used for alignment against the human reference genome hg19. Variants will be called with the 'tvc' variant caller with 'somatic low-stringency' configuration using *Ampli1™* BED regions and HotSpot variants included in the *Ampli1™* CHP custom β. This configuration is optimized for allele frequencies >2% and high sensitivity to minimize false negatives. The algorithm included in the TorrenSuite makes several filtering steps according to strand bias, read quality, and minimal coverage. Obtained variants were annotated in order to identify related COSMIC and dbSNP IDs. Further selection of variants is based on following inclusion criteria: coverage >20x and variant allele frequency (VAF) >10% for pools and bulk, VAF >30% for single cells, and VAF 0% for reference sample (i.e. buffy coat or normal tissue). Allelic dropout (ADO) rate was calculated as follows: homozygous SNPs in every sample, present as heterozygous in reference, divided by the heterozygous SNPs in reference. False positivity rate (FPR) was calculated: number of heterozygous variants (VAF >10%, or >30% for singles) in every sample divided by the number of homozygous events (VAF 0%) of the reference sample in the whole dataset (4, 5). As the majority of the FP errors occur at random sites in the genomes of single cells, it allows the FPR to be mitigated by calling mutations in two or more single cells (5). To maximize specificity amplified products of single WBC were used as negative controls. Subsequently, protein configurations were determined using PolyPhen and SIFT.

Shallow WGS

Shallow WGS was performed to determine the copy number profile. *Ampli*1™ WGA products were sheared using the Covaris system (microTUBE-15, PN520145) and subsequently purified using Ampure beads (Agencourt AMPure XP PCR pruification). Libraries were generated using the ThruPlex dual indexes kit according to manufacturer's instructions (Sopachem, Eke, Belgium). Quality of the Ampure purified libraries were evaluated using the Qubit 2.0 Fluorometer and the 2100 Bioanalyzer, and subsequently pooled and sequenced using paired-end chemistry on a Illumina HiSeq 4000 with a read length of 150 base pairs and at an estimated coverage depth of 0.5x (6, 7). Raw reads were analysed using FASTQC 0.11.5 (Babraham institute, UK). Reads were trimmed using Trimmomatic-0.36, to remove the adaptors and low quality base pairs (sliding window 5:20, average PHRED score ≥ 30,

average length ≥ 40 bp). Trimmed reads were analysed using FASTQC 0.11.5, and subsequently mapped to reference genome Hg19 using BWA-MEM 0.7.12, resulting in three SAM files (paired reads and single forward or reverse reads). SAM files were converted to BAM files using SAMTOOLS 1.3.1, where forward and reverse reads with MAPQ >20 were merged and sorted. BAM files were converted to BED files using BEDTOOLS 2.25.0, and subsequently subjected to the Ginkgo pipeline (Cold Spring Harbor, USA) (8), especially designed for copy-number alteration (CNA) analysis of single cells, although also very capable of analysing bulk data, according to the authors. Single-cell CNAs were identified with Ginkgo using variable-length bins of around 500 kb. After binning, data for each cell was normalized and segmented using a diploid reference sample (.bed file of the buffy coat) to normalize bin counts and eliminate additional biases uncorrected by GC normalization (or: using default parameters). Sensitivity was evaluated by assessing the recall of the CNAs and segment breakpoints at the different sequencing depths. Gains and losses within single cells are assigned to regions with copy numbers ≥3 and ≤1 respectively (as cancer cells can be triploid or more), for pools the cut-off is ≥2.3 and ≤1.7 respectively (which can result from a mix of cells with altered and normal CN). Amplifications and deletions have copy numbers ≥4 or ≤0 respectively. Alterations are referred to as recently proposed by Ben-David and Amon (9): (i) the term aneuploidy to describe all CNAs affecting either entire chromosome arms or whole chromosomes; (ii) the term CNAs to describe all sub-arm gains or losses larger than 10 kilobases (kb); and (iii) the term indels to describe all other CNAs.

Quality control

Samples were subjected to deep amplicon sequencing using the Ion AmpliSeq™ Cancer Hotspot Panel v2 covering 50 oncogenes and tumour suppressor genes. A high average read depth and width was obtained (**Table s1**). As each single cell provides only two (or a few more in case of cancer genomes) template DNA molecules for WGA, errors that occur in the initial rounds of amplification are inherited by all subsequent molecules (5). Major sources of technical errors that occur during WGA include allelic dropout (ADO) and introduction of false positive (FP) variants (10, 11). ADO rate across individual CTCs and WBCs and the mean FP rate was calculated and compared to previously reported data (5, 12). Variants were only called when absent in sorted leukocytes and present in ≥2 samples to eliminate false discovery, discarding the vast majority of variants (5).

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HER-2 status of Circulating Tumor Cells in a Metastatic Breast Cancer cohort: a comparative study on characterization techniques

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HER-2 status of Circulating Tumor Cells in a Metastatic Breast Cancer cohort: a comparative study on characterization techniques

ABSTRACT

Background:
Personalized targeted treatment in metastatic breast cancer relies on accurate assessment of molecular aberrations, e.g. overexpression of Human Epidermal growth factor Receptor 2 (HER-2). Molecular interrogation of circulating tumor cells (CTCs) can provide an attractive alternative for real-time biomarker assessment. However, implementation of CellSearch®-based HER-2 analysis has been limited. Immunofluorescent (IF) image interpretation is crucial, as different HER-2 categories have been described. Major questions in CTC research are how these IF categories reflect gene expression and amplification, and if we should consider 'medium' HER-2 expressing CTCs for patient selection.
Methods:
Tumor cells from spiked cell lines (n=8) and CTCs (n=116 samples) of 85 metastatic breast cancer patients were enriched using CellSearch®. Comparative analysis of HER-2 expression by IF imaging (ACCEPT, DEPArray™, and visual scoring) with qRT-PCR and *HER-2/neu* FISH was performed.
Results:
Automated IF HER-2-profiling by DEPArray™ and ACCEPT delivered comparable results. There was a 98% agreement between 17 trained observers (visual scoring) and ACCEPT considering HER-2^{neg} and HER-2^{high} expressing CTCs. However, 89% of HER-2^{med} expressing CTCs by ACCEPT were scored negative by observers. HER-2^{high} expressing tumor cells demonstrated *HER-2/neu* gene amplification, whereas HER-2^{neg} and HER-2^{med} expressing tumor cells and CTCs by ACCEPT were copy-number neutral. All patients with HER-2-positive archival tumors had ≥1 HER-2^{high} expressing CTCs, while 80% of HER-2-negative patients did not. High relative gene expression of HER-2 measured on enriched CTC lysates correlated with having ≥1 HER-2^{high} expressing CTCs.
Conclusion:
Automated images analysis has enormous potential for clinical implementation. HER-2 characterization and clinical trial design should be focused on HER-2^{high} expressing CTCs.

INTRODUCTION

Breast cancer is a heterogeneous disease, with distinct subgroups based on histological type, grade, and hormone receptor status. Human epidermal growth factor receptor 2 (HER-2) overexpression accounts for 10-15% of the primary invasive breast cancers and is associated with a more aggressive phenotype and inferior prognosis. In patients with advanced disease, clinically relevant discrepancies can arise in HER-2 expression status compared to the localized setting (1-3). Furthermore, patients often develop multiple lesions that might be composed of various tumor subclones harboring different molecular characteristics (4). As the HER-2 status can be subjective to temporal heterogeneity, in part influenced by prior therapies, it stands to reason that repeated analysis is a prerequisite for precision medicine. However, the acquirement of metastatic tissue is not always feasible and not without risk for the patient (5).

Circulating Tumor Cells (CTCs), isolated from the blood of patients with metastatic cancer, hold considerable promise to provide a convenient and safe alternative for real-time and repeated tumor profiling. Before molecular characterization of CTCs can be used to discover predictive biomarkers, e.g.

HER-2 receptor status, in-depth testing of analysis methods is essential. The CellSearch® system is an FDA-cleared and widespread implemented platform for enumeration of CTCs, and HER-2-positive CTCs have been detected using HER-2 immunofluorescence (IF) phenotyping. Visual scoring of HER-2 on CellSearch® IF images by individual observers has been performed in several studies (6-8). Image interpretation is crucial, especially when using CTCs in interventional trials testing HER-2-directed therapies. Although trained observers can reach acceptable agreement using a predefined definition (9), visual scoring is not objective and independent image review is laborious. Recently, an objective analysis software for CellSearch® IF images: Automated CTC Classification Enumeration and PhenoTyping (ACCEPT) has been made available, which is able to divide CTCs in HER-$2^{neg(ative)}$, HER-$2^{med(ium)}$, and HER-2^{high} expression (8). One major question in CTC research is how these IF categories reflect gene expression and amplification. One study demonstrated that HER-2-positive CTCs based on visual scoring were HER-2 gene amplified (10). Still the value of HER-2^{med} expressing CTCs has to be studied, as patients harboring these CTCs might as well benefit from HER-2-directed therapies. In this study we compare ACCEPT results with other IF imaging, quantitative reverse transcription (qRT)-PCR, and fluorescent *in-situ* hybridization (FISH).

METHODS

A detailed description of all materials and methods is provided in supplementary methods. A schematic overview of all samples and the workflow is depicted in Figure 1.

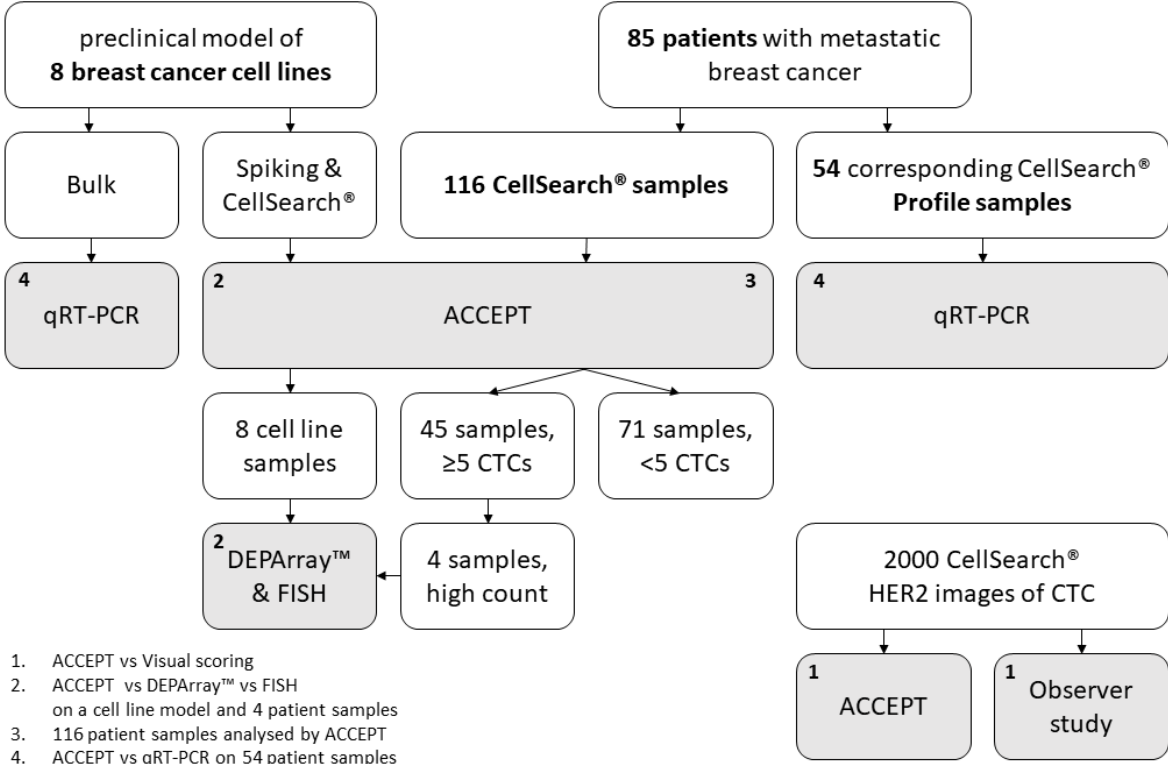


Fig 1. Overview of all samples and workflow.

Samples

The preclinical model utilizes eight breast cancer cell lines with increasing levels of HER-2 expression and/or amplification: MDA-MB-436, MCF-7, BT-20, MDA-MB-453, KPL-4, IBC-3, SUM190, and SKBR-3 (8, 9, 11-16). Cultured tumor cells were spiked in 7.5mL Cell Save®-collected healthy donor blood and subjected to the CellSearch® CTC procedure (Menarini Silicon Biosystems Inc., Huntingdon Valley, PA, USA), with addition of the HER-2 phenotyping reagent (Menarini Silicon Biosystems Inc.). Briefly, the CellTracks Autoprep® immunomagnetically enriches EpCAM-positive cells from blood and stains them with the nuclear dye DAPI, phycoerythrin conjugated antibodies against cytokeratin 8, 18 and 19 (CK-PE) and allophycocyanin conjugated antibody against the leukocyte specific marker CD45 (CD45-APC). The enriched cells are contained in a cartridge. Similarly, CTCs were enriched from 7.5 ml blood samples (n=116) of 85 patients starting a new line of systemic therapy for metastatic breast cancer (MBC), who were recruited between 2012 and 2015 at the Oncology Center of GZA Hospitals Sint-Augustinus (Antwerp, Belgium), after written informed consent (Study UA A11-18)(S1 and S2 Tables). In total, 45/116 (38.8%) samples contained ≥5 CTC/7,5 ml blood. For the inter-observer concordance study, 17 international pathologist and scientists scored 2000 CellSearch® HER-2-FITC thumbnail images of CTCs acquired from MBC patients who were enrolled, after written informed consent, in CTC studies at the Erasmus MC (Rotterdam, The Netherlands) (METC 2016-313 and METC 2009-405).

IF imaging

Image-based HER-2 fluorescent intensities were analyzed using three methodologies (S3 Table). First, visual scoring, which classifies the cells into negative, 2+, and 3+ was employed, as previously described (9). Using an online survey platform, CellTracks Analyzer II® thumbnail images of CTCs (n=2000) were reviewed by 17 international scientists and pathologists, who were trained to perform the visual HER-2 scoring. The obtained scores were benchmarked against the automated scoring results by ACCEPT (8). ACCEPT was used to automatically analyze the raw TIFF images of every fluorescent filter (DAPI, PE, APC, and FITC) taken by the CellTracks Analyzer II® (Menarini Silicon Biosystems Inc.) (8). CTC identification and HER-2 IF intensity classification (HER-2^{neg}, HER-2^{med}, and HER-2^{high}) was performed with gating and HER-2-FITC cut-off settings as previously described (8, 17). Briefly, CTC gates are defined as: Mean Intensity CD45 ≤ 5, Mean Intensity DNA > 45, Mean Intensity CK > 60, 16 ≤ Size CK ≤ 400, DNA overlay CK > 0.2; and HER-2 cut-offs are: HER-2^{neg} (Mean Intensity HER-2 = 0), HER-2^{med} (<100), and HER-2^{high} (≥100). Thirdly, to validate objective IF scoring by ACCEPT, 7 CellSearch®-enriched tumor cell lines and 4 CTC samples with high count were transferred to the DEPArray™ V2 system (Menarini Silicon Biosystems Inc.), as we have described previously (18). Briefly, the loaded sample is automatically injected into the microchamber of a cartridge where single cells are trapped in one of 16,000 electrical cages. IF images of the entire surface area are taken and cells are automatically detected by the system, generating an image library and 40 parameters per individual cell. HER-2 scoring was performed using the relative fluorescent units (RFU) of the HER-2-FITC signal after background subtraction (i.e. Mean Intensity-bgsub parameter). A cut-off for HER-2 positivity was defined at >1185 RFUs. Using this cut-off, 95% of the analyzed cells within the theoretically expected HER-2-positive and -negative cell lines classified as positive and negative, respectively.

FISH

A *HER-2/neu* FISH protocol was established using cell line models. CellSearch®-enriched tumor cells (8 spiked cell lines) and patient CTCs (4 samples) were spinned on a Superfrost Plus slide (Fisherbrand) using a Slide carrier with a 1ml One-Funnel Cytochamber (cat. 1662 and 1663 resp., Hettich) and fixed

in acetone at 4˚C for 5 minutes. FISH on slide was performed using the DAKO IQFISH kit (Agilent), with adjusted protocol as described in supplementary methods. Before and after FISH, slides were scanned on the BioView® scan device with a specialized CTC protocol (BioView®, Israel), in order to detect and map the tumor cells in the leukocyte background. HER-2 status was assessed according to the manufactures guidelines (Agilent).

qRT-PCR

Besides HER-2 image analysis, CellSearch Profile®-enriched tumor cell fractions (cell lines: n=7, patient samples: n=54) were subjected to HER-2 expression analysis, as described previously (19). Samples were taken simultaneously with CellSearch® CTC samples, to facilitate comparison between gene expression and IF. Briefly, 25% of the isolated RNA from the enriched fraction was subjected to complementary DNA (cDNA) synthesis and pre-amplification, using the RevertAid H Minus First Strand cDNA synthesis kit and TaqMan PreAmp master mix, respectively (Thermo Fisher Scientific #K1632 and #4488593). Pre-amplified cDNA was diluted 15x with 1xTE-buffer, after which qRT-PCR was performed for *ERBB2* as target gene*,* 3 housekeeping genes (*SDHA*, *HMBS* and *HPRT1*) to control for sample loading and RNA integrity, epithelial (*EPCAM, KRT19*) and leukocyte (*PTPRC*) markers to control for presence of epithelial and leukocyte content. *ERBB2* Cq value of every sample was normalized to the epithelial signal within that sample (dCq). All samples were further normalized to the calibrator (ddCq).

Statistics

Correlations between the HER-2 analysis methods ACCEPT, DEPArray™, and qRT-PCR was calculated using Pearson's correlation coefficient. Fisher's exact test was used to compare ACCEPT and qRT-PCR results within HER-2-negative and HER-2-positive patient groups.

RESULTS

HER-2 protein expression and gene amplification

HER-2 IF image interpretation is crucial, especially when using CTCs in interventional trials testing HER-2-directed therapies. In our international observer study, 17 trained readers performed visual HER-2 scoring (negative, or positive: 2+ or 3+) of 2000 patient-derived thumbnail CellSearch HER-2 images, which was compared to the new objective ACCEPT algorithm (HER-2^{neg}, HER-2^{med}, and HER-2^{high}). For 1535 CTCs (77%) a high concordance (i.e. >75%) between observers was reached, which was predominantly driven by agreement on HER-2 negativity (Fig 2A). According to ACCEPT, 860 (43.8%) CTCs were HER-2^{neg}, 608 (31.0%) were HER-2^{med}, and 495 (25.2%) were HER-2^{high} expressing CTCs. 37 CTCs were not detected by ACCEPT. When comparing the 1535 highly concordant scored CTCs with ACCEPT results, observers tend to score HER-2 expression on these CTCs lower than ACCEPT does (Fig 2B). Especially HER-2^{med} expressing CTCs were frequently scored as negative cells by observers. When merging the 2+ and 3+ scored CTCs into one HER-2-positive group, high concordance (i.e. >75%) was reached for 1843/2000 (92%) CTCs. 460/468 (98%) HER-2^{high} expressing CTCs were scored as positive by the observers, and 816/831 (98%) HER-2^{neg} CTCs were scored negative by the observers. Again, ACCEPT HER-2^{med} CTCs were scored negative in 457/511 (89%) cases (Fig 2C). These dim expressing CTCs are covered in the negative category of the visual scoring system, as it includes 0 and 1+ scores. Furthermore, there was a significant difference between the mean IF intensity of the HER-2^{med} CTCs scored negative (47.9 +/- 15.5) versus positive (82.1 +/- 13.1) on visual scoring. Inter-observer variability (Kappa test) was 0.636 for visual scoring in negative, 2+, and 3+ groups, and 0.785 for scoring

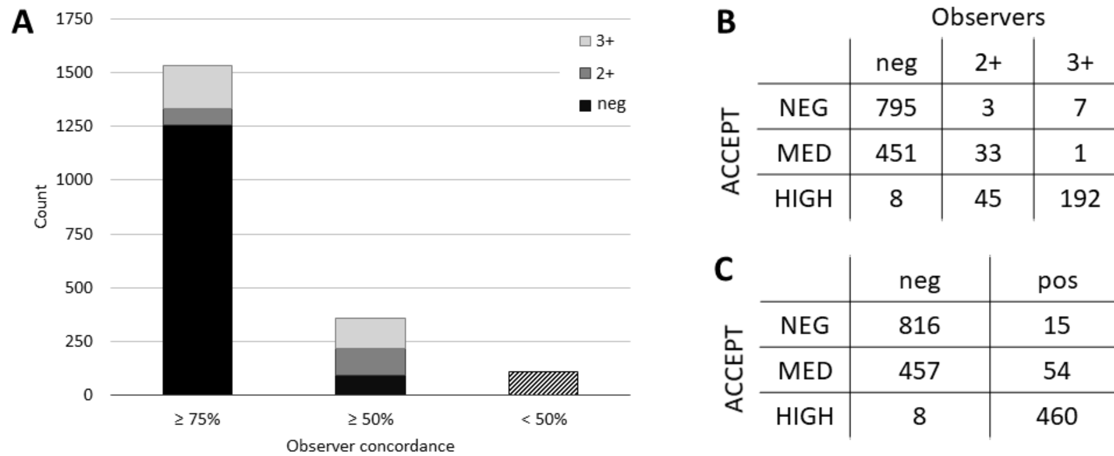


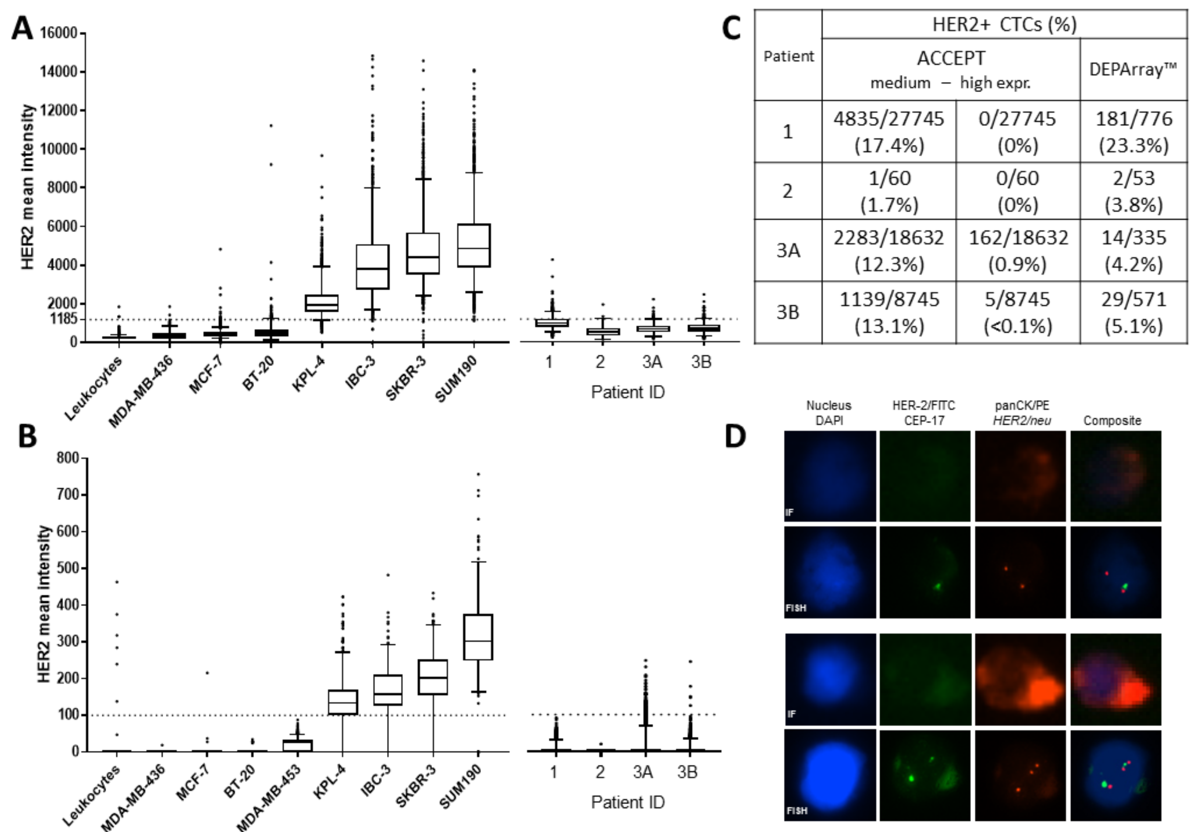
Fig 2. HER-2 scoring of 2000 CTC thumbnail IF images by 17 trained observers (0, 2+, 3+) versus ACCEPT (HER-2^{neg}, HER-2^{med}, HER-2^{high}). A. ≥75% concordance between observers was reached for 1535 CTCs. For 358 CTCs agreement was reached between >50% of observers, and for 107 CTCs (dashed box) no agreement was reached **B.** HER-2 scores of 1535 CTCs given by ≥75% of observers versus ACCEPT. HER-2-negative CTCs according to observers were mainly scored HER-2^{neg} or HER-2^{med} by ACCEPT. HER-2^{high} expressing CTCs by ACCEPT were predominantly scored 2+ or 3+ by the observers. **C.** HER-2 scores of 1810 CTCs given by ≥75% of observers versus ACCEPT. HER-2^{high} expressing CTCs by ACCEPT were predominantly scored positive by the observers.

in negative versus positive groups, which are both considered as 'good' according to the Koch and Landis classification (20). Overall, we found high agreement between observers and ACCEPT regarding HER-2^{neg} and HER-2^{high} expressing CTCs, however ACCEPT HER-2^{med} CTCs appear negative on visual scoring. To further investigate this, we performed IF image analysis and FISH on a cell line model.

Two automated image analysis methods, i.e. DEPArray™ and ACCEPT, were used to measure HER-2 IF signal on CellSearch-enriched samples of eight breast cancer cell lines with incremental HER-2-FITC intensity, categorized as HER-2^{neg} (MCF-7, MDA-MB-436, and BT-20), HER-2^{med} (MDA-MB-453), and HER-2^{high} (KPL-4, SUM190, IBC-3, and SKBR-3) according to literature (8, 9, 13) (Figs 3A and 3B). ACCEPT analysis of CellSearch Analyzer II® raw images demonstrated absence of HER-2-FITC signal in 8409/8434 (99.7%) leukocytes and 496/509 (97.4%) negative cell line cells (Fig 3B). MDA-MB-453 cells were HER-2^{med} expressing in 368/640 (57.5%), the rest being HER-2^{neg}. Within the HER-positive cell lines KPL-4, IBC-3, SKBR-3, and SUM190 we observed an incremental increase in the median HER-2-FITC intensity, with 415/542 (76.6%), 187/212 (88.2%), 189/208 (90.9%) and 220/228 (96.5%) cells, respectively, being classified as HER-2^{high} expressing cells (Fig 3B).

Overall, when comparing both DEPArray™ and ACCEPT cell line data, a comparable gradient in the mean HER-2 expression levels was observed (Pearson r = 0.96, p = 0.0001). As the DEPArray™ system is able to recover individual CTCs, the found cut-off can be used to sort samples into different HER-2 categories for downstream analysis.

To infer whether increased HER-2-FITC IF signals find their origin in gene amplification, we applied *HER-2/neu* FISH on the CellSearch®-enriched fractions. In all samples, analyzed leukocytes (n=20) demonstrated a copy-number neutral *HER-2/neu* status (S1 Fig). A similar observation was made in HER-2-negative cell lines MDA-MB-436, MCF-7, and BT-20, (n=10 per cell line). High gene amplification was detected in all visualized cells of HER-2-positive cell lines KPL-4, IBC-3, SKBR-3, and



Patient	HER2+ CTCs (%)		
	ACCEPT medium – high expr.		DEPArray™
1	4835/27745 (17.4%)	0/27745 (0%)	181/776 (23.3%)
2	1/60 (1.7%)	0/60 (0%)	2/53 (3.8%)
3A	2283/18632 (12.3%)	162/18632 (0.9%)	14/335 (4.2%)
3B	1139/8745 (13.1%)	5/8745 (<0.1%)	29/571 (5.1%)

Fig 3. HER-2 IF and FISH on a cell line model and 4 patient samples. A. HER-2 mean intensity background subtracted (bgsub) for donor leukocytes, 3 HER-2-negative cell lines (MDA-MB-436, MCR-7, BT-20) and 4 HER-2-posititve cell lines (KPL-4, IBC-3, SKBR-3, SUM190), and 4 patient samples measured by DEPArray™. The cut-off between HER-2-negative and -positive cells was defined at 1185 RFU. **B.** HER-2 mean intensities for donor leukocytes, 8 cell lines, and 4 patient samples measured by ACCEPT. HER-2^{neg}, HER-2^{med} and HER-2^{high} expressing cells are defined as mean intensity = 0, ≤100, and >100 respectively (8). **C.** Table showing the number and percentage of HER-2-positive CTCs in 4 patient samples based on ACCEPT and DEPArray™. HER-2-positive cells are subdivided in HER-2^{med} and HER-2^{high} expressing cells using ACCEPT. **D.** BioView IF and FISH images of DEPArray™-sorted HER-2-positive and -negative CTCs from patient 1. HER-2-negative CTCs are copy-number neutral (example shown). 7/24 (29%) HER-2-positive CTCs revealed a HER-2/CEP17 ration of 3:2, still being FISH negative (example shown).

SUM190 (n=7 for KPL-4, n=10 for IBC3, SKBR-3, and SUM190), with mean HER-2/CEP17 ratios of 5.5 (KPL-4), 6.3 (IBC-3), 8.3 (SKBR-3), and 4.5 (SUM190). Medium cell line MDA-MB-453 had on average 6 HER-2 and 3 CEP17 copies, being scored as borderline (S1 Fig).

Cell line results were confirmed in an explorative study on four patient samples with high CTC count. The majority of CTCs were HER-2-negative using DEPArray™ and ACCEPT (Fig 3C). With DEPArray™ image analysis, 23.3% of CTCs in patient sample 1 exceeded the HER-2-positivity threshold. A comparable HER-2^{med} expressing CTC rate was detected by ACCEPT. In patient samples 3 and 4 we observed 2283 (12.3%) and 1139 (13.1%) HER-2^{med} expressing CTCs, respectively. In all CellSearch patient samples no HER-2 amplification was observed. Additionally, CTCs from patient 1 just exceeding the positivity threshold were DEPArray™-sorted. *HER-2/neu* FISH analysis showed no amplification in these CTCs, although 7/24 (29%) cells visualized, revealed a HER-2/CEP17 ratio of 3:2 (i.e. non-amplified according to HER-2-FISH guidelines) (Fig 3D).

Our finding that HER-2^{med} expressing cells are mainly scored as negative using visual scoring, and are not HER-2-amplified, is in line with results from a large patient cohort where only positive CTCs by visual scoring were HER-2-amplified (10).

HER-2 analysis in a MBC patient cohort

Using the objective analysis software of ACCEPT, we studied the distribution of HER-2^{neg}, HER-2^{med}, and HER-2^{high} expressing CTCs in 45 CellSearch® CTC samples containing ≥5 CTCs from 35 MBC patients. 11/45 (24%) samples came from 10 patients with a HER-2-positive primary tumor and/or metastasis, and 34/45 (76%) samples came from 25 patients with a HER-2-negative primary tumor and/or metastasis (Fig 4, S1 Table). In the HER-2-negative patient group, all first blood samples were taken at the start of a new line (1st-3rd) of therapy for MBC. None of these patients received any anti-HER-2 directed therapy. From HER-2-postitive patients, all samples (except from patient 2000) were taken at first line of therapy for MBC and none of them were at that moment treated with anti-HER-2 directed therapy, due to various reasons (i.e. *de novo* MBC; adjuvant trastuzumab had already stopped; or in 1 patient no anti-HER-2 directed therapy had been added to the adjuvant treatment). Sample 2000_1 was taken at the start of the second line of therapy, however the first line did not include trastuzumab. During further treatment this patient did receive anti-HER-2 directed therapy, as was the case when sample 2000_2 was taken (S2 Table). We observed in both groups heterogeneous HER-2 expression patterns. All 10 (100%) patients with HER-2-positive MBC, had ≥1 HER-2^{high} expressing CTCs, while in the HER-2-negative MBC patients, this was in 5/25 (20%) patients (Fisher exact: $p < 0.0001$). Overall, 37% of patients harbored >10% HER-2^{high} expressing CTCs. However, when combining HER-2^{med} and HER-2^{high} expressing CTCs, this was 94% of patients. This is comparable with recent data of 132 patients (39% HER-2-positive and 61% HER-2-negative patients), where 89.4% of patients had HER-2^{med} and/or HER-2^{high} expressing CTCs (8).

Focusing on patients with multiple sampling, comparable HER-2^{neg}, HER-2^{med}, and HER-2^{high} CTC counts were observed. However, in patient 2000, diagnosed with HER-2-positive *de novo* MBC, HER-2^{high} expressing CTCs were eliminated after anti-HER-2 directed therapy. Similarly, in patient 3495, who was diagnosed with HER-2-negative (IHC 2+/FISH-negative) *de novo* MBC, 20% of the CTCs were HER-2^{high} expressing at the start of an aromatase inhibitor. Although the CTC burden was comparable, HER-2^{high} expressing CTCs disappeared completely after 6 weeks on therapy (Fig. 4, S2 Table).

Surprisingly, in all 71 samples containing <5 CTCs, we found no HER-2^{neg} CTCs and solely HER-2^{med} and HER-2^{high} expressing CTCs, independent of the primary tumor or metastasis status (S2 Fig). A similar observation was made by Zeune *et al*, with samples containing solely HER-2^{med} and/or HER-2^{high} CTCs all having ≤6 CTCs in total (8). When analyzing leukocytes, we found in all samples up to 5% of leukocytes did express some HER-2. This physiological phenomenon will not affect IF image-based HER-2 analysis of CTCs, as CTCs and leukocytes are measured individually (21).

We aimed to validate the ACCEPT results by applying gene expression analysis on enriched CTC fractions of blood samples taken simultaneously. ACCEPT image analysis and qRT-PCR data on CellSearch Profile®-enriched CTC fractions were available for 7 cell lines and 54 patient samples. *ERBB2* (*HER-2/neu*) relative gene expression (RGE) levels in cell lines (S3 Fig) correlated with mean intensity HER-2 IF data, as measured by DEPArray™ ($r = 0.97$, $p = 0.0002$) and ACCEPT ($r = 0.96$, $p = 0.0007$) image analysis. *ERBB2* expression, corrected for CTC content, in 20 patient samples with ≥5 CTCs (range 5-17502) demonstrated variable *ERBB2* RGE (Fig 5), but correlated well with ACCEPT data ($r = 0.8255$, $p < 0.00001$). Focusing on HER-2^{high} expressing CTCs, comparative analysis with *ERBB2* RGE data demonstrated how 3/10 (30%) samples with low *ERBB2* RGE (<0.2, i.e. below median RGE) contained

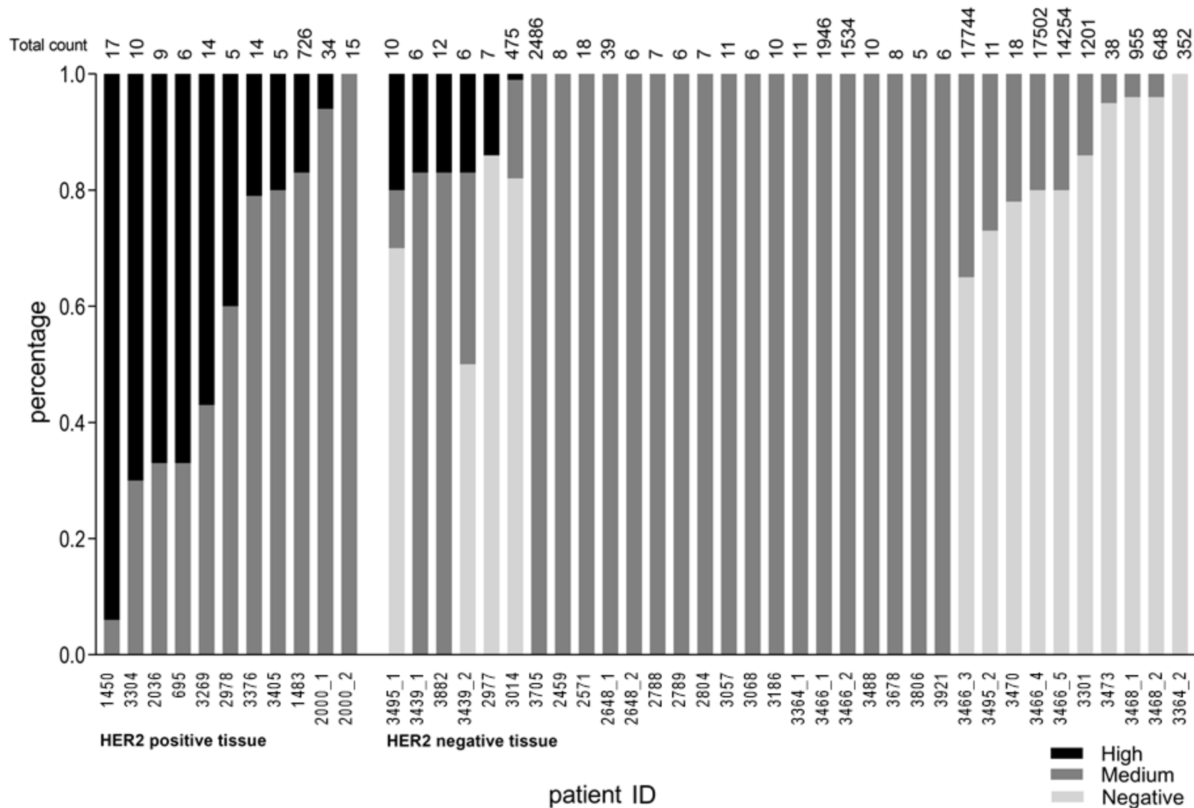


Fig 4. HER-2 IF scoring by ACCEPT of a MBC patient cohort. Percentage of HER-2^{neg}, HER-2^{med}, and HER-2^{high} expressing CTCs in a MBC cohort divided in patients with HER-2-positive or -negative tissue (primary tumor and/or metastasis) samples. Total CTC count per sample is depicted on top. HER-2^{high} expressing CTCs are present in at least 1 sample of 10/10 (100%) HER-2-positive patients, and 5/25 (20%) HER-2-negative patients.

HER-2^{high} expressing CTCs (range 1-2), whereas HER-2^{high} expressing CTCs (range 1-16) were present in 7/10 (70%) samples with high *ERBB2* RGE (>0.2) (Fisher exact: $p = 0.1789$). When focusing on the samples with a highest *ERBB2* expression (RGE >0.34, i.e. above the third quartile) 5/5 patients harbored >20% (i.e. above the third quartile) HER-2^{high} expressing CTCs, while in the other group this was in 1/15 of patients (Fisher exact: $p = 0.0004$). Moreover, 5/5 patients with the highest *ERBB2* expression had ≥1 HER-2^{high} expressing CTC, while this was 5/15 in the group with a lower *ERBB2* RGE (Fisher exact: $p = 0.016$). We did not observe a correlation between ACCEPT and qRT-PCR data in samples with <5 CTCs. Taken together, this demonstrates that in patients with ≥5 CTCs, qRT-PCR on CellSearch®-enriched samples can identify samples containing HER-2^{high} expressing CTCs.

DISCUSSION

Personalized targeted treatment of patients with MBC relies on the accurate assessment of specific molecular aberrations in tumor cells, e.g. the overexpression of the transmembrane HER-2 receptor. To circumvent potential clinically-relevant discordances in HER-2 receptor status between archival primary tumor tissue and metastatic lesions, the molecular interrogation of FDA-cleared CellSearch®-enriched CTCs can provide an attractive alternative for real-time biomarker assessment (1-3).

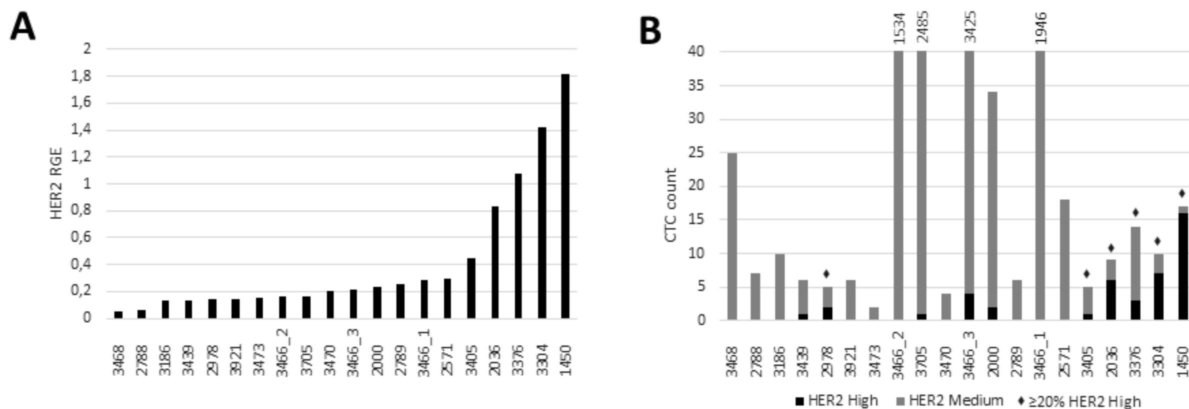


Fig 5. HER-2 protein (ACCEPT) versus mRNA (qRT-PCR) expression of MBC patient samples. A. Increasing *ERBB2* (HER-2) relative gene expression (RGE) corrected for CTC content of 20 patient samples with ≥5 CTCs. **B.** Corresponding HER-2^{med} and HER-2^{high} expressing CTC count by ACCEPT of 20 patient samples with ≥5 CTCs. For four samples the total number of HER-2^{med} expressing cells is given on top. For six samples ≥20% of total CTC count were HER-2^{high} expressing CTCs (♦).

However, implementation of CellSearch®-based HER-2 analysis using visual scoring has been limited. Most recently an objective analysis software has been made available (8), which we compared to other CTC analysis techniques.

Our observer study demonstrated high agreement between the observers and ACCEPT considering the HER-2^{neg} and HER-2^{high} expressing CTCs, while HER-2^{med} expressing CTCs by ACCEPT were scored negative by the observers in 89% of CTCs. Moreover, we show that HER-2^{med} expressing cell line cells and patient CTCs, did not show HER-2/*neu* gene amplification, which is in agreement with literature, were MDA-MB-453 was scored IHC and FISH negative (8, 9, 11-13, 22). Both results are in line with data from a large patient cohort where negative CTCs by visual scoring were HER-2 copy number neutral (10). As patients only receive HER-2-directed therapy when HER-2 overexpression is proven on tissue samples (i.e. IHC 3+ or FISH+), one might argue on the clinical benefit of treating patients harboring HER-2^{med} expressing CTCs.

When inferring the prevalence of HER-2^{neg}, HER-2^{med}, and HER-2^{high} expressing CTCs in our patient cohort, we found that one third harbored >10% HER-2^{high} expressing CTCs, while almost all patients harbored HER-2^{med} expressing CTCs. This is comparable with recent data on a similar cohort of 132 patients (8). In daily clinic, HER-2 overexpression (IHC 3+ or FISH+) is only present in a minority of patients with primary invasive breast cancer, although a higher incidence of HER-2-positivity is seen in MBC (26,3% in stage IV versus 15% in stage I-III patients) (23). This prevalence is in line with the percentage of patients with HER-2^{high} expressing CTCs in both our and the MBC cohort examined by Zeune *et al* (8). Taken together, we suggest that HER-2^{high} expressing CTCs might be more clinically relevant than HER-2^{med} expressing CTCs.

Besides all HER-2-positive patients, also 5/25 HER-2-negative patients harbored ≥1 HER-2^{high} expressing CTCs based on ACCEPT. This suggests either a shift in HER-2 status in these 5 patients, or outgrowth of a minor HER-2-positive subclone not detected with FISH on tissue samples. The latter has been demonstrated with FISH on DEPArray™ sorted primary tumor samples (24). One should realize that HER-2 expression on tissue samples is often heterogeneous and are given IHC scores of 1+ or 2+. In our cohort, 3 out of 5 patients with discrepant HER-2 status were assigned IHC 1+ or 2+, suggesting

some HER-2-positive tumor cells were already present at baseline. In general, IHC status (i.e. more homogeneous 0 or 3+, or more heterogeneous 1+ or 2+) of the archival tumor was not related to the degree of heterogeneity we found in the CTC samples. Acquisition (i.e. clonal selection/expansion) of HER-2 gene amplification in CTCs has reported to be associated with cancer progression (25). Still, 80% of patients with a HER-2-negative primary tumor did not harbor any HER-2^{high} expressing CTCs. We argue that in these patients a major clinical impact of HER-2-directed monotherapy cannot be expected.

Clinical trials incorporating quantitative HER-2 analysis on CTCs might learn us the clinical validity of both HER-2^{med} and HER-2^{high} expressing CTCs. The ongoing DETECT III trial aims to demonstrate the benefit from Lapatinib therapy in initially HER-2-negative patients, who are HER-2-positive on CTCs (7). The CirCe T-DM1 trial showed that *HER-2/neu* gene amplification in CTCs from 7 HER-2-negative MBC patients occurs in a minor CTC subpopulation (26). Overall a low response rate was reported (1/7), questioning the clinical utility of anti-HER-2 therapy in patients with HER-2 amplification in a minor subset. Another phase II trial tested effectiveness of Lapatinib in MBC patients with HER-2-negative primary tumors and HER-2-positive CTCs analyzed by visual scoring of CellSearch® images and FISH (27). 7/96 patients, harboring 2-5 CTCs, were eligible (i.e. ≥50% of CTCs were HER-2-IF positive, and 1 sample was FISH-positive). No objective tumor responses occurred in this population, underlining the importance of patient selection for such trials. Based on our findings this should be patients with ≥5 CTCs and at least one HER-2^{high} expressing CTC. To enhance clinical utility of CTC-based therapy selection, it is important to consider improved quality control, validation, and standardization for HER-2 characterization and scoring on CTCs, as is required for HER-2 diagnostics on tissue. Objective image analysis is key start.

Liquid biopsies have the major advantage that they can be taken easily and repeatedly. The ability to detect *ERBB2* gene amplifications in plasma has already been proven (28), however no trials testing anti-HER-2 directed therapy in MBC based on HER-2 alterations in cell free (cf)DNA have been performed. Although, efforts have been made in gastric cancer (29). For both cfDNA and CTCs (independent of the enrichment technique, i.e. EpCAM based or marker free) applies: a standardized biomarker should be tested in the right patient population in a four-armed randomized trial (30, 31) to proof its utility in distinguishing between patients that will or will not benefit from specific therapies.

CONCLUSIONS

Our data shows that HER-2 characterization on CTCs should be focused on HER-2^{high} expressing CTCs in patient samples containing ≥5 CTCs. Although CTC-derived HER-2 expression in patients is heterogeneous, the prevalence of patients with ≥1 HER-2^{high} expressing CTCs better reflects the incidence of HER-2-positive MBC seen in the clinic. Additionally, we have demonstrated that only HER-2^{high} expressing tumor cells harbor amplification of the *HER-2/neu* gene, and samples containing HER-2^{high} expressing CTCs show high relative gene expression of HER-2 on qRT-PCR. For both these downstream techniques, prior CTC enrichment is necessary, involving extra cost and labor. Therefore, straightforward automated images analysis has enormous potential for clinical implementation. When focusing on the right patient population, CTC-direct anti-HER-2 therapy might proof itself in clinical trials.

Acknowledgements

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Conflict of interest

The company HistoGeneX NV provided support in the form of salaries for authors ES and AEM. This commercial affiliation does not alter our adherence to PLOS ONE policies on sharing data and materials. The authors declare no further conflict of interest.

Ethics approval and consent to participate

The ethical committee of the Antwerp University Hospital (UZA) and University of Antwerp (UA) approved this non-interventional study (UA A11-18). Images used in the observer study were obtained from samples of patients enrolled in studies of the Erasmus Medical Center Rotterdam (METC 2016-313 and METC 2009-405). Informed written consent was obtained from all patients.

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List of Abbreviations

ACCEPT: Automated CTC Classification Enumeration and PhenoTyping (software)
CTC: circulating tumor cell
ER: estrogen receptor
ERBB2: erythroblastic oncogene B 2 (gene, also *HER-2/neu*)
FISH: fluorescent *in situ* hybridization
HER-2: human epidermal growth factor receptor 2
IF: immunofluorescence
IHC: immunohistochemistry
MBC: metastatic breast cancer
qRT-PCR: quantitative reverse transcription polymerase chain reaction
RFU: relative fluorescent units
RGE: relative gene expression

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SUPPORTING INFORMATION

Patient	190	388	695	889	957	1450	1483	1529	1782	1944	2000	2036	2139	2280	2459	2535	2571	2648	2734	2788	2789	2802
PT-IHC	0	0	3+	0	0	2+	1+	0	1+	2+	2+	3+	2+	NA	1+	1+	1+	2+	0	0	2+	2+
PT-FISH	NA	NA	1	NA	NA	1	1	NA	0	0	1	1	1	NA	NA	NA	NA	0	NA	NA	0	0
MET-IHC	NA	1+	NA	NA	NA	NA	2+	0	2+	NA	NA	NA	NA	2+	NA	NA	1+	NA	NA	2+	1+	NA
MET-FISH	NA	NA	NA	NA	NA	NA	0	NA	1	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	0	0	NA
Tumor	neg	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	pos	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg

Patient	2804	2880	2923	2977	2978	2989	2991	3014	3057	3063	3068	3122	3134	3157	3174	3185	3186	3269	3272	3289	3294
PT-IHC	2+	1+	2+	0	2+	NA	3+	NA	1+	2+	0	0	1+	2+	2+	3+	0	3+	0	2+	2+
PT-FISH	0	NA	1	NA	1	NA	0	NA	NA	0	NA	NA	0	1	0	1	NA	1	NA	0	0
MET-IHC	NA	NA	3+	NA	2+	2+	NA	2+	1+	2+	NA	1+	NA	NA	2+	NA	0	NA	NA	NA	NA
MET-FISH	NA	NA	0	NA	0	0	NA	0	NA	0	NA	NA	NA	NA	0	NA	NA	NA	NA	NA	NA
Tumor	neg	neg	pos	neg	pos	neg	pos	neg	neg	neg	neg	neg	neg	pos	neg	pos	neg	pos	neg	neg	neg

Patient	3301	3304	3336	3357	3364	3376	3380	3404	3405	3439	3441	3447	3466	3468	3469	3470	3473	3488	3495	3507	3515
PT-IHC	2+	3+	3+	2+	1+	3+	1+	2+	3+	NA	0	1+	0	0	3+	2+	2+	0	2+	2+	1+
PT-FISH	0	1	1	1	NA	1	NA	1	NA	NA	NA	0	NA	NA	NA	0	0	NA	0	1	NA
MET-IHC	1+	NA	NA	NA	NA	NA	NA	NA	3+	1+	NA	1+	NA	NA	NA	NA	NA	1+	NA	NA	NA
MET-FISH	NA	NA	NA	NA	NA	NA	NA	NA	1	0	NA	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
Tumor	neg	pos	pos	pos	neg	pos	neg	pos	pos	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	pos	neg

Patient	3546	3548	3557	3614	3626	3647	3675	3678	3680	3705	3707	3713	3741	3779	3806	3811	3844	3882	3921	3936	4176
PT-IHC	0	1+	2+	2+	1+	0	NA	NA	NA	NA	0	3+	NA	2+	NA	NA	1+	0	0	2+	NA
PT-FISH	NA	NA	0	1	NA	NA	NA	NA	NA	NA	NA	1	NA	0	NA	NA	NA	NA	NA	1	NA
MET-IHC	1+	2+	NA	2+	NA	1+	NA	1+	NA	1+	NA	NA	NA	NA	1+	2+	2+	NA	NA	NA	2+
MET-FISH	0	0	NA	1	NA	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	0	NA	NA	NA	0
Tumor	neg	neg	neg	pos	neg	neg	NA	neg	NA	neg	neg	pos	NA	neg	neg	neg	neg	neg	neg	pos	neg

S1 Table. HER-2 tissue status. IHC (0-3+) and FISH (0=negative, 1=positive) results for primary tumor (PT) and metastatic tissue (MET) per patient.

patient_ID	Adj_therapy	Adj_anti-HER-2	LineMBC_at_Blooddraw	anti-HER-2_at_Blooddraw
695	y	n	1	n
1450	y	y	1	n
1483	y	y	1	n
2000_1	prim_MBC		2	n
2000_2	prim_MBC		5	y
2036	y	y	1	n
2978	prim_MBC		1	n
3269	y	y	1	n
3304	prim_MBC		1	n
3376	prim_MBC		1	n
3405	n (DCIS)		1	n

*1st line no anti-HER-2

HER-2 postitive tissue

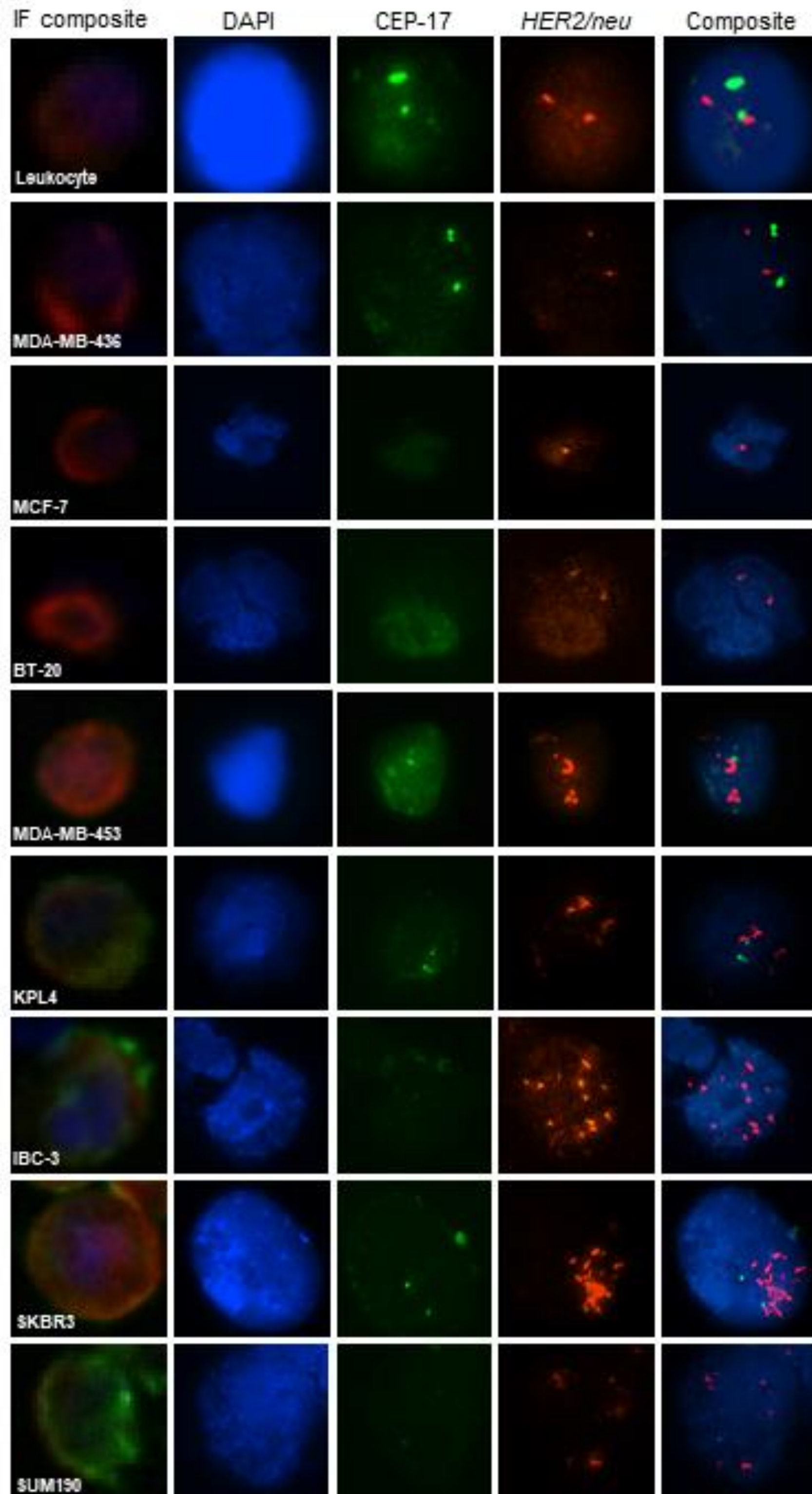
patient_ID	LineMBC_at_Blooddraw	patient_ID	LineMBC_at_Blooddraw	patient_ID	LineMBC_at_Blooddraw
2459	1	3186	1	3468_1	1
2571	2	3301	1	3468_2	1_FU
2648_1	1	3364_1	1	3470	1
2648_2	1_FU	3364_2	1	3473	1
2788	1	3439_1	1	3488	1
2789	1	3439_2	1_FU	3495_1	1
2804	1	3466_1	1	3495_2	1_FU
2977	3	3466_2	1_FU	3678	2_PD
3014	3	3466_3	1_FU	3705	1
3057	3	3466_4	1_FU	3806	1
3068	1	3466_5	1_PD	3882	1
				3921	1

HER-2 negative tissue

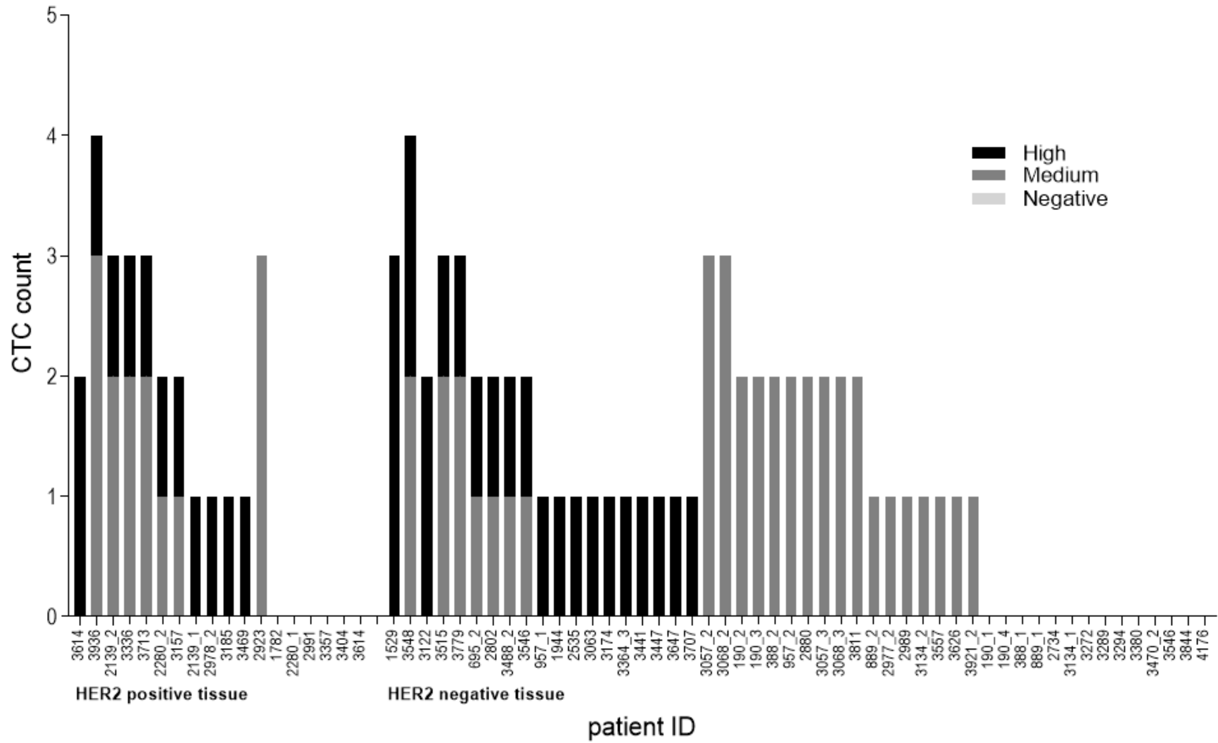
S2 Table. Line of treatment at time of CTC enumeration. In the HER-2-negative patient group, all first blood samples were taken at the start of a new line (1st-3rd) of therapy for MBC. None of these patients received any anti-HER-2 directed therapy. From the HER-2-postitive patients, all samples were taken at first line of therapy for MBC, but sample 2000_1 (start of the second line), and none of them were at that moment treated with anti-HER-2 directed treatment. Sample 2000_2 was taken at the start of the fifth line of therapy, after prior anti-HER-2 directed therapy. Abbreviations: Adj, adjuvant; prim_MBC, *de novo* Metastatic Breast Cancer; DCIS, ductal carcinoma *in situ*; FU, follow-up; PD, progressive disease.

Tool	Source	HER2 classes	HER2 cut-off	reference
ACCEPT	Raw cartridge images of CellSearch® Analyzer II	negative, medium, high	mean intensity: 0, 0-100, ≥100	8
Visual	Image gallery of CellSearch® Analyzer II	0, 1+, 2+, 3+	Semi-quantitative scale	15
DEPArray™	DEPArray™ parameters	negative, positive	mean intensity-bgsub: 1185 RFU	

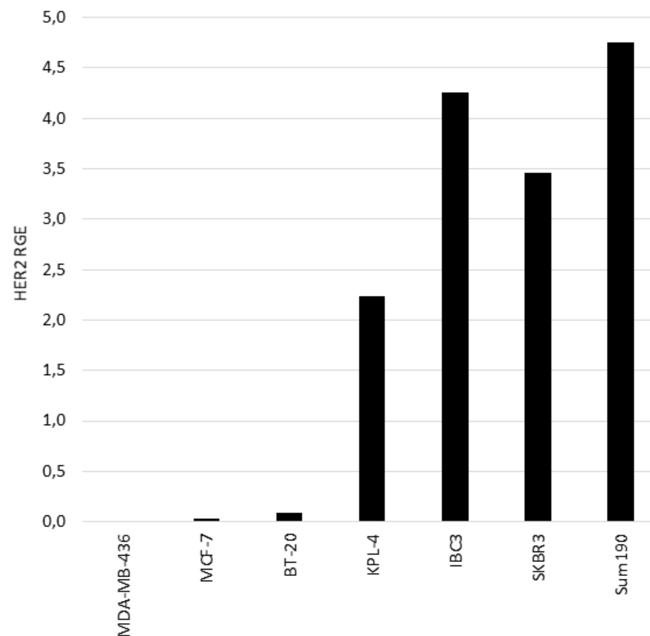
S3 Table. Methodologies and cut-offs used for image-based analysis of HER-2 fluorescent intensities.



S1 Fig. BioView IF and FISH images of leukocytes and cell line cells. IF composite image is taken before FISH. Secondly, Nucleus/DAPI, CEP-17/SpectrumGreen, *HER-2-neu*/SpectrumOrange, and the composite images are shown. Leukocytes, MDA-MB-436, MCF-7, and BT-20 cells demonstrated a copy-number neutral *HER-2/neu* status. Mean HER-2/CEP17 ratios for amplified cell lines were 5.5 (KPL-4), 6.3 (IBC-3), 8.3 (SKBR-3), and 4.5 (SUM190). Medium cell line MDA-MB-453 had on average 6 HER-2 and 3 CEP17 copies.



S2 Fig. ACCEPT results in <5 CTC patient samples. HER-2^{neg}, HER-2^{med}, and HER-2^{high} expressing CTC count in a MBC cohort with samples <5CTC, divided in patients with HER-2-positive or -negative tissue (primary tumor and/or metastasis) samples.



S3 Fig. HER-2 gene expression. *ERBB2* relative gene expression (RGE) corrected for housekeeping gene expression, of bulk samples from 7 cell lines.

SUPPLEMENTARY METHODS

Samples

The preclinical model consists of eight breast cancer cell lines with increasing levels HER-2 expression and/or amplification: MDA-MB-436, MCF-7, BT-20, MDA-MB-453, KPL-4, IBC-3, SUM190, and SKBR-3 (8, 9, 11-16). SUM190 and IBC-3 were cultured in Ham's F12 Nutrient Mixture (Life Technologies: 21765-029) with 1% penicillin/streptomycin/L-glutamine (Life Technologies: 10378-016), 1% anti/anti (Life Technologies: 15240-062), 5% FBS (Life Technologies: 10270-106), 1% HEPES buffer 1M (Life Technologies: 15630-056), 1µg/ml hydrocortisone (Sigma-Aldrich: H4001-1G), and 5µg/ml insulin solution (Sigma-Aldrich: I9278-5ML). MCF-7, MDA-MB-436, BT-20, and KPL-4 were cultured in RPMI (Life Technologies: 11835-063), and SKBR-3 in McCoy's (Life Technologies: 16600-082), all with 1% penicillin/streptomycin/L-glutamine, 1% anti/anti, and 10% FBS. Tumor cells were spiked in CellSave® tubes containing 7.5 ml donor blood and subjected to the CellSearch® system (Menarini Silicon Biosystems Inc., Huntingdon Valley, PA, USA), with addition of a FITC-bound HER-2 antibody (Menarini Silicon Biosystems Inc.). Similarly, CTCs were enriched from 7.5 ml blood samples (n=116) of 85 patients with metastatic breast cancer (MBC), who were recruited between 2012 and 2015 at the Oncology Center of GZA Hospitals Sint-Augustinus (Antwerp, Belgium), after written informed consent (Study UA A11-18)(Supplementary Table S2). In total, 45/116 (38.8%) samples contained ≥5 CTC/7,5 ml blood. For the inter-observer concordance study, 17 international pathologist and scientists scored 2000 CellSearch® HER-2-FITC thumbnail images of CTCs acquired from MBC patients who were enrolled, after written informed consent, in several CTC studies at the Erasmus MC (Rotterdam, The Netherlands). For this part we included all single, fully visible CTC images of selected patients (known to be CTC-HER-2 positive or negative) regardless of image quality.

HER-2 IF scoring methods

Image-based HER-2 fluorescent intensities were analyzed using three methodologies.

Tool	Source	HER-2 classes	HER-2 cut-off	reference
Visual	Image gallery of CellSearch® Analyzer II	0, 1+, 2+, 3+	Semi-quantitative scale	9
ACCEPT	Raw cartridge images of CellSearch® Analyzer II	negative, medium, high	mean intensity: 0, 0-100, ≥100	8
DEPArray™	DEPArray™ V2 parameters	negative, positive	mean intensity-bgsub: 1185 RFU	

First, a semi-quantitative scale, which classifies the cells into 0, 1+, 2+, and 3+ was employed, as previously described (9). 0 and 1+ cells are considered negative. They have a pixelated cell staining with a grey and pixelated background. While 2+ and 3+ cells have sharp edge and fluent cell staining with a dark to completely black background respectively. Using an online survey platform, CellTracks Analyzer II® CTC images (n=2000) were reviewed by 17 international scientists and pathologists, who were trained to perform the visual semi-quantitative HER-2 scoring. The obtained scores were benchmarked against the automated scoring results by ACCEPT (8).

Secondly, ACCEPT was used to automatically analyze the raw TIFF images from the CellTracks Analyzer II® (Menarini Silicon Biosystems Inc.)(8). CTC identification, and classification of HER-2 intensity levels (negative, medium, and high) was performed with gating and HER-2-FITC cutoff settings as previously described (8, 17). It generates multiple parameters on shape and fluorescent intensities per individual cell. Cut-offs were set for CTCs (CD45-APC mean intensity ≤ 5, DAPI mean intensity >45, CK-PE mean intensity >60, CK-PE size 16-400, CK-PE overlay with DAPI >0.2) and leukocytes (CK-PE ≤ 5, DAPI mean intensity >45, CD45-APC mean intensity >5, CD45-APC size 16-400, CD45-APC overlay with DAPI >0.2). HER-2 scoring is divided in negative (HER-2 mean intensity is 0), medium (HER-2 mean intensity is ≤100), and high (HER-2 mean intensity is >100).

Thirdly, CellSearch®-enriched CTC samples were transferred to the DEPArray™ system (Menarini Silicon Biosystems Inc.), as described previously (18). Briefly, the loaded sample is automatically injected into the microchamber of a cartridge where single cells are trapped in one of 16,000 electrical cages. IF images of the entire surface area are taken and cells are automatically detected by the system, generating an image library and 40 parameters per individual. This can be used for further cell sorting.. HER-2-FITC scan filter configuration was set to an exposure time of 800ms with a gain of 5%, empirically defined using cell line samples, and maintained throughout all preclinical and clinical experiments. HER-2 scoring was performed using the relative fluorescent units (RFU) of the HER-2-FITC signal after background subtraction (i.e. Mean Intensity-bgsub parameter). A cut-off for HER-2 positivity was defined at >1185 RFUs. Using this cut-off, 95% of the analyzed cells within the theoretically expected HER-2-positive and -negative cell lines classified as positive and negative, respectively.

HER-2 qRT-PCR on CellSearch® enriched samples

CellSearch® CTC and CellSearch Profile® samples were taken simultaneously to facilitate comparison between IF and gene expression. CellSearch Profile®-enriched tumor cell fractions were subjected to HER-2 expression analysis, as described previously (19). Tumor cell fractions were lysed in 250µL RNeasy RLT+ buffer (Qiagen BV, The Netherlands) and stored at -80°C until RNA isolation. RNA was isolated from these lysates with the AllPrep DNA/RNA Micro Kit (Qiagen). Complementary DNA (cDNA) synthesis and *ERBB2* pre-amplification was performed on 25% of the isolated RNA, using the RevertAid H Minus First Strand cDNA and TaqMan PreAmp amplification kit, respectively (Thermo Fisher Scientific #K1632 and #4488593, Merelbeke, Belgium), in a GeneAmp® PCR System 9700 (Life technologies). Pre-amplified cDNA was diluted 15x with 1xTE-buffer, after which qRT-PCR was performed to quantitate *ERBB2* transcripts. Additionally, 3 housekeeping genes (*SDHA*, *HMBS* and *HPRT1*) were used to control for sample loading and RNA integrity. Epithelial (*EPCAM, KRT19*) and leukocyte (*PTPRC* coding for CD45) markers were used to control for presence of epithelial and leukocyte content. PCR reactions (40 cycles) were performed using TaqMan Gene Expression Assays with Universal PCR Master Mix No AmpErase UNG on a 7900HT Fast Real-time PCR System (all Applied Biosystems). A calibrator (positive control) sample was used in each run to assess inter-run variability. Negative controls included a NTC and -RT sample. Only samples with a Cq value of <35 for each of the 3 reference genes, were considered of sufficient quality and quantity. *ERBB2* Cq value of every sample was normalized to the epithelial signal within that sample (dCq). All samples were further normalized to the calibrator (ddCq). RGE was calculated as 2^-ddCq.

HER-2 FISH on CTC

A FISH protocol was established using the preclinical cell line model. CellSearch® enriched cells were spinned on a Superfrost Plus slide (Fisherbrand) using a Slide carrier with a 1ml One-Funnel Cytochamber (cat. 1662 and 1663 resp., Hettich, The Hague, The Netherlands). Cells were fixed on the slides in acetone at 4˚C for 5 minutes. FISH on slides was performed using the DAKO IQFISH kit (Agilent, Diegem, Belgium), including a pretreatment step, denaturation and hybridization, a stringent wash, mounting, and reading. A detailed prescription of the protocol is written in the table below. Before and after FISH, slides were scanned on the BioView® with CTC protocol (BioView®, Israel), in order to detect and map the tumor cells in the leukocyte background before the FISH protocol was applied. Amplification status was scored by the algorithm included in the BioView® software. Furthermore, four patient samples were subjected to HER-2 FISH analysis on BioView®.

Step	Reagent	Concentration	Interval/T	Manufacturer/batch
Slide preparation				
Cytospin			5 min at 800g	Fisherbrand, Superfrost Plus Hettich, #1662 and #1663
Cell fixation	Aceton	1:1	5 min at 4°C	
Add coverslip and scanning	Glycerol	1:1		
Pretreatment				
Remove coverslip				
Cell permeabilization	Dako pretreatment solution 20x	1:20	5 min at 97°C	Agilent, #K5731
Wash	Dako wash buffer 20x	1:20	2x3 min at RT	Agilent, #K5731
Pepsin incubation	Pepsin	1:1	2-3 min at 37°C	Agilent, #K5731
Wash	Dako wash buffer 20x	1:20	2x3 min at RT	Agilent, #K5731
Dehydration	Ethanol	70%, 85%, 100%	3x2 min at RT	Fisher, #BP2818-4
HER-2/CEP17 probe mix	HER2/CEP17 PathVysion probe mix	2:1		Abbott, #06N46-030
Add coverslip and sealant	Fixogum	1:1		Kreatech, #LK-071A
Denaturation and hybridization				
Preheat hybridization chamber			37°C	
Denaturation			5 min at 75°C	
Hybridization			3h at 37°C	
Stringent Wash				
Remove coverslip	Dako stringent wash buffer 20x	1:20		Agilent, #K5731
Wash #1	Dako stringent wash buffer 20x	1:20	10 min at 63°C	Agilent, #K5731
Wash #2	Dako stringent wash buffer 20x	1:20	2x3 min at RT	Agilent, #K5731
Dehydration	Analytical grade ethanol, 100%	70%, 85%, 100%	3x2 min at RT	Fisher, #BP2818-4
Mounting and reading				
Nuclear counterstaining	DAPI	1:1		Abbott, #06N46-030
Add coverslip and sealant	Fixogum	1:1		Kreatech, #LK-071A

A large-scale retrospective study in metastatic breast cancer patients using circulating tumor DNA and machine learning to predict treatment outcome and progression-free survival

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ABSTRACT

Purpose

Monitoring levels of circulating tumor-derived DNA (ctDNA) represents a non-invasive snapshot of tumor burden and potentially clonal evolution. Here we describe how a novel statistical model that uses serial ctDNA measurements from shallow whole genome sequencing (sWGS) in metastatic breast cancer patients produces a rapid and inexpensive assessment that is predictive of treatment response and progression-free survival.

Patients and Methods

A cohort of 188 metastatic breast cancer patients had DNA extracted from serial plasma samples (total 1098, median=4, mean=5.87). Plasma DNA was assessed using sWGS and the tumor fraction in total cell free DNA estimated using ichorCNA. This approach was compared with ctDNA targeted sequencing and serial CA 15-3 measurements. The longitudinal ichorCNA values were used to develop a Bayesian learning model to predict subsequent treatment response.

Results

We identified a transition point of 7% estimated tumor fraction to stratify patients into different categories of progression risk using ichorCNA estimates and a time-dependent Cox model, validated across different breast cancer subtypes and treatments, outperforming the alternative methods. We then developed a Bayesian learning model to predict subsequent treatment response with a sensitivity of 0.75 and a specificity of 0.66.

Conclusion

In patients with metastatic breast cancer, sWGS of ctDNA and ichorCNA provide predictive real-time data on treatment response across subtypes and therapies. A prospective large-scale clinical trial to evaluate clinical benefit of early treatment changes based on ctDNA levels is now warranted.

INTRODUCTION

Breast cancer is the most common cancer diagnosis and the fifth leading cause of cancer death worldwide. Treatment options for patients with metastatic breast cancer have greatly increased but there remains an unmet need to monitor therapy response in real time[1–3]. Accurate real-time methods of monitoring treatment response are required to minimize time spent on ineffective therapies and improve access to more effective therapy. CA15-3, a tumor marker available in the clinic is often used to monitor response but has limited sensitivity and dynamic range[4]. We have shown it has inferior performance when compared with circulating tumor DNA (ctDNA)[4]. ctDNA assays can also provide a rapid, non-invasive and dynamic way of tracking genomic evolution and detecting the emergence of resistance mutations which could prompt therapy change[5–7].

Breast cancer genomic landscapes are dominated by chromosomal copy number aberrations (CNAs), with around 85% of tumor gene expression changes driven by these CNAs [8–10]. CNAs can be profiled using shallow whole genome sequencing (sWGS) of plasma DNA as a rapid and cheap method to characterize CNAs in ctDNA. Crucially, the detection of ctDNA in plasma using sWGS does not rely on any prior knowledge of the originating tumor genome.

Here, we evaluated the utility of ctDNA quantification using sWGS to predict treatment response in a consecutive cohort of metastatic breast cancer patients. We assessed the performance of established analysis tools to measure ctDNA levels, including ichorCNA[11], z-score[12] and t-MAD[13] and developed a Bayesian learning model that uses data from serial ctDNA measurements to dynamically predict treatment response. We also compared this approach to the use of a ctDNA targeted sequencing panel and measuring CA15-3 in the same plasma samples to determine how these different methods performed in predicting treatment response.

PATIENTS AND METHODS

Patient Cohort and Sample Collection

A cohort of 188 patients with metastatic breast cancer was recruited into the DETECT clinical study at Cambridge University Hospitals (CUH), UK between 2012-2019. Eligible patients were those women with metastatic breast cancer undergoing treatment. Serial blood samples were collected at specific time points as shown in **Figure 1**. For patients on chemotherapy, blood samples were taken prior to the next cycle of therapy and for a minimum of four cycles. For patients on continuous treatments such as endocrine therapy, blood samples were taken at routine clinic visits (typically every 3-6 months). Cohort composition for each analysis is shown in **Figure 1a-c**.

Sample Processing and analysis

In total 1,098 blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes and processed within 1 hour for plasma and buffy coat separation (Supplementary Methods). DNA was extracted from plasma and buffy coat and sequencing libraries for sWGS were prepared using 5 ng of cfDNA from each sample and 50 ng of DNA from buffy coat using the ThruPLEX® Tag-seq Kit (Takara Bio, Inc., Shiga, Japan) as described in the manufacturer's instructions. The sequencing libraries were purified and quantified as detailed in the Supplementary Methods.

Targeted sequencing of 20 breast cancer specific genes (NGTAS), as described in Gao,*et. al*.[14], was also performed using 5ng DNA and library preparation as above for sWGS. Samples were then amplified in triplicate with specific primers using the Fluidigm Access array™ platform.

All libraries were sequenced using an Illumina HiSeq 2000, at a mean depth of 0.1x for sWGS and >100x for NGTAS.

Serum Ca15-3 levels collected as part of routine clinical care were analyzed at the Cambridge University Hospitals biochemistry laboratory (accredited by the United Kingdom Accreditation Service).

Bioinformatic analysis

Sequencing data was processed and analyzed as described in the Supplementary Methods. The copy number profiles produced using QDNAseq[15] were used as input into three algorithms for tumor fraction estimation (ichorCNA[11], z-score[12] and t-MAD[13], see Supplementary Methods). Mutational profiling was performed using the NGTAS pipeline[14], with the maximum variant allele frequency (VAF) of any somatic mutation detected used for assessing its predictive performance.

RECIST Criteria

Progression-free survival (PFS) for each line of treatment was calculated using RECIST 1.1[16] guidelines to determine Progression Free Survival 2 (PFS2) as described previously (the time from the start of a line of treatment until objective progression on medical imaging using computed tomography (CT) of

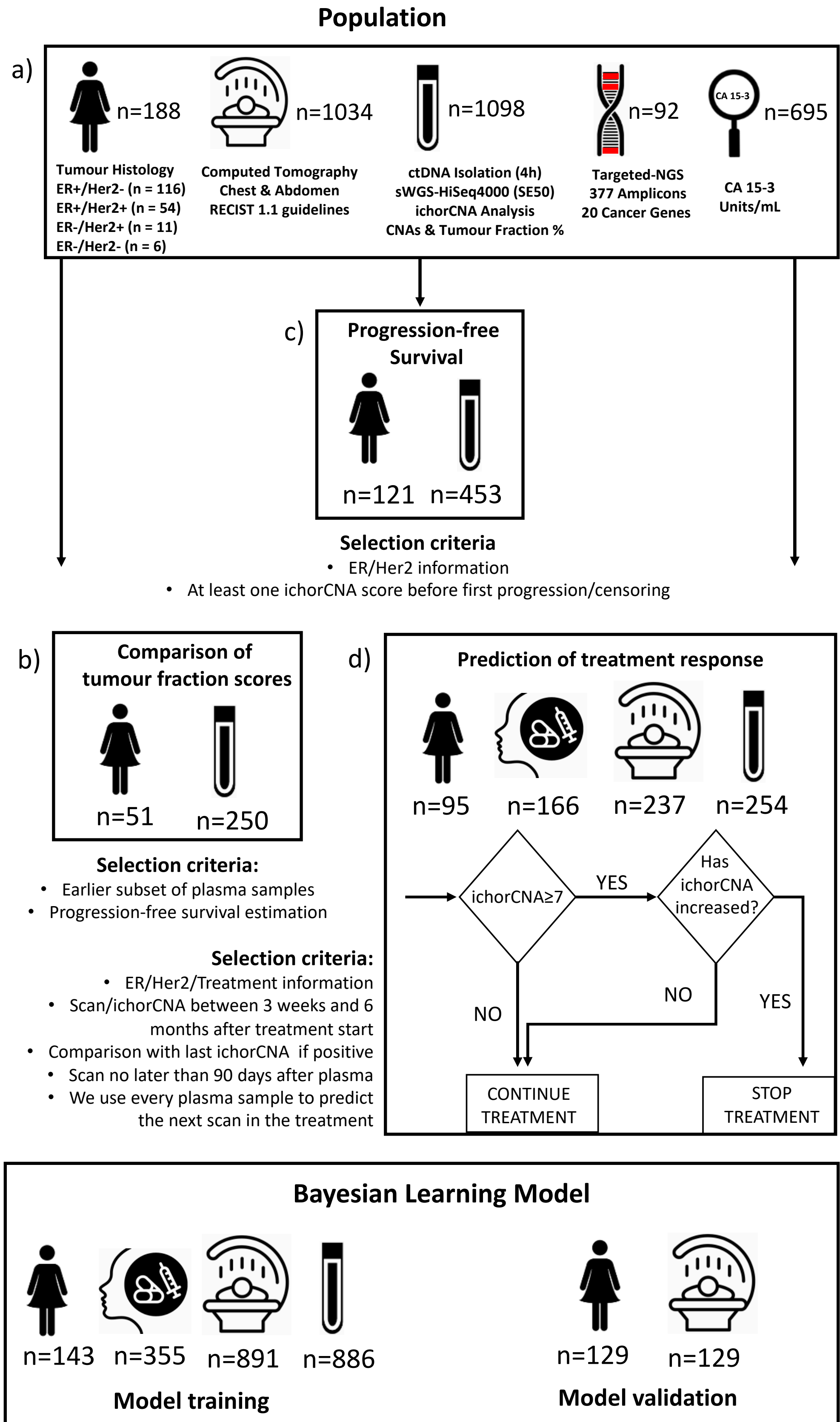


Figure 1. Clinical cohort and sample analysis with histology, sample timelines and treatment types. **A.** Patient numbers, histology and sample processing. **B**. Timeline of ctDNA collection per patient **C.** Treatment types assessed. *one patient was excluded as no relevant CT staging scan. 29 samples were not used for sWGS library preparation due to the very low levels of DNA in these samples (<5ng DNA total).

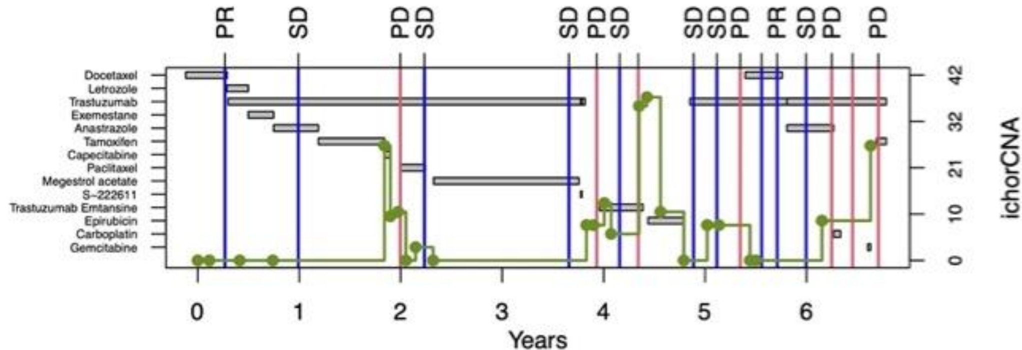
the chest and abdomen, or death)[17,18]. For each line of treatment, the CT scan prior to the start of this line of treatment was used as baseline. Imaging of the head was not included in the assessment of progression in this study. Longitudinal data available for two representative patients is shown in **Figure 2**.

RESULTS

ichorCNA best predicts progression-free survival

Several methods for ctDNA fraction estimation using sWGS CNA data of DNA extracted from plasma have been proposed, including ichorCNA[11], z-scores[12] or t-MAD[13]. In order to identify which method performed best at estimating tumor burden in plasma, we used a discovery dataset consisting of the first 51 patients totaling 250 plasma samples. We built univariable time-dependent Cox models for PFS using each measure (ichorCNA, t-MAD, or z-score) individually. Using the c-index[20] to assess the three models showed ichorCNA performed best (ichorCNA c-index=0.71, se=0.05; t-MAD c-index=0.68, se=0.06; z.OR c-index=0.61, se=0.06).

We subsequently determined a threshold to identify patients at high risk of progression in order to facilitate clinical implementation. We used a spline term to model the effect of ichorCNA score on the hazard of progression and then fitted a segmented linear regression. This revealed a linear increase in the risk of progression followed by a changepoint at a score of 6.4% (**Supplementary Figure 1**, whole cohort, n=121). As expected, this categorization of the score showed lower predictive power than the continuous model (c-index=0.64, se=0.04, vs. 0.71, n=51), though the introduction of a static threshold



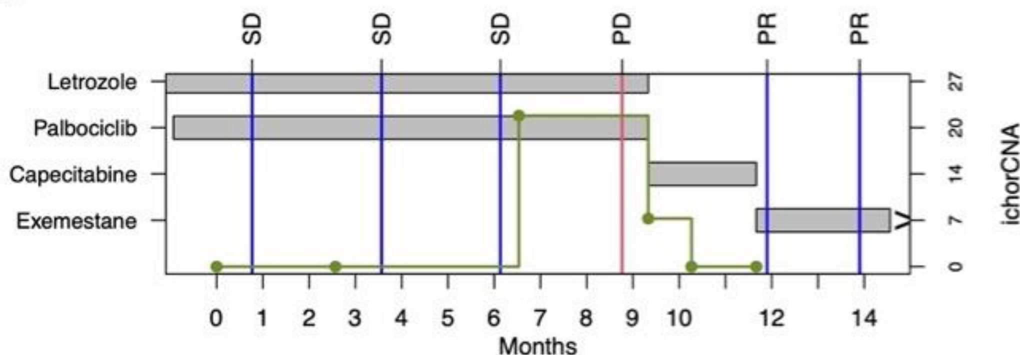


Figure 2. Profiles of two patients, showing the complexity of the longitudinal data available for each patient. Treatment regimes, CT Scans (PD=Progressive disease, SD=Stable disease, PR=Partial response) and ichorCNA scores are shown. Treatments ongoing are labelled with a > symbol.

makes clinical implementation easier. By choosing a threshold of 7%, the hazard ratio of progression for high ichorCNA score (≥7%) was 5.97 [3.72, 9.59] for the whole cohort. When we stratified tumors by subtype, we observed differences in predictive ability. In ER+HER2- patients, the hazard ratio was 5.60 [2.80, 11.18] and the expected time until progression for patients with a 'low risk' ichorCNA score (<7%) was 19.8 months, versus 8.2 months if the ichorCNA score was high. The prognostic effect was even higher for HER2+ patients, with a hazard ratio of 7.55 [3.39, 16.82] and a difference in the expected time of progression of 31.1 vs. 5.0 months. **Figure 3** shows the predicted survival curves for two patients with high and low ichorCNA scores.

We observed that ichorCNA can also be used to predict overall survival. Using a continuous score with a linear term (p=9.77x10-8, hazard ratio: 1.08, [1.05, 1.11]) and the common threshold of 7%, a higher ichorCNA value was associated with an increased risk of death (p-value: 0.001, hazard ratio: 7.40, [2.38, 23.00]). This result highlighted the ability of ichorCNA to predict prognosis and the utility of our proposed threshold.

Comparison with targeted sequencing (NGTAS) and CA15-3

Targeted mutational sequencing data was also available in 92 samples obtained from 22 patients (described in Gao *et. al.*[14]). In this smaller sub-cohort and using the maximum VAF observed in the sample, we did not observe a significant effect on the hazard of progression (p=0.24) while the ichorCNA remained significant (p= 0.034). Combining the two scores into the same model did not improve the fit (p=0.44).

We also looked at the ability of CA15-3 to predict response. For this analysis we compared data from a sub-cohort of 66 patients where 695 CA15-3 measurements were available with the corresponding ichorCNA values for the matched patient samples. Acknowledging the limitations of comparing both datasets (different number of patients and different sampling intervals, with the CA15-3 and the ichorCNA not always taken on the same day), a similar model using CA15-3 showed lower performance in terms of c-index (0.60, se=0.074) than the model using ichorCNA scores.

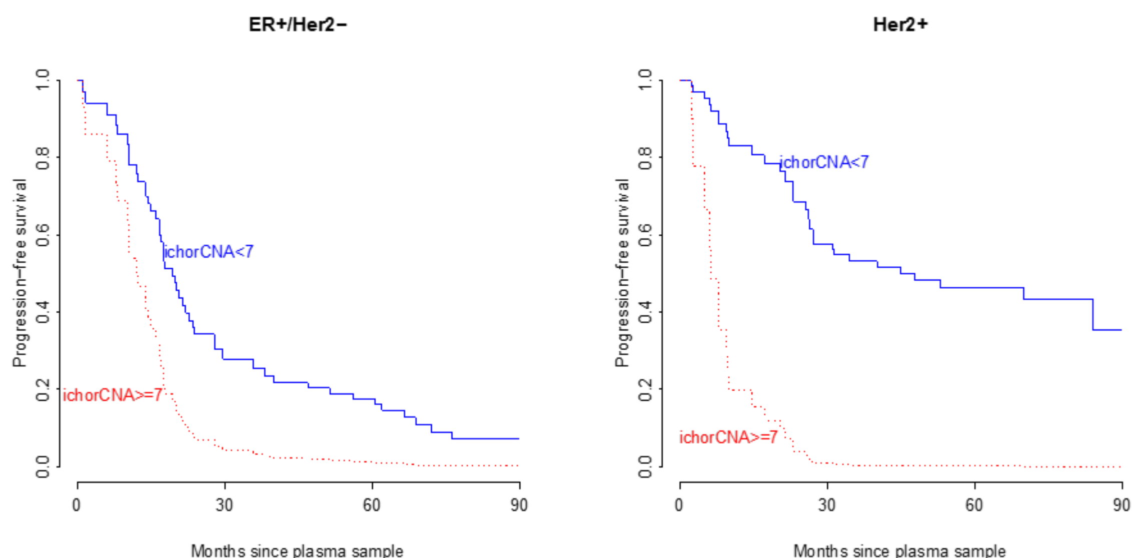


Figure 3. Predicted progression-free survival curves for two patients (ER+/HER2- and HER2+) with low and high ichorCNA scores. The hazard ratio for each subtype has been obtained from a different model fitted for each disease subtype.

Considering a threshold of 0.01% VAF for NGTAS and 31U/ml (positivity threshold) for CA15-3, there was 78% concordance between NGTAS and ichorCNA, and a 60% concordance between ichorCNA and CA15-3. **Figure 4** shows the instances where these measures showed discrepant results. Although the sampling times were different, comparing these values with the closest CT scan gave a better performance to the maximum VAF from NGTAS based on the area under the curve, but with a very small number of observations (0.78, n=15, ichorCNA=0.63, n=96 and CA15-3=0.59, n=81). Given these results, we decided to focus on ichorCNA for the rest of the study.

ichorCNA ctDNA fraction predicts treatment response

Using the ichorCNA 7% threshold we evaluated its ability to predict subsequent response or resistance to treatment at each time point. We used the following rules: (i) for a prediction of response ichorCNA <7% or ≥7% if a decrease from the previous time point, (ii) for a prediction of progression ichorCNA ≥7% and an increase (or no change) from a previous time point (when available). The preceding time points needed to be on the same treatment to be relevant for decision-making. The application of these rules produced a sensitivity of 0.42 and a specificity of 0.90. The median time prior to prediction of progression for concordant decisions (stopping treatment) was 36 days (versus 29 days for discordant). For concordant decisions about continuing treatment the median time prior to the CT scan was 40 days (versus 38 days for discordant). These differences in the time where the decisions were made were not statistically significant and would not explain the difference in predictive ability.

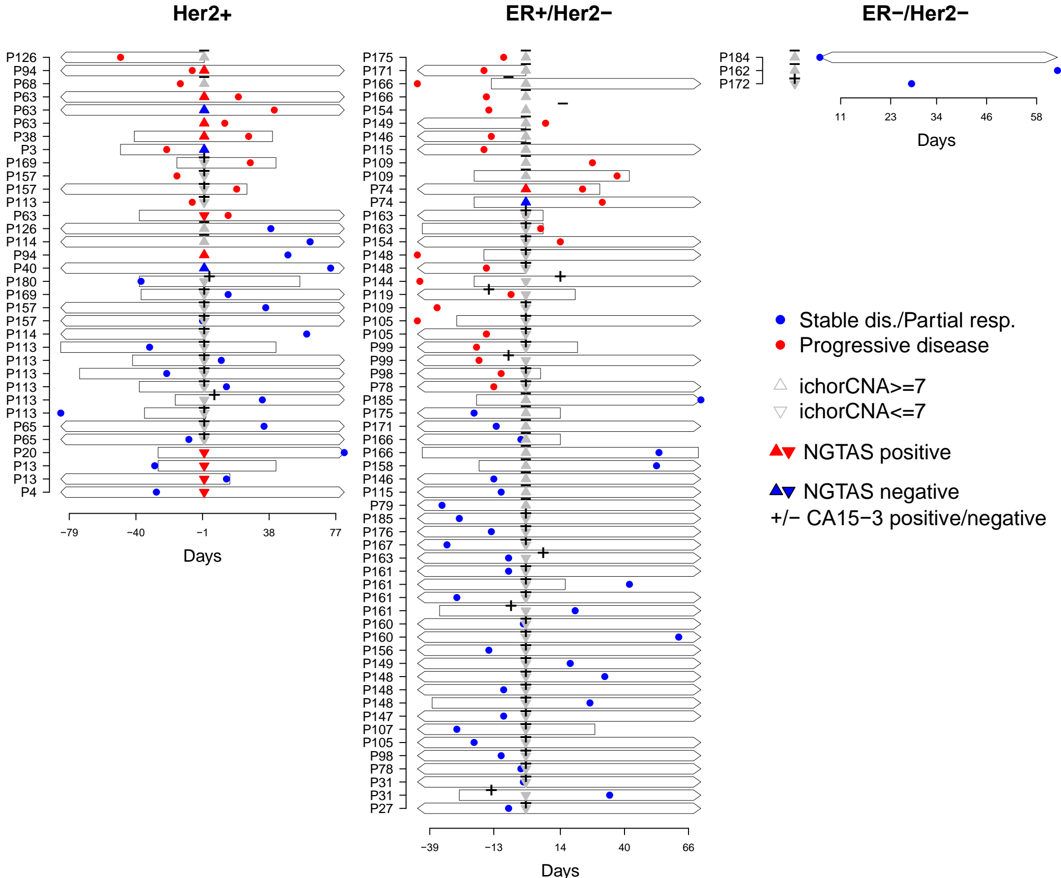


Figure 4. Discrepant results of ichorCNA measured with sWGS, mutant VAF measured with NGTAS and CA15-3. 96 instances where the CT Scan was done less than 90 days from or to the plasma sample and the CA15-3 was taken 15 days apart from the plasma sample measure are considered, Discrepancies are considered based on the 6 threshold for ichorCNA, 30 for CA15-3 and 1% for VAF.

	Specificity			
	71%	69%	**66%**	61%
Sensitivity	68%	69%	**75%**	76%

Table 1. Sensitivity at various levels of specificity (last observation not included for each patient)

A Bayesian machine learning model (BAY-ML) to predict treatment response

Motivated by these findings, we developed a novel statistical model to predict treatment response (based on RECIST criteria) using the full history of ichorCNA scores and CT imaging (**Figure 5a**). The model comprises two components, one that includes the characteristics common to the cohort (ER/HER2 status and treatment regime) and another that models the patient specific longitudinal ctDNA scores and disease progression measurements on CT. The model is fitted using a two-stage approach: in the first stage, the evolution of the repeated ctDNA measurements is summarized by random effects obtained by fitting a linear mixed effects model, and in the second stage, the resulting random effects are used as covariates in a logistic regression to predict the risk of progression. Both steps include a set of independent variables, such as the treatment regime and the tumor (see Supplementary methods). The model adapts to each patient learning from common cohort's effects such as the current treatment or the tumor subtype in both the ichorCNA trajectories and the probability of progression, but the model also learns from specific features of the patient. Leaving the final observation out for each patient (see Supplementary methods for details), we evaluated the sensitivity for predicting progressive disease when using ctDNA information at several clinically relevant specificity thresholds (**Table 1**). Leaving the last observation out in the model estimation, at

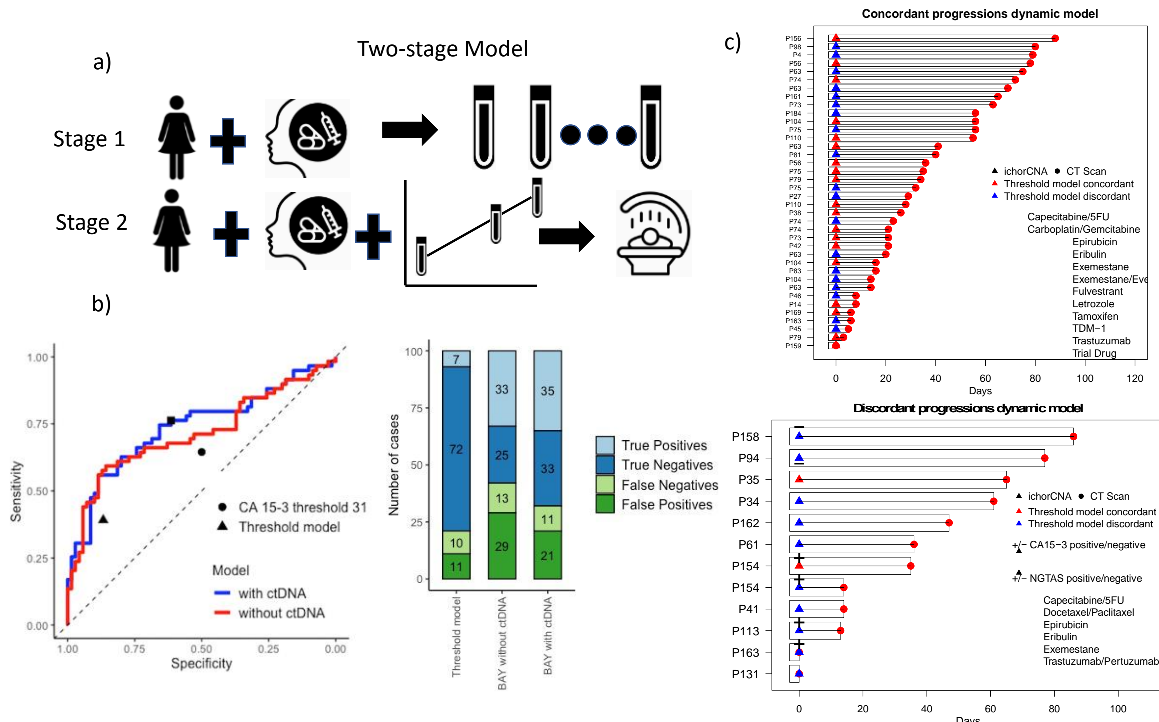


Figure 5. BAY-ML model. A) Visual summary of the two-stage model. B) Left: Receiver operator characteristic (ROC) curve of our dynamic predictive model. The predictions were obtained with the last CT scan for each patient, left out when the model was fit. Right: Number of true/false positives and negatives over 100 patients when the simplest threshold model and when the longitudinal ctDNA scores are considered or not into the BAY-ML model. C) Instances where the model correctly predicted progression and instances where it did not, comparing the available information at that moment.

66% specificity, the sensitivity for detecting PD was 75%, significantly higher than our previous model based on a simple stopping rule. **Figure 5b** shows the receiver operator characteristic (ROC) curve for the model, highlighting the improvement in predictive performance, particularly in sensitivity, when iterative ichorCNA data is included.

Treatment types

We looked at the predictive capability of ichorCNA for different treatment types. Predictions of response to targeted therapy (mainly CDK4/6 inhibitors and anti-HER2 therapy) and chemotherapy showed a higher concordance with CT results than endocrine treatment alone though this would need to be substantiated with larger data sets for statistical significance (see **Figure 6**). Blood test sampling was also less frequent for patients on endocrine treatment alone which could also explain this apparent difference.

Discordant result analysis

In order to understand better the limitations of ichorCNA we evaluated decisions whereby the ichorCNA values did not agree with disease status as measured using RECIST 1.1. The ichorCNA score was high (predictive of treatment failure) but the subsequent CT showed RECIST stable disease in 24 instances. In 11/24 cases (46%), progressive disease was observed on the subsequent CT scan, suggesting early detection of progression by ctDNA, with a median lead time of 4.8 months. In 2/24 cases disease progression was observed in the brain (not evaluated as part of the RECIST criteria for this study).

In the cases where progression occurred on a CT scan but were not detected by ichorCNA (22 instances in total), in 5 of these cases this followed treatment with palliative radiotherapy which we hypothesize may have led to a reduction in ctDNA levels, separate to other systemic treatments. In 2/22 of cases a mixed response was seen, and it may be that having additional information from ctDNA could have aided the decision-making process. For an additional 6/22 cases low volume changes were seen (<10mm in <2 lesions) which may have had limited clinical impact. In 60% of these cases clinical management continued unchanged.

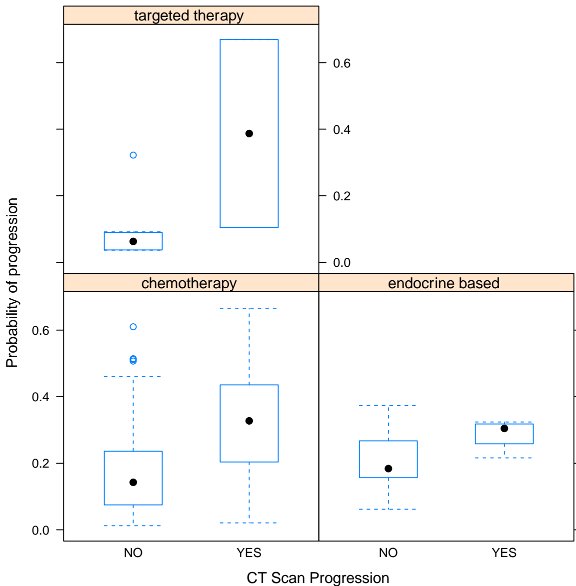


Figure 6. Analysis showing the association between the probability of predicting progressive disease and the result of the CT scan depending on different treatment types.

DISCUSSION

We have demonstrated the real-world performance of ichorCNA in predicting treatment response for metastatic breast cancer patients and have used a novel Bayesian machine learning approach which uses longitudinal data, improving its predictive capability. This approach has several advantages over other methodologies. Firstly, all data is useful (including ichorCNA = zero) as it evaluates changes across the whole genome, having an important advantage over targeted sequencing, whereby a lack of somatic mutations in ctDNA only means that those specific mutations have not been detected. Moreover, within our study the average turnaround time, including library preparation and sequencing, was less than one week, and the assay we used was cheaper than currently available alternatives, with a typical cost of £100 per sample including processing costs. This compares favourably to commercially available ctDNA panel tests e.g Guardant360 (98 genetic alterations) at ~£4,000 per sample and MSK-IMPACT™ panel (468 genes) at ~£2,000 per sample. In addition, ctDNA monitoring could reduce the need and frequency of CT scanning if levels continued to be low.

Altogether, this shows that the method we describe in this study can be cheaply deployed within the clinic for therapy monitoring in real time. Importantly, the prediction capability was agnostic of breast cancer subtype and treatment regime, though it may be that predicting response to targeted therapy and chemotherapy may be slightly more reliable than for endocrine treatment alone. This may reflect the biology of disease as patients on targeted treatment and chemotherapy are likely to have more aggressive disease or have a higher tumor burden potentially making ctDNA levels higher and more dynamic. Moreover, cytostatic treatments are probably less likely to cause large changes in ctDNA compared with cytotoxic treatments. For a subset of cases, we also had data on CA15-3 levels and somatic mutations from a targeted sequencing panel. From this analysis ichorCNA was more accurate than CA15-3, and targeted sequencing data did not improve the predictive power with the caveat these comparisons were in smaller numbers of patients.

Due to lower patient numbers, we were less able to comment on any specifics for triple negative breast cancer. A prior study in metastatic triple negative breast cancer patients found a similar link between PFS and ichorCNA score[19] and we do not believe there would be any significant difference for this subtype though the optimal threshold may be different. This also highlights the potential benefits of using machine learning to improve predictive power. We initially developed a simple stopping rule that is easy to implement in the clinic that is prognostic of progression and can predict treatment response with moderate success. We improved on this using a dynamic model that uses the history of the patient to predict more accurately the probability of progression under a given treatment.

Thinking more broadly about estimating ctDNA it could be that this is a better marker of overall disease activity than purely measuring disease on a CT scan. In a recent publication evaluating the use of ctDNA in the neoadjuvant setting it was shown that the presence of residual ctDNA post operatively was more predictive of relapse than pathological complete response[20]. We hypothesize that using the approach we describe here could provide benefits in terms of both quality of life, by reducing unnecessary toxicity, and increased access to more efficacious treatments in a timely fashion. This is also a realistic prospect as genomic assessment using ctDNA is rapid and relatively inexpensive making it accessible to public health systems. Our findings need to be fully evaluated in a prospective randomized clinical trial to assess the use of ctDNA to make treatment decisions, comparing it also to endpoints such as quality of life and overall survival.

DATA AVAILABILITY

Shallow whole genome sequencing data for all samples has been deposited at the European Genome-Phenome Archive (EGA), which is hosted by the EBI and the CRG, under accession number.

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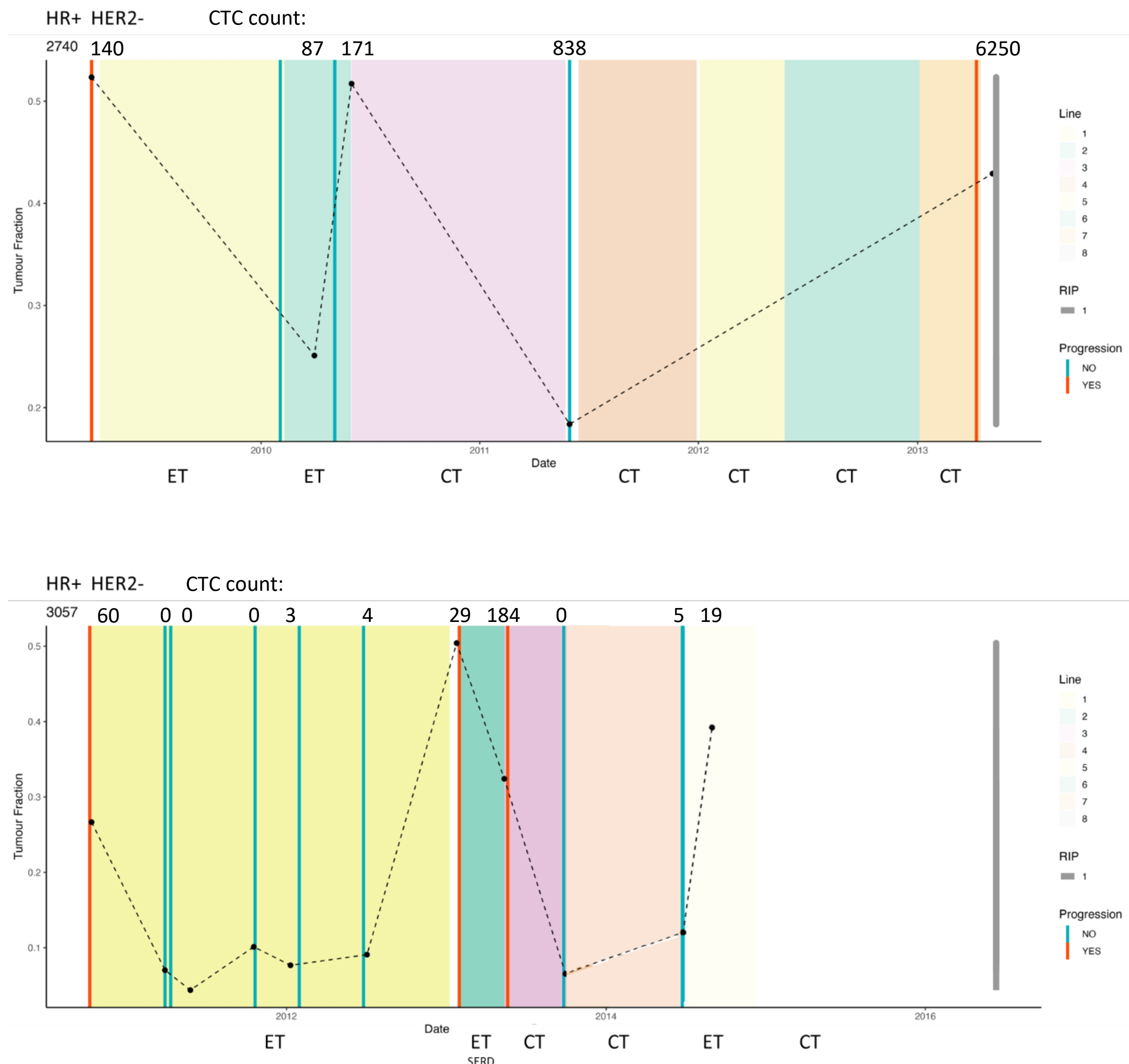
ADDENDUM

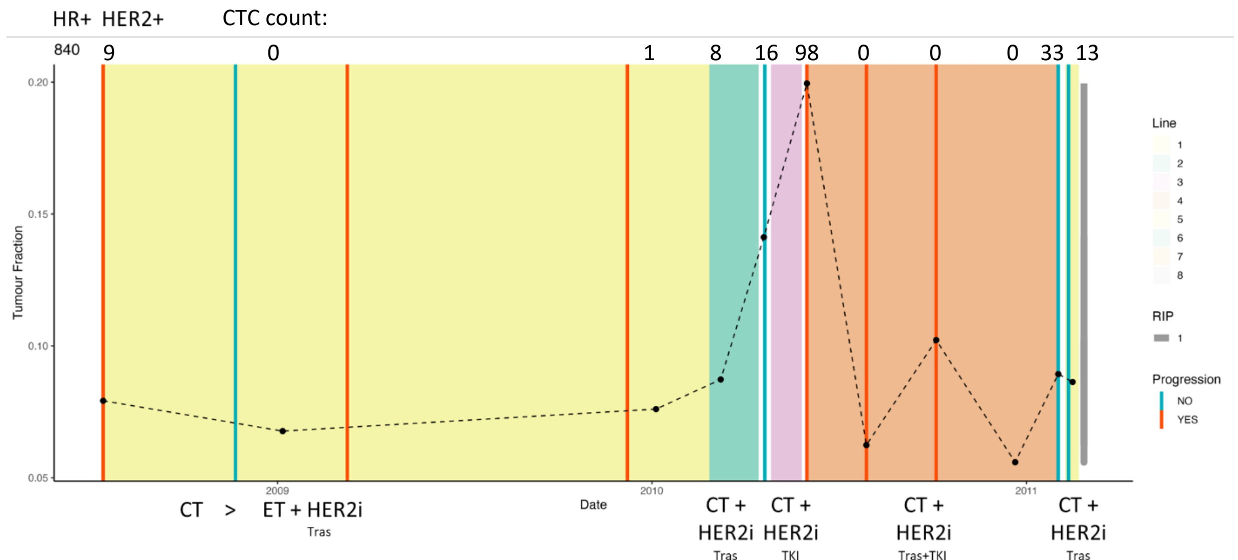
For above study, longitudinal ichorCNA values of retrospectively collected plasma samples from the Sint Augustinus Antwerp-cohort were used for a training set to develop the Bayesian learning model to predict subsequent treatment response.

From 74 patients 814 plasma samples and 74 buffy coats were sequenced. Figures below demonstrate **preliminary data** of tumor fraction per sample. Colored blocks depict various lines of therapy in the metastatic setting and vertical lines are scan evaluations. The five graphs of **figure 1** show often decrease in tumor burden (CNA values) after the start of a therapy line, and subsequent increase at time when treatment switch is needed. **Figure 2** depicts increase in tumor burden at time of progressive disease on scans, just before therapy switch. **Figure 3** shows the response on therapy.

Limitations of this work is the diverse population in this retrospective Antwerp-cohort, as well as infrequent sampling. Ideally, samples should be taken at baseline, around 8 weeks into therapy, and at time of progressive disease (i.e. the new baseline of the next line of therapy). Missing data can interfere with the predictive value ichorCNA values like depicted in the last graph of **figure 1**.
(Abbreviations: HR hormone receptor, ET endocrine therapy, CT chemotherapy, i inhibitor)

ADDENDUM FIGURE 1

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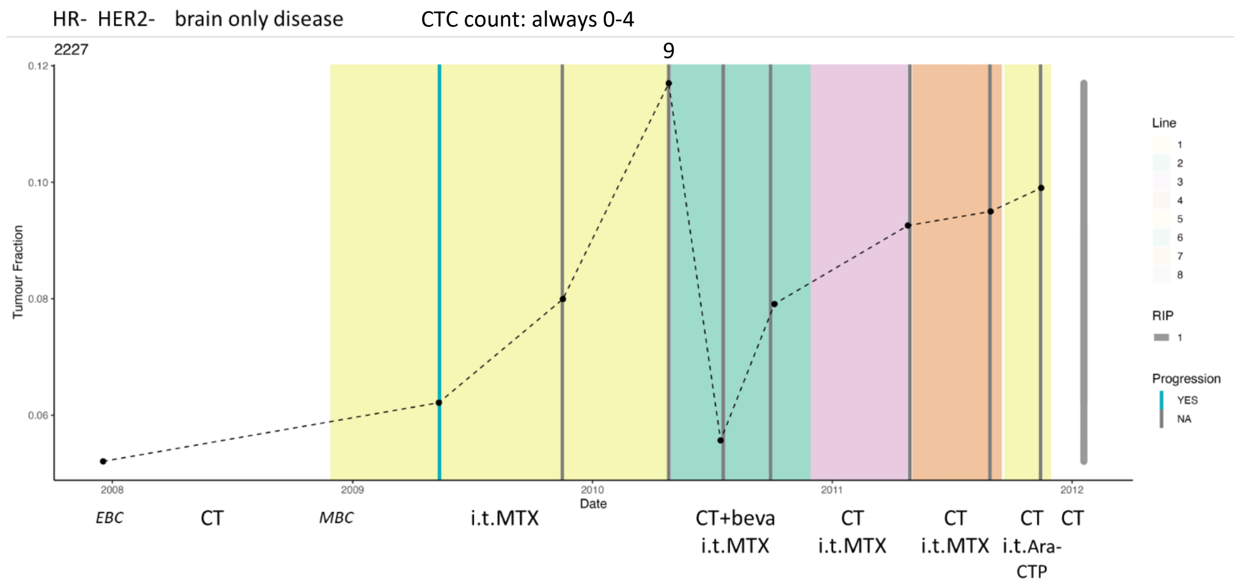
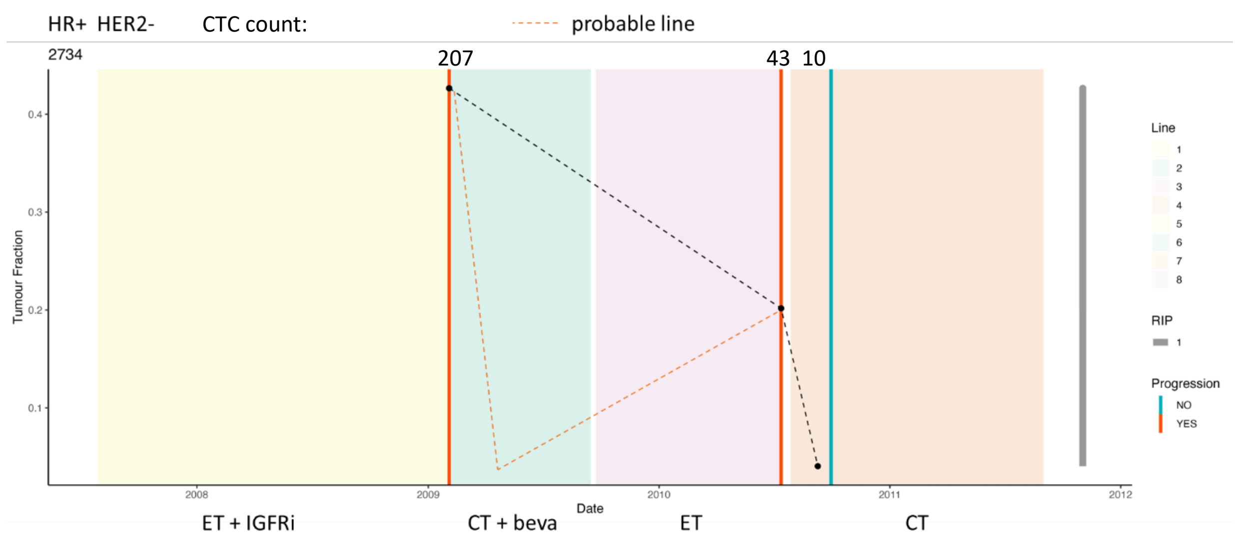
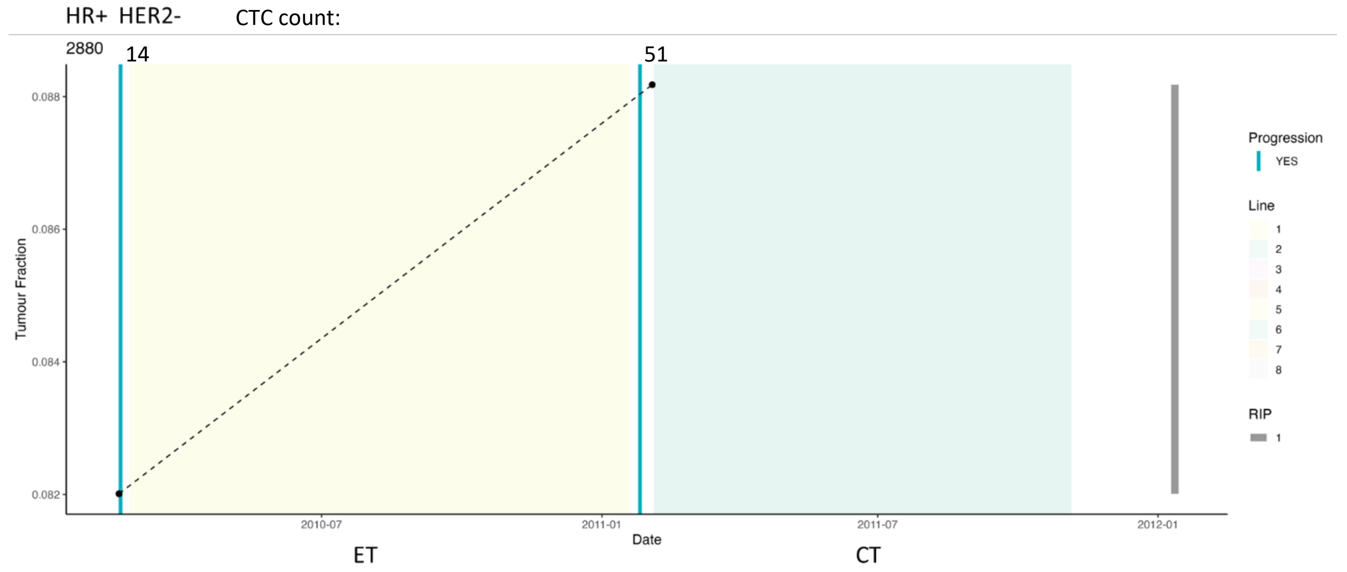
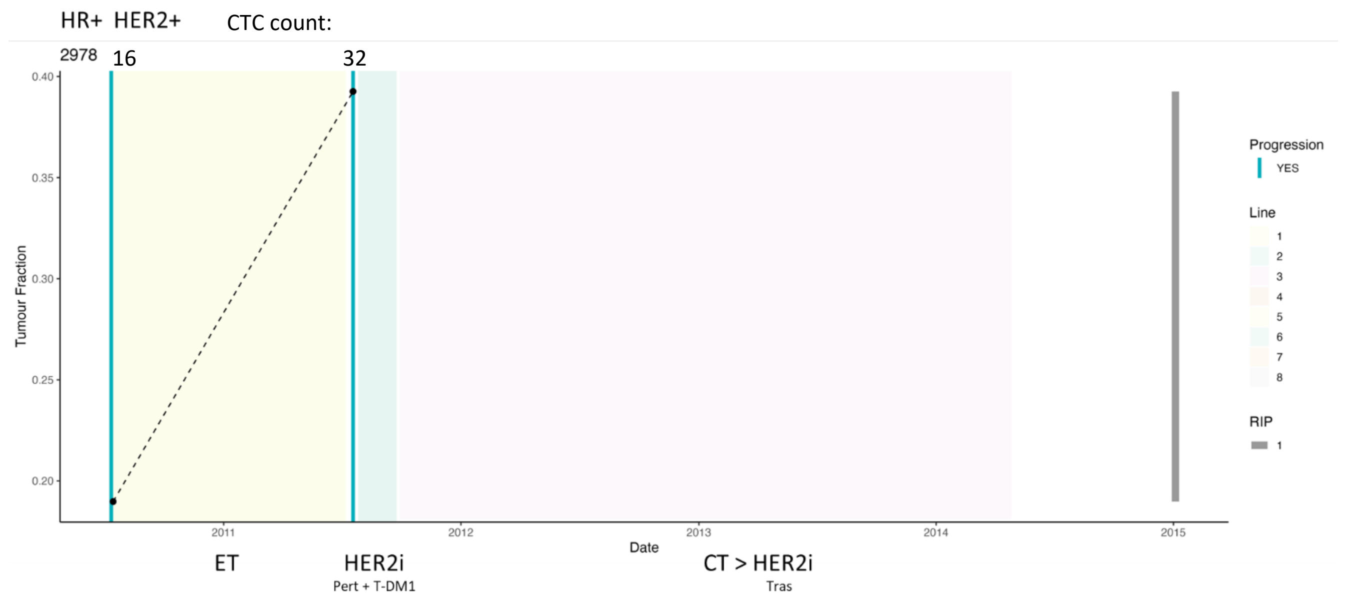
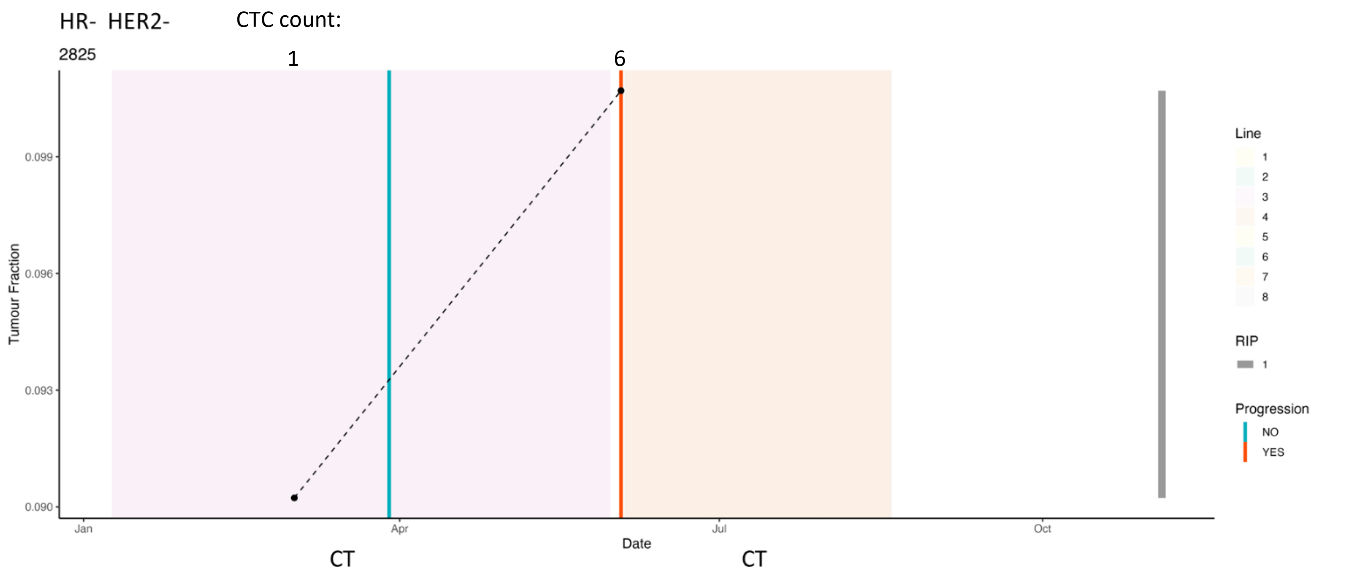

HR- HER2- brain only disease
CTC count: always 0-4
2227
9
Tumour Fraction
Date
EBC
CT
MBC
i.t.MTX
CT+beva
i.t.MTX
CT
i.t.MTX
CT
i.t.MTX
CT
i.t.Ara-CTP
CT
Line
RIP
Progression
YES
NA


HR+ HER2-
CTC count:
probable line
2734
207
43
10
Tumour Fraction
Date
ET + IGFRi
CT + beva
ET
CT
Line
RIP
Progression
NO
YES

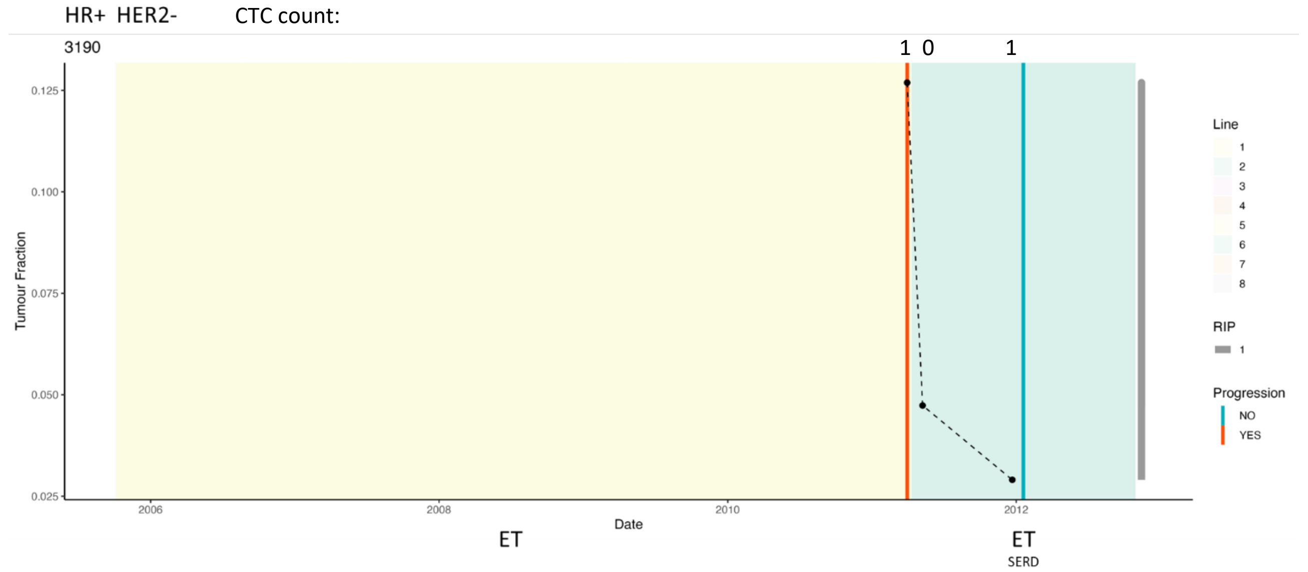
ADDENDUM FIGURE 2

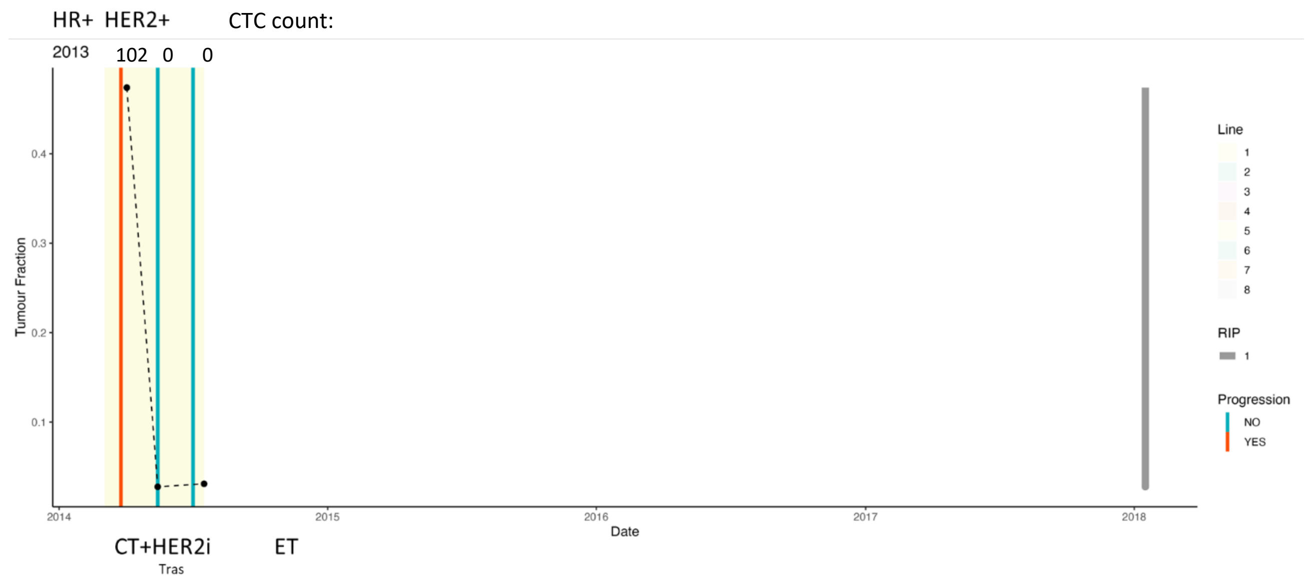


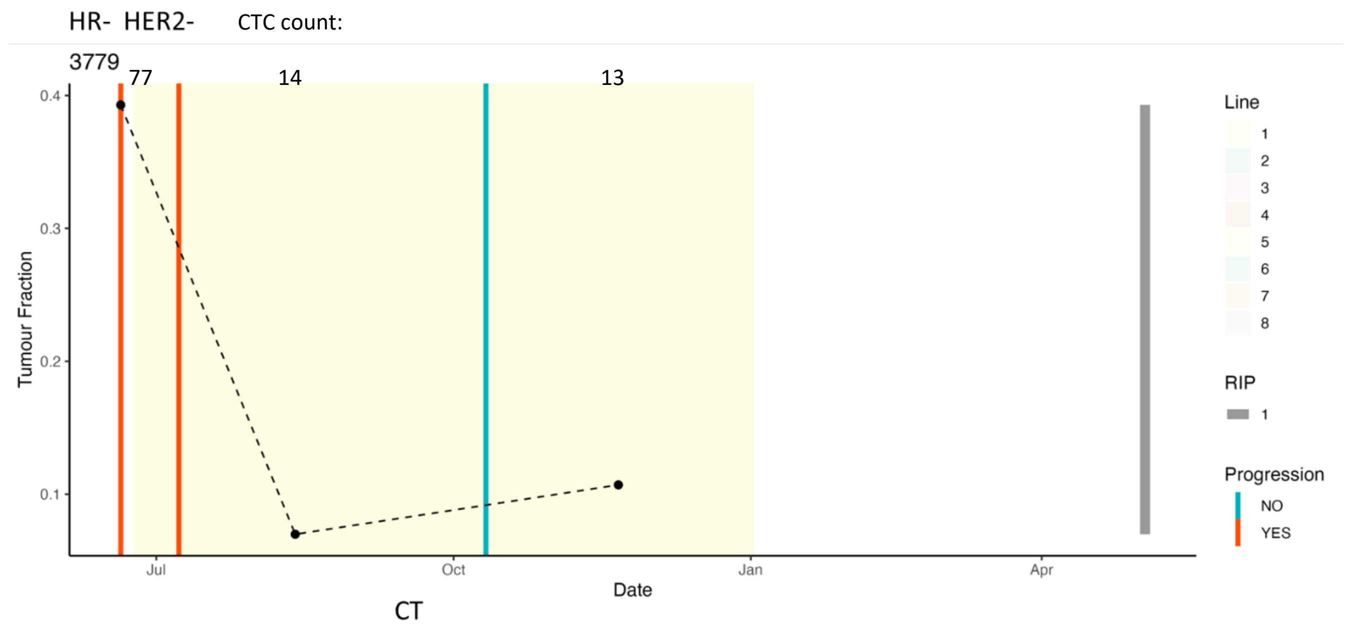




ADDENDUM FIGURE 3







6

General discussion

GENERAL DISCUSSION

Technical considerations

Circulating tumour cells (CTCs) and circulating tumour DNA (ctDNA), but also RNA, exosomes, proteins are all present in liquid biopsy samples. An exploration of the advantages and disadvantages of each substrate and how to better incorporate them into clinical application is needed to achieve more clinical validity.

Various CTCs detecting technologies have emerged. Epithelial marker-based CTC detection technologies, such as the CellSearch system, have been extensively validated. Although its drawbacks have become increasingly acknowledged, since CTCs are frequently studied as part of the metastatic cascade in the early stages of breast cancer. These studies focus at the phenotypic changes like epithelial-to-mesenchymal transition (EMT), CTC clusters, and interactions between CTCs and peripheral blood cells (2), indicating insufficient capture efficiency of EpCAM-based methods. Evolution in multiple-marker and marker-independent CTC enrichment has already increased yield and diversity of CTCs (3, 4, 5, 6). Still, mesenchymal marker-based detection technologies may also be contaminated by non-CTCs, as do physical-property-based CTC detection technologies (6). In the last decade efforts have been made to optimize microfluidic- and nanotechnology-based CTC detection technologies, though they are still lacking large-scale clinical validation (6). An extensive summary of novel enrichment technologies can be found in table 2 of (6). For the research described in this thesis, the epithelial marker-based CellSearch system was used. We have studied CTCs in overt metastatic disease, where abundant shedding of these cell represent different tumour sites, justifying the use of EpCAM and cytokeratin for selection of CTCs. In general high cell detection efficiency and contamination removal capability are the two key strengths of a successful CTC detection technology.

Efforts have been made to increase the yield of CTCs in liquid biopsies for downstream analysis. In early cancer stages, studies have been performed with local blood draw such as in the liver veins in colorectal cancer and pulmonary veins in lung cancer (7, 8, 9, 10, 11). Furthermore, diagnostic leukapheresis (DLA) has been applied to increase the yield (12). Many studies are currently focussing on single cell sequencing requiring further sorting of CTCs, that consequently causes cell loss. Isolation of single CTCs can be performed with micromanipulation or FACSorting, though these techniques are very inefficient. New nano-chips for direct single CTC sorting without prior enrichment are being developed (13). Still, various studies have been conducted using the DEPArray system (14, 15). In **chapter 2** we performed a power analysis on the number of CTC that should be analysed to be able to capture the full heterogenetic landscape with its subclones, and applied that by sequencing many pure DEPArray sorted pools of CTC and DTC of three patients with high CTC count (**chapter 3**).

Studies across multiple tumour types have demonstrated the feasibility of molecular analysis of CTCs. In order to use CTC profiling for understanding tumour heterogeneity, disease evolution (through serial sampling), and clinical management, technical improvements are needed. Efforts have been made to improve and standardize both amplification methods (16, 17, 18) and sequencing techniques (19, 20, 21) as well as subsequent bioinformatics and data interpretation (22, 23, 24, 25). This all contributes to more reliable detection of aberrations in CTC research. Mainly the limited amount of genomic DNA, RNA, and protein content of CTCs is a bottleneck for exploring their genome, transcriptome, epigenome, and proteome properties. Various whole genome amplification methods have been developed, with in mind to minimize introduction of artifacts. Still, part of SNV

found in CTCs, especially when not detected in other samples, likely represent passenger mutations with little clinical significance and should be interpreted with caution. Sequencing of WBC samples has shown that the number of private mutations was at least one order of magnitude lower as compared to CTCs (15). Nonetheless, the emerging genome and transcriptome studies of CTCs have recently profited from the fast-evolving technology of single-cell sequencing (6, 26). Moreover, isolation of viable CTCs for culture, three-dimensional organoid cultures, and generating CTC-derived Xenografts (CDXs) constitute new tools for drug development, understanding of drug resistance, exploring the biology of advanced cancers, and identifying novel biomarker signatures. However, the culture of CTCs is very challenging due to limited methods that are available to isolate enough viable CTCs and difficulties mimicking a favourable circulatory microenvironment for CTC survival (6)

In all, the study of CTCs is attractive, and CTC detection and downstream analysis may likely become an essential component of cancer management in the future. On the other hand, cell free (cf)DNA can be obtained with straightforward DNA extraction from plasma, that will deliver fragments within a small size range that do not need cutting or shearing for downstream analysis. This makes cfDNA research far less laborious and expensive compared to CTC studies. ctDNA is detectable in the majority (85.7%) of MBC patients (27) and only requires standard laboratory equipment facilitating its use in daily clinical practice (28).

Nonetheless, cfDNA studies are compromised by very low concentrations of ctDNA, therefor needing highly sensitive and specific methods for detection of cancer alterations like somatic mutations or copy number alterations (CNA) (29, 30). These include digital PCR (31), BEAMing (32), Safe-SeqS (33), Capp-Seq (34) and TamSeq (35) for targeted mutation detection, or whole-genome next generation sequencing (NGS) (36) for copy number analysis. PCR-based methods have a very low limit of detection, are cost-effective and require low hands-on time, making them well suited for large sample series, although they can only detect a limited number of predefined alterations (37). Contrarily, targeted or genome-wide NGS requires more laborious, comes at a higher cost, and generally requires more cfDNA. However, depending on sufficient sequencing depth, NGS can detect every alteration present within the included amplicons (37). In **chapter 5** we describe that with our workflow, the study could be fast (less than one week turnaround time) and cheaply (£100 per sample, compared to £2000-4000 for commercial kits) deployed within the clinic for therapy monitoring in real time.

Implementing ctDNA into the clinic requires standardized procedures for collection and cfDNA extraction. A recent systematic review comparing 33 cfDNA studies in metastatic breast cancer (MBC) discusses the need for cell stabilizing tubes to minimize the leakage of genomic DNA from leukocytes into the plasma thereby decreasing the ctDNA fraction (37). However, most of the included studies made use EDTA tubes (37) –as did we in **chapter 5**– rather than the preservative-based tubes commonly used for ctDNA studies now (i.e. Streck and CellSave tubes) (38). Still, EDTA tubes are reliable with swift processing time, according to ASCO/CAP guidelines (39). Isolation of cfDNA in the 33 studies was predominantly (16 studies) performed by the QIAamp Circulating Nucleic Acid kit (37). This kit showed the highest cfDNA recovery rate in a recent multicenter comparison, which in combination with semi-automated extraction protocols performs most consistently in extracting cfDNA (40). In our study we did use this kit on the QIAsymphony instrument.

Lastly, data analysis and reporting needs to be standardized for successful future clinical implementation of ctDNA for longitudinal monitoring of treatment response. ctDNA fraction can be monitored using VAF or the absolute number of mutant molecules per defined unit (37), whereas for CNAs ploidy profile determines ctDNA fraction. A previous study has compared several

bioinformatics pipelines and found that ichorCNA provided the most stable ploidy profile, with similar purity estimates to ABSOLUTE/FACETS (41). We used a novel Bayesian machine learning approach which uses longitudinal data, improving its predictive capability. Herewith all data is useful (including ichorCNA = zero) as it evaluates changes across the whole genome, having an important advantage over targeted sequencing, whereby a lack of somatic mutations in ctDNA only means that those specific mutations have not been detected. Each of the approaches (mutations, CNA, methylation, cfDNA concentration) for longitudinal monitoring of treatment response have their own strengths and limitations, which are extensively reviewed by Jongbloed *et al* (37).

Subclone analysis and tumour evolution

It is well established now that breast cancer evolves over time under the selection pressure of various factors including treatments received by the patients (42). Breast cancer is mostly a copy number driven disease as activation of oncogenes and inactivation of tumour suppressor genes is key in cancer development. Furthermore, different routes of tumour cell dissemination contribute to heterogeneity (43). Various studies have been performed assessing clonality based on CNA and mutations in both single or pooled CTCs and cfDNA.

Like the CTC research described in **chapter 2**, two very recent studies (2022) used the same CellSearch®/DEPArray™/Ampli1 workflow to decipher clonality in breast cancer CTCs (14, 15). In our study we extensively looked into the CTC as well as DTC profile of three patients, in order to conclude on spatiotemporal heterogeneity. 136 samples of singles and pools (varying 5-150 cells per pool) were sequenced. On mutational level, two patients harboured a truncated PIK3CA mutation, common in ER+ breast cancer (44, 45), detected in the primary tumour and all CTC and DTC samples. Rothé *et al*, profiling 11 singles and 10 pools of CTC, describe a similar finding of the same patient-specific driver mutations in bulk tissue and the majority (~80%) of CTCs in 2 out of 3 patients (15). Homogeneous CNA profiles were found in bulk tissue and CTCs for these two patients as well. Whereas three distinct clones with different CNA profiles and driver mutations (defined by mutations in *ESR1* and *TP53*, respectively) were identified for the third patient (ER+). However, only three CTCs were sequenced. In our targeted panel sequencing data we ended up with an abundance of passenger mutations or single nucleotide variants (SNV), suggesting every patient harbouring over a dozen unique cells within the mayor subclone lineages.

In our study, a baseline intra-patient homogeneity was also seen on CNA level. All samples including the primary tumour had an prominent 8q amplification as well as deletions at chromosome 11q and 17p, with loss of important tumour suppressor genes (like *TP53* on 17p13), known to emerge in early stages of breast cancer and are associated with relapse and worse clinical outcome (46, 47, 48, 49). This general homogeneity with many CNA is coherent with time of sampling, i.e. end stage metastatic disease. Fernandez-Garcia *et al* sequenced 58 CTC samples (majority singles) and 21 WBC samples and found many of the breast cancer- or patient-specific CNA present in all samples within one patient, with only a scarce CNAs marking subclonality (14). Identically, here most of the ten MBC patients had progressive drug-resistant metastatic breast cancer. On top of the homogeneous background, we found a few newly acquired CNA in the liquid biopsy samples compared to the primary tumours. For patient 1, amplification in part of 18q (apoptosis-related genes *Bcl-2* and *NOXA*) was only present in CTCs and cfDNA, while not in the primary tumour nor pleural fluid DTC. Oppositely, partial 5q gain (*FGFR4*) was only seen in two CTC samples and almost all DTCs. Patient 2 had full clonal CNA profile in CTC samples, while samples of patient 3 had various additional amplifications in 1q, 5q, 12, and 16p, in most of the bone marrow DTCs at baseline, then

emerging in the cerebrospinal fluid, to only become dominant in the CTC compartment after second progression. Similarly in prostate cancer, using diagnostic leukapheresis (DLA), analysis of hundreds of CTCs unravelled a marked tumour heterogeneity including subclonal CNAs that were not easily distinguished from bulk analysis of tumour biopsies (50). Still CTC represent the metastatic sites. Comparison of individual and pooled CTCs from MBC patients with their metastatic tissue counterparts revealed 85% concordance in at least one or more recurrent somatic mutations and CNAs (51). On a transcriptomic level, that has the potential to unravel altered molecular pathways during tumour evolution, a pioneering single-cell study in CTCs from MBC patients demonstrated a remarkable intra-patient heterogeneity in expression of 87 cancer-associated and reference genes (52). Also in other tumour types like prostate cancer and melanoma, single cell RNA-sequencing revealed networks of tumour evolution (43).

The enormous advances made in high-throughput sequencing methods have mainly highlighted ctDNA as a non-invasive biomarker able to recapitulate some of the branched subclone(s) seeding the tumour relapse in patients with lung cancer (53, 54). However, ctDNA sampling strategy could present some caveats in terms of providing a detailed phylogeny due to a potential over-representation of DNA from dying cells (43). Moreover, the low concentration of some ctDNA variants requires a substantial sequencing depth combined with the need for multiplexing to encompass tumour heterogeneity, which is a technical challenge (55). A pilot study on five patients with MBC compared the mutational content of CTCs at single-cell resolution and ctDNA (56). In all patients, ctDNA profiles provided an accurate reflection of mutations seen in individual CTCs.

ctDNA offers a less laborious method to repeatedly interrogate tumour genomes, providing opportunities to track clonal dynamics induced by metastasis and therapeutic selective pressures in various tumour types over time (57, 58, 59). In an effort to exploit the use of ctDNA for determining how a patient's cancer is evolving, deep WGS of various metastatic lesions (via a rapid autopsy programme) and cfDNA was performed for two patients (60). Reconstruction of the metastatic cascade revealed that early monoclonal seeding was the dominant pattern of metastatic spread, as previously reported in colorectal cancer (61, 62), with evidence of polyclonal seeding restricted to one liver and one ovary sample. In the second patient, monoclonal and early seeding was also dominant (60). In both patients, the ctDNA reflected predominantly active metastatic sites, as seen on PET/CT. Plasma of patient 1 predominantly reflected metastatic liver disease, especially the treatment-resistant *ESR1* mutant clone under hormone therapy, consistent with clinical course (progressive liver disease and death due to liver failure) (60). In the sub-analysis of the PALOMA-3 trial they found similar results with *PIK3CA* mutation being clonal, while *ESR1* mutations are frequently sub-clonal (lower variant allele frequencies), and became undetectable during both therapy regimens (29). Whole exome and 396-gene panel sequencing on an extensive ctDNA sample series from seven patients with metastatic triple-negative breast cancer (TNBC) treated with Cabozantinib, showed distinct clonal populations specific to each patient (41). They were able to build phylogenetic trees revealing alterations in hallmark breast cancer drivers, including *TP53*, *PIK3CA*, *CDK4*, and *PTEN*. In some patients shifts in SNVs were seen under treatment, while copy number profiles remained stable across the seven patients (41). This may reflect that large-scale CNA events occur early in TNBC development (63).

In conclusion, both CTCs and ctDNA can be used to study clonal evolution during treatment and at progression without the need for repeated biopsies, which may not even be feasible if the tumour is in an inaccessible site.

Serial sampling and disease monitoring

Monitoring levels of ctDNA represents a non-invasive snapshot of tumour burden and is able to predict treatment response and progression-free survival. In a recent overview of 33 studies using cfDNA to measure treatment response, two types of assessment were used: ctDNA dynamics related to PFS versus real-time response (RECIST or tumour markers like CA 15-3) (37). The majority of studies use a single marker for ctDNA detection. Mutations in *PIK3CA*, *ESR1* and *TP53* were most frequently assessed. On average, these were present in 10-50% of the included patients (37). This indicates that even in selected populations only around half of the patients could be monitored by use of mutations in a single gene. The association with treatment response was less evident for dynamics in *ESR1* mutations (37), which is consistent with the findings described in the paragraph above. The use of gene panels could broaden the applicability of a test since the mutational landscape in MBC is heterogeneous. The studies that did use a combination of genes, observed an association between the dynamics in mutations and treatment response during monitoring (37). One of these, exploring resistance to anti-HER2 therapy, showed that mutational tumour burden index (mTBI, i.e. mean VAFs of mutations as measure for ctDNA levels) was superior to single gene mutations for assessing therapeutic response (64). Personalized mutation panels may allow for higher sensitivity detection of known variants in ctDNA, however fail to capture the development of new alterations over time, limiting their utility to largely retrospective analyses. Of notice, half of the studies excluded patients without detected alterations at baseline, making it less applicable to the general patient population (37). Studies using CNAs to evaluate treatment response can be based on gene specific or genome-wide cfDNA aneuploidy-score. The level of *HER2* amplification in the cfDNA was associated with treatment response during HER-targeted therapy (64, 65, 66). Contrary, for shallow WES or WGS only minimal cfDNA input is necessary. However, similar to mutation based ctDNA detection, various patients were excluded due to low aneuploidy scores for all analysed time points despite progressive disease (30).

In our study (**chapter 5**) we performed shallow WGS on 1098 samples of 188 MBC patients. Longitudinal ctDNA levels (ichorCNA values) provided predictive real-time data on treatment response across subtypes and therapies, outperforming ctDNA targeted sequencing and serial CA 15-3 measurements. However, it may be that predicting response to targeted therapy and chemotherapy may be slightly more reliable than for endocrine treatment alone. This may reflect the biology of disease as patients on targeted treatment and chemotherapy are likely to have more aggressive disease or have a higher tumour burden potentially making ctDNA levels higher and more dynamic. Moreover, cytostatic treatments are probably less likely to cause large changes in ctDNA compared with cytotoxic treatments. Using similar workflow in seven patients with metastatic TNBC treated with Cabozantinib (a multi-tyrosine kinase inhibitor; cytostatic), no significant relationship between early tumour fraction change and RECIST measured outcomes was found (41). A prospective large-scale clinical trial to evaluate clinical benefit of early treatment changes based on ctDNA levels is now warranted. Thinking more broadly about estimating ctDNA it could be that this is a better marker of overall disease activity than purely measuring disease on a CT scan.

Baseline CTC count has been known to correlate with PFS and OS for many years now (67). Besides, CTC count can be used to stratify patients with MBC, irrespective of disease subtype, line of therapy, and site of disease into two groups: indolent versus aggressive disease (68). A recent study (2,202 samples from 469 MBC patients) found that the CTC trajectory patterns during the course of treatment was a better predictor of PFS and OS compared to baseline CTC and combined CTC (baseline-end of cycle 1) models (69). This was preceded by two smaller studies in patient with MBC

treated with hormonal therapy and bevacizumab, or chemotherapy respectively. The first showed failure to clear CTCs during treatment was associated with significantly increased risk of progression and death (70). The other stating that changing CTC levels significantly correlated with response to therapy as measured by radiologic RECIST criteria and serum CA 15-3 level changes (71). One study even reported a higher sensitivity of CTCs than imaging examination in a few cases (5). This makes CTCs as well as cfDNA possibly a biomarker for early response evaluation in MBC, which should be further investigated in large prospective trials.
With the growing body of evidence concerning the prognostic value of CTCs, clinicians began to investigate interventions able to increase survival in patients with poor prognosis associated with high CTC count or an unfavourable CTC variation, as reviewed by Vasseur *et al* (72). In the pre-CKD4/6 inhibitor era, the first trial to compare clinician-based choice of first-line therapy (chemotherapy versus endocrine therapy in ER+HER2- MBC) to a CTC-based choice, was the STIC-CTC trial (NCT01710605). The CTC-driven choice was noninferior in terms of PFS (15.5 months in the CTC arm versus 13.9 months) (73). The AMBRE trial (NCT04158362) is currently recruiting. They aim to evaluate CTCs a secondary outcome when comparing first-line chemotherapy to endocrine therapy plus the CDK4/6 inhibitor abemaciclib. Two trials have explored CTC trajectory for decision making on early therapy switch in subsequent lines in MBC. SWOG S0500 (NCT00382018) randomly assigned patients to early change or continuation of first-line chemotherapy in the presence of persistently high CTC count (>5 CTC/7.5ml on CellSearch). Early switching to an alternate cytotoxic therapy was not effective in prolonging OS (10.7 months in the standard arm versus 12.5 months in the CTC arm) (74, 75). A similar study, CirCe01 (NCT01349842), was also negative (76). In metastatic TNBC Cristofanilli *et al*. have initiated a phase II trial evaluating the combination of pembrolizumab and carboplatin in high-risk patients (i.e. ≥ 5 CTCs/7.5 mL at baseline) (NCT03213041).

In contrast to MBC, ctDNA is less frequently detected in non-metastatic, early breast cancer (EBC). Zhou *et al.* reported that 86% of stage IV MBC patients carried tumour-derived mutations in blood, compared to only 58% of stage I–III patients (27). Another study, using digital droplet PCR, found that ctDNA was detectable in >75% of patients with advanced cancers, like MBC. In patients with EBC, ctDNA detection rate was 50% (77). Over all tumour types, ctDNA was often present in patients without detectable circulating tumour cells, suggesting that these two biomarkers are distinct entities (77). There are major opportunities to further develop liquid biopsies in EBC, like monitoring of primary resistance or even recurrence (**Figure 1**).

In both colorectal and oesophageal cancer relapse could be detected by ctDNA levels months ahead as compared to conventional imaging follow-up (78, 79). The recent CHiRP study showed that ctDNA successfully identified minimal residual disease in patients with hormone receptor-positive (HR+), HER2- EBC who are at high risk for recurrence, at least 5 years post diagnosis (80). Personalized ctDNA assays, based on WES of the archival primary tumour, were made for 83 patients. Six patients (7.2%) developed distant metastatic recurrence, all of whom were ctDNA-positive (total of eight positive patients) before overt clinical recurrence, with median ctDNA lead time of 12.4 months (80). Additionally, ctDNA detection shortly after neo-adjuvant chemotherapy for EBC was also associated with relapse, especially in patients with non-pathological complete response (pCR) (81, 82). A retrospective analysis of ctDNA samples from the i-SPY2 trial (NCT01042379) in the neoadjuvant setting showed that the presence of residual ctDNA post operatively was more predictive of relapse than pCR (83). Publications from before 2022 on ctDNA in EBC follow-up and detection of minimal residual disease are nicely reviewed by Sant *et al* (84). c-TRAK-TN

(NCT03145961), a multi-centre phase II trial, with integrated prospective ctDNA surveillance by digital PCR, enrolled patients with early TNBC and residual disease following neoadjuvant chemotherapy or stage II/III with adjuvant chemotherapy (85). ctDNA+ patients were initially randomized 2:1 to intervention (pembrolizumab) versus observation. 45 of 161 patients were ctDNA+. 72% (23/32) of patients allocated to intervention had metastases on staging at time of ctDNA+. Five patients commenced pembrolizumab in EBC, though none achieved sustained ctDNA clearance. This emphasizes the need for larger interventional trials for testing the clinical utility of ctDNA monitoring in this setting (85). Currently the ZEST phase III trial (NCT04915755) is recruiting 800 patients for niraparib versus placebo after (neo)adjuvant chemotherapy, in HER2- patients with $BRCA^{MT}$ ctDNA or TNBC patients with $BRCA^{WT}$ ctDNA.

CTC detection mid- or post therapy in EBC have been shown to harbour both prognostic relevance towards DSF and OS, as well as information for monitoring therapeutic effects (86). In a large trial with 1,697 patients, CTC count was shown to possibly predict a benefit of radiotherapy in the adjuvant setting of EBC (87). The detection of CTCs before the start of neoadjuvant chemotherapy adversely affected metastatic-free and overall survival (REMAGUS02-study) (88). This was confirmed by a meta-analysis of 21 studies (89). Additionally, SUCCESS-A and ECOG-ACRIN-E5103 trials were clearly able to show an increased risk for recurrence in patients with persistence of CTCs two years and even five years after (neo)adjuvant chemotherapy (90, 91, 92). A very recent trial measured pre- and post-therapy CTC levels in 1220 patients with EBC. CTC positivity at baseline was associated with shorter OS, while CTC status at follow-up (post-therapy) predicted disease recurrence. CTC positivity predicted early (within 5 years) but not late recurrence, emphasising the need for investigations into novel adjuvant therapeutic approaches (93).

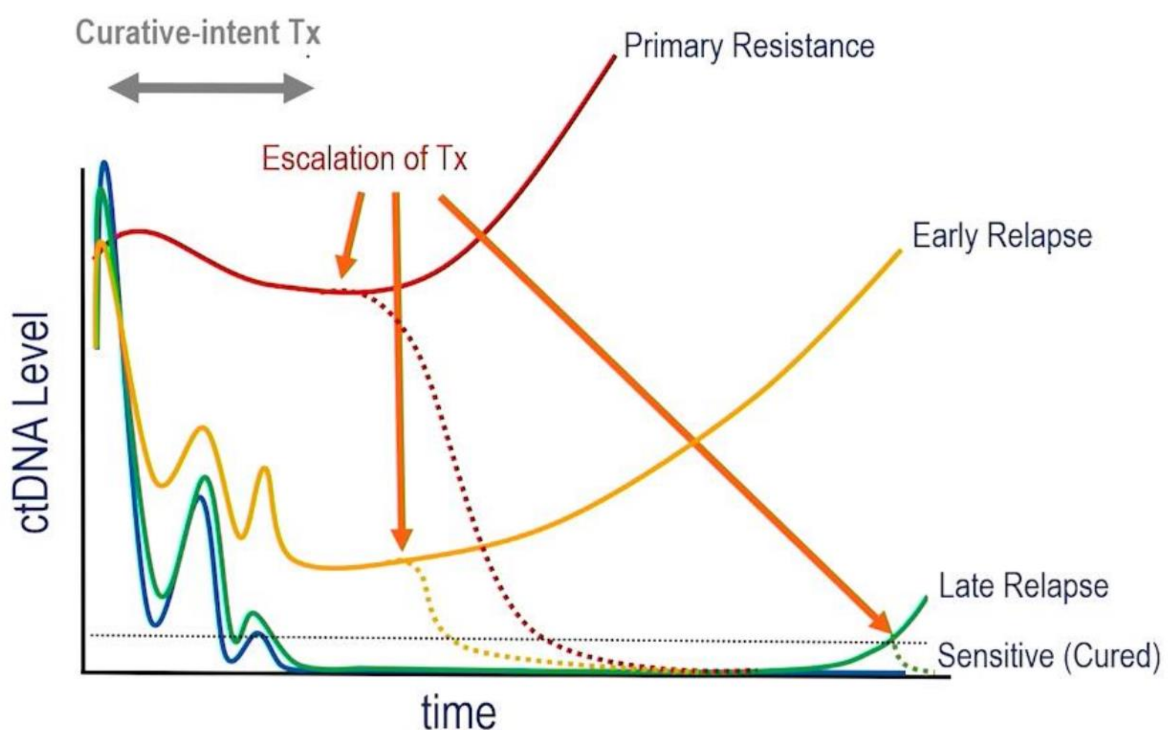


Figure 1. Theoretical model on ctDNA levels over time in localized cancer. ctDNA levels might predict primary therapy resistance, or early versus late relapse in early breast cancer, emphasising the need for escalation of therapy (Tx). Adapted from ESMO webinar series on liquid biopsies.

Actionable targets in breast cancer

Biomarkers predicting therapy response are frequently assessed using tumour biopsies, reflecting only parts of a patient's disease at a specific moment in time (94). It is well-known that targetable molecules can change during the course of the disease. Already 15 years ago CTCs were used in serial analysis to identify emergence of activating mutations in the *EFGR* gene in lung cancer patients receiving EGFR-targeting therapy, conferring a mechanism of acquired resistance (95). Meanwhile CTCs have shown to be useful in understanding and predicting acquired resistance to therapies in various cancer types, and might be used to circumvent this. In the TRACERx trial (NCT01888601), primary tumours of hundred non-small cell lung cancer (NSCLC) patients were sequenced, as well as cfDNA and CTCs obtained at multiple time points during therapy, to identify targetable driver events (96, 97). The subsequent DARWIN trial (NCT02183883) aims to evaluate whether targeting driver events, detected by the TRACERx trial, has a different clinical outcome in patients harbouring a dominant versus subclonal driver mutation (98). Similarly, the Prostate-Biomarker (ProBio, NCT03903835) study is a large international multi-centre, outcome-adaptive, multi-arm, ctDNA biomarker-driven platform trial for tailoring treatment decisions in metastatic castration resistant prostate cancer (mCRPC) (99, 100).

In breast cancer, HER2 status has been extensively evaluated in both CTCs and cfDNA. Several studies have demonstrated the feasibility of determining the HER2 status of CTCs in MBC using CellSearch® (101, 102, 103, 104). HER2 protein detection using immunofluorescence by the CellSearch® system has been under debate, since only a limited correlation was observed between the HER2 status of CTCs and of the primary tumour (e.g. 33% of patients with a HER2-negative tumour had HER2-positive CTCs and 42% of patients with a HER2-positive tumour had exclusively HER2-negative CTCs) (105). This was confirmed by others (14, 15, 106). To address this issue, we analysed thousands of CTCs from 85 MBC patients (all subtypes) with >5 CTCs, on HER2 protein level (immunofluorescence) and compared this to *ERBB2* gene expression (qrt-PCR) and amplification (FISH); see **chapter 4**. We argue that immunofluorescent scores should divided in HER2 negative, medium and high expressing CTCs. One third of patient samples harboured >10% $HER2^{high}$ expressing CTCs, while almost all patients harboured $HER2^{med}$ expressing CTCs. This first prevalence is in line with the incidence of HER2-positivity is seen in MBC (26,3% in stage IV) (107). Furthermore, there only was a correlation between having >10% $HER\text{-}2^{high}$ expressing CTCs and *ERBB2* gene overexpression. No patients switched from HER2-positive primary to negative based on CTCs, while five HER2-negative patients (20%) had ≥1 $HER\text{-}2^{high}$ expressing CTCs. These five patients could be eligible for anti-HER2 directed therapy.

The DETECT III trial (NCT01619111) aimed to demonstrate the benefit from lapatinib therapy in HER2-negative MBC patients, who are HER2-positive on CTCs (108). 105 patients were randomized between standard chemotherapy with or without lapatinib. They showed promising preliminary results of improved OS with the addition of lapatinib (109). Further validation in larger patient cohorts is needed. The CirCe T-DM1 trial (NCT02975142) showed that *ERBB2* gene amplification in CTCs from 7 HER2-negative MBC patients occurs in a minor CTC subpopulation (110). Overall a low response rate to trastuzumab-emtansine was reported (1/11), questioning the clinical utility of anti-HER-2 therapy in patients with HER-2 amplification in a minor subset. Similar results were found in a trail measuring HER2 protein expression on CTCs from HER2-negative patients using the PRO Onc assay (NCT01048099). Only 1/14 patients treated with trastuzumab-pertuzumab had stable disease. Another phase II trial (NCT00820924) tested effectiveness of Lapatinib in MBC patients with HER2-negative primary tumours and HER2-positive CTCs analysed by visual scoring of CellSearch® images

or FISH (111). 7/96 patients, harbouring 2-5 CTCs, were eligible (i.e. ≥50% of CTCs were HER2 positive on immunofluorescence, and 1 sample was FISH-positive). No objective tumour responses occurred in this population, underlining the importance of patient selection for such trials. Based on our findings this should be patients with ≥5 CTCs and at least one HER-2^{high} expressing CTC. From all above studies, only DETECT III shows preliminary but positive results. This result was also shown in a small study that randomized 11 HER2-negative and 60 HER2-positive patients (112). They demonstrated increased PFS in patients with HER2+ CTCs when treated with anti-HER2 therapy compared to those not receiving this. Notably, up to 52% of patients with HER2+ primary tumours harboured HER2- CTCs and anti-HER2 therapy in this group did not significantly improve median PFS (112). One such case is also been described in **chapter 3**.

Further investigations aimed at evaluating the impact of HER2-expression or *ERBB2* copy number heterogeneity detected in CTCs on anti-HER2 treatment response is warranted, even in patients with tumours classified as HER2-negative and especially in the light of the recent introduction of the “HER2-low” category (113) and the DESTINY-Breast06 trial, randomising patients with HER2-low disease for Trastuzumab-deruxtecan versus standard of care (NCT04494425).

Finally, efforts have been made to look into HER2 gene mutations in ctDNA. Acquired HER2 mutations confer sensitivity to HER2-targeted therapies such as neratinib in HER2-negative MBC, as shown in the plasmaMATCH trial (NCT03182634) (114). Ma and colleagues found several HER2 mutations in ctDNA of HER2-negative MBC patients that demonstrated neratinib sensitivity (115). These mutations decreased during treatment with neratinib and increased at progression. Then, also new mutations emerged, including T798I, which is known for neratinib resistance (116). In women with HER2-positive MBC, the HER2 V777L mutation induces acquired resistance to trastuzumab (65, 117).

CTC and cfDNA profiling can identify diverse intra- and interpatient molecular mechanisms of endocrine therapy resistance (**Figure 2**). Several studies have investigated *PIK3CA* mutations in CTCs and cfDNA (44, 118, 119, 120, 121). The majority of *PIK3CA* mutations are truncal mutations, but others are subclonal (29, 122, 123). *PIK3CA* mutations are associated with worse prognosis (121), although they confer sensitivity to PI3K inhibitors (PI3Ki) such as taselisib, alpelisib, buparlisib, and copanlisib (114, 124, 125, 126). A subgroup analysis of the phase III SOLAR-1 trial (NCT02437318)

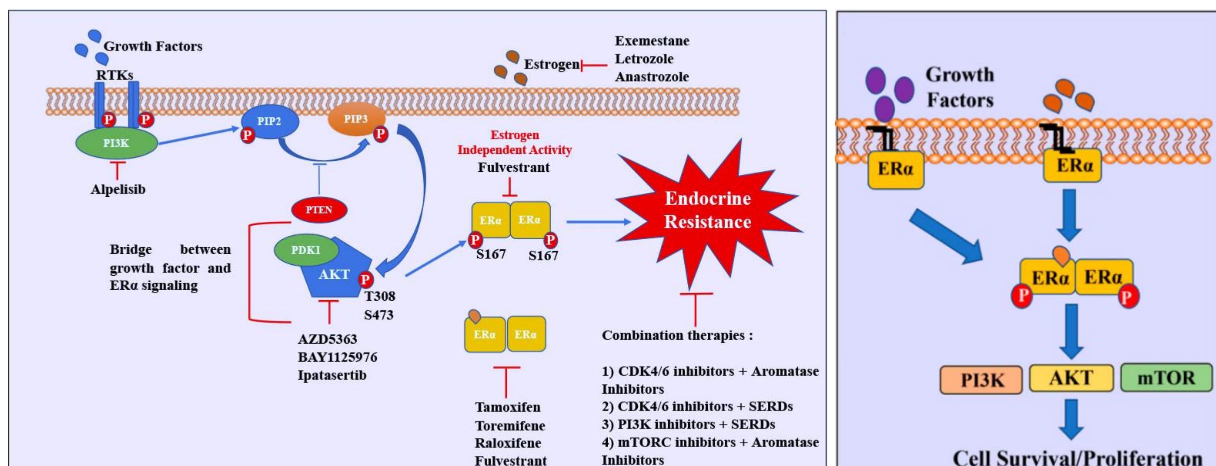


Figure 2. PIK3CA/PTEN/AKT and Estrogen Receptor (ER) pathways, and their inhibitors. LEFT. Activated PI3K-AKT-mTOR can enhance genomic actions of ERα: activation of PI3K leads to activation of AKT, which can directly phosphorylate ERα to promote ligand-independent activity and endocrine therapy resistance. RIGHT. Membrane anchored ERα can also activate various cytoplasmic kinases including PI3K-AKT-mTOR pathway through nongenomic actions and these actions occur rapidly (within five minutes) after encountering the ligand. Adapted from (1).

showed that patients with a *PIK3CA* mutation detected in ctDNA benefit more from addition of alpelisib compared to all patients in which *PIK3CA* mutations were detected in tumour (125, 127). One study demonstrated that *PTEN* loss-of-function mutations found in cfDNA promotes PIK3α-independent activation of AKT, causing resistance to alpelisib (128). *AKT1* mutations are truncal and respond to capivasertib, an AKT kinase inhibitor (114). Other frequently used drugs in ER+ MBC are CDK4/6 inhibitors like palbociclib, ribociclib, or abemaciclib. In the PALOMA-3 trial (NCT01942135, HR+ MBC after progression on endocrine therapy), early response to cytostatic palbociclib and fulvestrant was correlated to early decrease of the *PIK3CA*-mutant ctDNA (29). This makes ctDNA dynamics of truncal *PIK3CA* mutations a predictive biomarkers for response to CDK4/6 inhibitors. Though also to fulvestrant alone (122).

The most frequently assessed genes in ctDNA to monitor therapy response include *PIK3CA* and *ESR1* (37). *ESR1* encodes for ER and its acquired mutations are found, often subclonal, in 30% of patients receiving endocrine therapy (114, 128, 129, 130, 131, 132). *ESR1* mutations are located in the ligand-binding domain and are hormone-independent activating mutations, driving resistance to aromatase inhibitors (A-I) (130, 133, 134). Although no relationship between *ESR1* dynamics in cfDNA and general treatment response has been found (29, 122), *ESR1* mutation rates decrease after treatment with a CDK4/6 inhibitor together with an A-I (135). The recently published PADA-1 phase III trial (NCT03079011) demonstrates a significant benefit for switching from first line palbociclib plus A-I to palbociclib plus fulvestrant when *ESR1* mutations emerge in cfDNA (136). Moreover, *ESR1* mutations detections before start of first line therapy is important, as these are twice as prevalent (7% vs. 3%) among patients who received an A-I in the adjuvant setting (137). BOLERO-2 (NCT00863655) included *ESR1* mutation detection in cfDNA as an exploratory endpoint. Sub-analysis showed these were associated with more aggressive disease (132). Currently, CICLADES (NCT03318263) enrolled 146 patients to monitor *ESR1*, *PIK3CA*, and *AKT* mutations for early detection of A-I resistance. INTERACT (NCT04256941), will screen patients for acquired *ESR1* mutations 12 months after initiation of an A-I and CDK4/6 inhibitor, and will switch them to fulvestrant if detected.

Functional retinoblastoma protein (encoded by the *RB1* gene) is a requirement for response to CDK4/6 inhibition *in vitro* (138). Analysis of the individual and pooled CTCs revealed loss of *RB1* (13q) in one patient that developed resistance to palbociclib (14). In the PALOMA-3 trial, RB1 mutations were present in 5% of the patients who progressed during treatment with palbociclib plus fulvestrant but not in those treated with a placebo plus fulvestrant (136, 137). However, these mutations are likely subclonal and of relatively low prevalence, suggesting that they are not a major mechanism of resistance to CDK 4/6 inhibitors.

In metastatic TNBC, patients are eligible for immune checkpoint inhibitors (ICI) when PD-L1 positive (139, 140) or PARP inhibitors when *BRCA1*/2 mutations haven been detected (141, 142, 143). *BRCA* mutations have been identified in liquid biopsies, though patients are only eligible when germline mutations are detected (141, 142, 143). Many studies have shown that PD-L1 expression can be assessed on breast cancer CTCs by immunofluorescence (144, 145, 146), and might represent an alternative to PD-L1 assessment on tissue (147). In NSCLC, patients with tumour response to ICI showed decreased levels of PD-L1 positive CTCs, and vice versa (148, 149). I-CURE-1 (NCT03213041, currently recruiting) is a single arm study to evaluate the impact on PFS of the combination pembrolizumab-carboplatin in patients with CTC+ HER2- MBC. In this study they will also include PD-L1 expression analysis on CTCs by immunofluorescence.

In conclusion and nicely depicted by Sant *et al* (84): *HER2* mutations predict sensitivity to HER2 inhibitors like lapatinib and neratinib. *PIK3CA* mutations are correlated to resistance to

endocrine therapy and CDK4/6 inhibitors, while they show sensitivity to PI3K inhibitors like alpelisib and others. *PTEN* and *AKT* mutations demonstrated sensitivity to capivasertib (AKT inhibitor), though *PTEN* loss of function mutations cause resistance to PI3K inhibitors. *ESR1* mutations are known for their role in resistance to aromatase inhibitors. All of these mutations are trackable in liquid biopsies.

Concluding remarks

To implement liquid biopsies into daily clinical practice, a test must have shown analytical and clinical validity, and clinical utility. In the first part of this discussion we have brought up some technical considerations to improve the accuracy of detecting or excluding relevant biomarkers, i.e. analytical validity (38, 40). A lot of effort has already been done to improve clinical validity of CTCs and cfDNA, for example specific copy number profiles or mutations corresponding to tumour subtype or acquired mutations after certain therapies. Clinical utility is the added value of the test for diagnosing or clinical decision making. CTC trajectory and ctDNA level monitoring have both been able to predict therapy response, PFS and OS. Targetable mutations can be detected in both CTC (6, 14) and cfDNA (37, 84). To further improve the applicability of liquid biopsies, they are increasingly incorporated in clinical trials for diagnosing and therapy decision making.

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Sophie & Sven, jullie zijn nu nog te klein om de impact van dit boekje volledig te begrijpen, hoewel ik jullie wijsneuzen zeker niet mag onderschatten. Sophie, als jij met de pop speelt, zeg je dat "de baby vannacht bij Sven moet slapen, want jij moet alsof in het ziekenhuis gaan werken"… Weet allebei dat één knuffel van jullie veel belangrijker is dan al het werk dat in dit boekje staat.

Curriculum Vitae

Anja, Aaltje Jantje Brouwer, was born in Wijster (The Netherlands) on 16 March 1986. After graduating grammar school at dr. Nassau College in both health-sciences and mathematical-sciences, she started her studies in Biomedical Sciences at Utrecht University in 2005. After earning a bachelor degree and performing full time lab work during one year master in Immunity and Infection, she started Medical School at the University of Antwerp in 2009. She graduated as a medical doctor with great distinction in 2014, and pursued her PhD in Medical Sciences at the Center for Oncological Research (CORE) at the University of Antwerp. She performed her research at the Translational Cancer Research Unit of GZA Hospital Sint-Augustinus and in part at the Cancer Research UK – Cambridge Institute. In parallel, she performed ten percent clinic at the Oncology department of Antwerp University Hospital and the Acute Medicine department of Cambridge University Hospitals Addenbrookes. In recent years, this thesis was finished during a full time training as resident in Medical Oncology at Ghent University.
Anja Brouwer is married to Martijn Schuijs and mother of two children: Sven & Sophie, born in 2017.

Grants & Awards

2015-2019	Personal research grant: Fellowship of Research Foundation Flanders (FWO)
2016	Award for oral presentation, BSMO conference, Brussels
2015-2018	Project grant: Stand up to Cancer (KotK-Belgium)
2015-2016	Personal research grant: 'FWO umbrella grant' – University of Antwerp
2014-2015	Personal research grant: Emmanuel Van Der Schueren – Stand up to Cancer

Courses

2022	ESMO Academy & Examination
2019	CMT regional training day
2016	ECG training course
2016	Statistics-UA course: R-workshop, Antwerp
2015	DEPArray V2 training course, Bologna
2014	VIB course: Building bioinformatics workflows, Antwerp
2012	EORTC course: Introduction in Clinical Trials, Brussel
2009	Laboratory Animal Sciences (PIL-course A/B/C), Utrecht

Skills

Languages	Dutch (mother tongue), English (Good, **IELTS: C1**)
Techniques	Cell culture, Transwell, Blotting, (RT-)qPCR, xCELLigence, CellSearch, DEPArray, FACS, MACS, Library prep, DNA sequencing (454, Illumina)

Experience abroad

- Internship in Surgery and Internal medicine: Oncological center Daniël den Hoed, Erasmus MC, Rotterdam, Netherlands. November 2012 – January 2013.
- Summer school: 9th ESO-ESMO Course on Oncology, Ioannina, Greece, 21-27 July 2012
    - Third place at the Clinical Oncology Examinations.
- Summer school: Cancer and Immunology research, Boston MA, USA, 10-20 April 2009.
    - Classes: Harvard Medical School (prof. Michael Carroll), MIT, Whitehead Institute, Broad Institute, Dana-Farber cancer Institute.

Interests

Next to my strong interest in medical sciences, I have spent part of my time in **various committees** of the medical faculty and the biomedical students society, e.g. Year-representative, Outreach Biomedical Sciences, introduction committees of both the Bachelor and the Graduate School of Life Sciences. Besides, I was a member of the youth sailing committee within our marina.
Furthermore, I enjoy **teaching**. I worked as a spokesperson for Biomedical Sciences and taught physics at a secondary school, worked as a `StuDoc´ teaching bachelor students medical skills, and have guided many bachelor and master students during my career.

Publications

Articles

- Anja Brouwer, Solon, Mario Ortega-Duran, Bram De Laere, Alistair Martin, Steven Van Laere, Emma Beddowes, Luc Dirix, Carlos Caldas, Oscar Rueda. **Monitoring plasma cfDNA copy number alterations during therapy in metastatic breast cancer.** In preparation.

- Anja Brouwer, Bram De Laere, Pieter-Jan van Dam, Dieter Peeters, Marc Peeters, Steven Van Laere, Luc Dirix. **Mutational and copy number analysis of many sorted circulating and disseminated tumour cells to interrogate subclonal evolution in advanced breast cancer.** Submitted to BJC.

- Emma Beddowes, Mario Ortega-Duran, Solon Karapanagiotis, Alistair Martin, Meiling Gao, Riccardo Masina, Ramona Woitek, James Tanner, Fleur Tippin, Justine Kane, Jonathon Lay, Anja Brouwer, Stephen-John Sammut, Suet-Feung Chin, Davina Gale, Dana Tsui, Sarah Jane Dawson, Nitzan Rosenfeld, Maurizio Callari, Oscar M Rueda, Carlos Caldas. **A large-scale retrospective study in metastatic breast cancer patients using circulating tumor DNA and machine learning to predict treatment outcome and progression-free survival.** Submitted to JCO.

- Anja Brouwer, Bram De Laere, Pieter-Jan van Dam, Dieter Peeters, Jasper Van Haver, Ellen Sluydts, Ali El Moussaoui, Pauline Mendelaar, Jaco Kraan, Marc Peeters, Steven Van Laere, Luc Dirix. **HER-2 status of Circulating Tumor Cells in a Metastatic Breast Cancer cohort: a comparative study on characterization techniques.** PloS One. 2019 Sep 4;14(9):e0220906.

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- Anja Brouwer, Bram De Laere, Dieter Peeters, Marc Peeters, Roberto Salgado, Luc Dirix, Steven Van Laere. **Evaluation and consequences of heterogeneity in the circulating tumor cell compartment**. Oncotarget 2016 Jul 26;7(30):48625-48643.

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- Bram De Laere, Dieter Peeters, Anja Brouwer, Roberto Salgado, Peter van Dam, Annemie Rutten, Gert Van den Eynden, Carsten Denkert, Laure-Anne Teuwen, Peter Vermeulen, Steven Van Laere, Luc Dirix. **Patients with metastatic hormone receptor-positive breast cancer express PIK3CA oncogene mutational heterogeneity in circulating tumor cells**. Journal of Translational Science 2016, August 15.

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- Pol Specenier and Anja Brouwer. **Matrix Metalloproteinases in Head and Neck Cancer.** World J Surg Med Radiat Oncol 2015;4:18-27.

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Orals

- Brouwer A, van Dam PJ, De Laere B, Peeters M, Van Laere S, Dirix L. Evaluation of HER2 Expression and Amplification on CTCs of 85 Metastatic Breast Cancer Patients Using CellSearch, DEPArray Sorting, and FISH Analysis. **DEPArray Meeting**, Bologna, Italy, 25-26 October 2016.

- Brouwer A, Peeters D, Peeters M, Van Laere S, Dirix L, Evaluation of subclonality in the CTC and DTC compartment of patients with MBC using AmpliSeq panel sequencing. Belgian Society of Medical Oncology (**BSMO**) **conference**, Zaventem, Belgium, 27 February 2016.

- Brouwer A, Peeters D, Peeters M, Van Laere S, Dirix L, Evaluation of subclonality in the CTC and DTC compartment of patients with metastatic breast cancer using DEPArray sorting and AmpliSeq panel sequencing. **DEPArray Meeting**, Bologna, Italy, 10-11 November 2015.

- Brouwer A, Peeters DJE, Op De Beeck K, Vandeweyer G, Pauwels P, Peeters M, Vermeulen PB, van Dam PA, Van Laere SJ, Van Camp G, Dirix LY. Whole exome sequencing of circulating and disseminated tumour cells in patients with metastatic breast cancer. Belgian Association for Cancer Research (**BACR**) **conference**, Université catholique de Louvain, Leuven, Belgium, 31 January 2015 + Belgian Society of Medical Oncology (**BSMO**) **conference**, Zaventem, Belgium, 7 March 2015.

- Brouwer A, Heterogeneity in the CTC compartment, **Post-graduate**, Wilrijk, Belgium, 20 Jan 2016.

Posters

- Brouwer A, De Laere B, van Dam PJ, Peeters M, Van Laere S, Dirix L. HER2 status of Circulating Tumor Cells in a Metastatic Breast Cancer cohort: a comparative study on characterization techniques. **EACR-ESMO Liquid Biopsie**s conference May 2019, Bergamo, Italy. Poster 20.

- Brouwer A, van Dam PJ, De Laere B, Peeters M, Van Laere S, Dirix L. Evaluation of HER2 expression and amplification on CTCs using DEPArray analysis and sorting followed by FISH. San Antonio Breast Cancer Symposium (**SABCS**) 2016, TX, USA: poster P3-05-10

- Brouwer A, De Laere B, van Dam PJ, Peeters M, Van Laere S, Dirix L. Evaluation of subclonality in the CTC and DTC compartment of patients with metastatic breast cancer using low pass whole genome and AmpliSeq panel sequencing. San Antonio Breast Cancer Symposium (**SABCS**) 2016, TX, USA: poster P1-06-01 & Belgian Association for Cancer Research (**BACR**) symposium, Brussels, 30 January 2016.

- Brouwer A, van de Wiel M, Peeters B, van Dam PJ, Peeters M, Van Laere S, Dirix L, Peeters D**.** Manual and digital detection of HER2 status of 2721 circulating tumour cells in patients with metastatic breast cancer. San Antonio Breast Cancer Symposium (**SABCS**) 2015, TX, USA: poster P3-04-01.

- Brouwer A, Peeters DJE, Op De Beeck K, Vandeweyer G, Pauwels P, Peeters M, Vermeulen PB, van Dam PA, Van Laere SJ, Van Camp G, Dirix LY. Whole exome sequencing of circulating and disseminated tumour cells in patients with metastatic breast cancer. San Antonio Breast Cancer Symposium (**SABCS**) 2014, TX, USA: poster P4-01-14 & **IMPAKT** 2015, Brussels: poster #252.

- Anja Brouwer, Nathalie Degrieck, Marika Rasschaert, Fabrice Lockefeer, Manon Huizing, Wiebren Tjalma. Differential diagnosis of mastitis: a case of a 33 year old woman presenting with mastitis. 4th International Inflammatory Breast Cancer Conference (**IBCC**), Antwerp, 6-7 May 2014.

- Anja Brouwer, Ridha Limame, Marc Peeters, Filip Lardon, Marc Baay. Real-time monitoring of monocyte-to-macrophage differentiation using impedance measurement. Belgian Association for Cancer Research (**BACR**) symposium, Antwerp, 2 March 2013.

“Life is like riding a bicycle. To keep your balance, you must keep moving.” Albert Einstein (1879 -1955)