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CHAPTER 1

BREAST CANCER

Overview

Breast cancer (BC) is the second most common cancer worldwide and the most commonly occurring malignancy in women (22.9% of female cancers) [1]. According to the data available, there are significant variations in incidence, mortality, and survival of patients among different countries and regions [2]. Although the incidence is higher in Western Europe and North America, it is rising in developing countries, because of increased life expectancy, urbanization, and the adoption of western lifestyles [3]. According to the American Cancer Society, the five-year survival rate has improved from 63% in 1960 to 90% at present [4], thanks to earlier diagnosis with mammogram screening, and improved surgery and adjuvant treatment. Indeed, in 2018, BC death rates have rapidly slowed to 6.6% [5]. However, survivors are at increased risk of recurrence, even 20 years after the initial diagnosis [6]; in addition, they show increased risk to gain weight and develop other comorbidities, such as cardiovascular diseases or metabolic disorders [7-9].

Cancer transformation is primarily due to biologic, genetic, and environmental factors such as smoking cigarette, inherited mutation of genes and harmful radiation exposure [10]. In 2020, there were 2.3 million women diagnosed with breast cancer and 685 000 deaths globally. As of the end of 2020, there were 7.8 million women alive who had been diagnosed with breast cancer in the past 5 years.

Breast cancer mortality changed little from the 1930s through to the 1980s, at which time significant improvements in survival were achieved through early detection programmes combined with different modes of treatment aiming at eradicating invasive disease [11]. In recent decades, the widespread use of mammographic screening [12] clinical and radiological examinations, as well as molecular subtype assessment by predictive biomarkers have increased the rate of early disease detection and treatment efficacy.

At the same time, the development of more effective adjuvant chemotherapeutic regimens [13], the extended use of endocrine therapies, and the standard application of targeted anti-HER2 agents have all contributed to improve outcome of women with primary breast cancer. Breast cancers are heterogeneous, showing variable morphologic and biological features and thus different clinical behaviour and response to treatment.

Cancer classification aims at providing accurate diagnosis and predicting tumor aggressiveness. Combined, these facilitate decision-making processes by medical oncologists. Staging of breast cancer is based on clinical and pathological factors and criteria, including tumor size, nodal status, and distant metastasis (TNM staging). The routine assessment of breast cancer also includes Estrogen receptor (ER α), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression/amplification status [14, 15].

1.1 ***Histological classification***

Breast cancer typically develops in normal breast cells such as mammary glands, lobules, and connective tissues and arises in the lining cells (epithelium) of the ducts (85%) or lobules (15%) in the glandular tissue [16].

Initially, cancerous growth is confined ("in situ"), asymptomatic and has minimal potential for metastatic spread. Over time, these in situ (stage 0) cancers may progress and invade the surrounding breast tissue (invasive breast cancer), then spread to the nearby lymph nodes (regional metastasis) or to other organs in the body (distant metastasis). If a woman dies from breast cancer, it is because of widespread metastasis.

There are over 20 different histologic types of invasive breast cancers. The most common is infiltrating duct carcinomas, which accounts for 70% to 80% of all invasive cancers, followed by invasive lobular carcinomas (around 10% of all invasive cancers) [15, 17, 18]. Classification into histologic types is based on a wide range of criteria, including tumor cell type (eg, carcinoma with apocrine features), extracellular secretion (eg, mucinous carcinoma), architectural features (eg, papillary carcinoma), and immunohistochemical profile (eg, carcinoma with neuroendocrine differentiation) [19].

However, this morphological classification cannot fully reflect the biological heterogeneity of breast cancers.

1.2 ***Immunophenotype***

ER, PR, and HER2 assessment is routinely use for breast cancer management. They are prognostic markers and important predictive factors for hormonal and anti-HER2-targeted therapy. ER and PR are expressed in ~75% of all breast cancer and when present they are bona fide indicators for responsiveness to hormonal therapy [15].

The majority of ER+ cancers are also PR+ and they are usually low grade and less aggressive. However, a small percentage of breast cancers show single hormone receptor positivity and these tumors seem to be more aggressive and less responsive to hormonal therapy [20, 21]. Moreover, 15% of breast cancers overexpress HER2 with amplification of the corresponding gene at 17q12 [22]. HER2 overexpression is associated with an aggressive clinical course and poor prognosis[23, 24], resistance to therapy with a high chance of metastasis and recurrence [25-27], but it is also predictive of response to anti-HER2 targeted treatments [28].

The remaining 10% to 15% of breast cancers that express none of these 3 markers are termed triple negative breast cancers (TNBC). These are in general high-grade and associated with a poor prognosis. Patients with TNBC do not benefit from the current targeted therapies [29], although recent years have witnessed considerable progress in this area. In the very days when this thesis is being written Sacituzumab Govitecan, and Antibody-drug Conjugate to TROP-2 is being granted EMA approval.

The information obtained through biomarkers is integrated with data from the TNM staging system [tumor size (T), the status of regional lymph nodes (N), and distant metastases (M)] to stratify the disease into 5 stages (0, I, II, III, and IV) and to provide more accurate prognostic info.

*1.3 **Molecular classification***

Breast cancer diagnostics have several components, e.g., clinical information and histopathologic analysis are supported by molecular classifications (Fig.1).

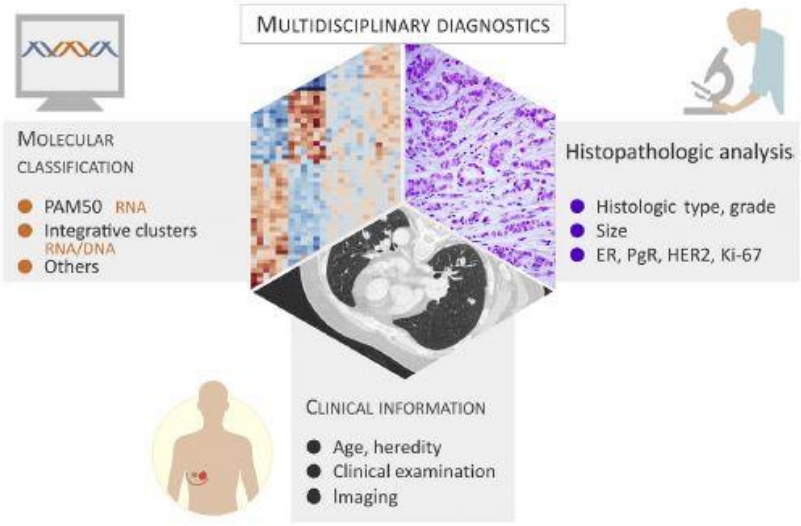

Fig. 1 *Multidisciplinary Diagnostics approach in breast cancer [30]*

Technological advancements and molecular techniques, such as systematic and sub-genomic gene expression profiling, have led to considerable refinement in breast cancer classification, prognostication, and prediction of response to therapy [31]. Many molecular bio-markers and patterns of gene expression are becoming available to break down breast cancer subtypes [32, 33]. Over the past decade several groups have pursued the development of multi-gene panel signatures that classify patients, such as *MammaPrint*, *Wang-76* and *Oncotype-DX* [34, 35] used as prognostic classifiers and predictor of response to treatment [36]. A particularly popular platform is the PAM50 (Prediction Analysis of Microarray using 50 classifier genes plus 5 reference genes) system that has been developed as a standardized method to categorize breast cancers [37].

High-throughput technologies have provided direct evidence of breast cancers' heterogeneity at the molecular level, and led to paradigmatic changes in our understanding of breast cancer biology. However, while the use of complex gene expression algorithms is precluded in clinical practice, a simplified analysis of 4-5 markers is now routinely adopted. A number of studies (too many to be extensively reported herein) have entirely re-classified breast cancers into 5 intrinsic subtypes by hierarchical clustering. Namely, the 5 subtypes are: luminal A, luminal B, HER2-overexpressing, basal-like breast cancers (BLBC), and normal-like tumors (Table 1).

Intrinsic Subtype	Gene Profile	Molecular Findings	IHC Phenotype	Histologic Subtypes
Luminal A	High expression of luminal epithelial genes and ER-related genes	Mutations in PI3KCA, MAPK3K1, and GATA3; CCDN1 amplification; no corresponding activation of PI3K pathways	ER+, PR ≥ 20%, HER2−, Ki67low	Tubular Carcinoma, low-grade IDC-NST, classic ILC
Luminal B	Lower expression of luminal epithelium and ER-related genes, but higher level of proliferation and HER2-related genes than luminal A	Similar to luminal A but with a higher prevalence of TP53 and RB pathways inactivation as well as Myc-related and FOXM1 related transcription	ER+, PR < 20%/ or HER2+/or Ki67high	IDC-NST, micropapillary carcinoma, pleomorphic ILC
HER2-OE	High expression of HER2-related genes; low expression of ER-related genes	HER2 amplicon and EGFR/HER2 signal protein signature	ER−, PR−, HER2+	High-grade IDC-NST, pleomorphic ILC
Basal like	High expression of basal epithelial and proliferation genes; low expression of HER2-related and ER-related genes	Mutations in TP53; losses in RB1 and BRCA1; amplification of MYC; high PI3K/AKT pathway activation	ER−, PR−, HER2-	High-grade IDC-NST, metaplastic carcinoma, medullary carcinoma, adenoid cystic carcinoma

Table 1 *Molecular subtypes of breast cancer*

gene expression profiling; IHC, immuno-histochemical; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2, IDC-NST infiltrating duct carcinoma, no special type; ILC, invasive lobular carcinomas [15]

In this clustering, the immunophenotypic classification by hormone receptors and HER2 status is prominent [18, 32, 33, 38]. ER expression stratifies breast cancers into 2 distinct clusters: ER+ and ER−. Luminal A and B subtypes are enriched with ER-positive cancers, whereas HER2-overexpressing, Basal like and normal-like tumors are ER− [32].

Luminal A is the most common molecular subtype (40-50% of invasive breast cancer). Generally, these tumors are low-grade, with the best prognosis among all intrinsic subtypes. Luminal B cancers tend to be higher grade and have a worse prognosis than luminal A. They show lower expression of ER-related genes, but higher expression in proliferation-related genes and variable expression of HER2-related genes than luminal A cancers.

Clinically, the luminal A group is likely to benefit from hormonal therapy alone, whereas luminal B tumors may candidate for additional chemotherapy.

The HER2-overexpressing subtype, comprising ∼15% of all invasive breast cancers, is characterized by the overexpression of HER2/HER2 signalling-associated genes and genes located with the HER2 amplicon on chromosome 17q12 [32]. HER2-overexpressing tumors are likely to be high grade, ER and PR− but there is a minor sub-population of HER2-positive cancers co-expressing ER that are classified as luminal B [39]. They run an aggressive clinical course, but nonetheless they are highly responsive to anti-HER2-targeted therapy, which resulted in a greatly improved outcome through targeted therapies.

Basal like breast cancers (BLBC) are associated with the expression of genes typical of the normal mammary basal/myoepithelial cells, including basal cytokeratin [14]. They also show overexpression of proliferation-related genes but lack ER, PR, and HER2- related gene expression [40]. Histologically, BLBC are usually high-grade, with a high proliferation index, and show a triple negative phenotype. BLBC patients have poor prognosis, and relapses may occur within 5 years from diagnosis [41].

Furthermore, HER2 and basal like groups are the major molecular subtypes identified among hormone receptor-negative breast cancer [34, 39, 42, 43].

The ‘normal-like cluster’ identified in initial studies is characterized by the expression of genes similar to normal breast epithelium. However, it is a controversial subgroup and has been later considered to be an artifact due to true normal epithelial cell ‘contaminating’ tumor tissues with a low malignant cell content [44].

Gene expression profiling could also identify additional rare subtypes, including claudin-low[45], molecular apocrine [46], and interferon-rich [33] breast cancers. However, these subtypes were only recognized by hierarchy clustering, and they lack representative signatures for unequivocal identification as separate entities [30].

Because of the cost and technical complexities, the daily clinical practice of systematic gene expression profiling is prohibitive, and the IHC-based surrogate molecular classification has been advocated, as per ASCO/CAP 2018 guidelines [47].

In this simplified but highly efficient and cost-effective classification, ER, PR, HER2, and Ki-67 are used to differentiate among clinically relevant subtypes:

(1) luminal A-like (ER+, PR≥20%, HER2−, Ki67< 20%),

(2) luminal B-like (ER+, PR< 20% and/or HER2+ and/or Ki67≥ 20%),

(3) HER2-overexpression (ER−, PR−, HER2+),

(4) basal-like (triple negative: ER−, PR−, HER2−).

Although IHC classification largely overlaps with gene expression profiling, some discrepancies do exist. Only ~80% of TNBC belong to the intrinsic BLBC subtype, and only 65% of HER2-positive tumors belong to the intrinsic HER2-overexpressing subtype [48]. Moreover, the cut off for Ki67 expression is still a matter of debate. The most recent St Gallen consensus adopted 20%: however, inter-laboratory variations in measurement and cut offs do exist. The other contentious issue is low ER expression (1% to 9%). These tumors are rare and are usually classified as luminal cancers on the basis of the current criteria even though they are more similar to BLBC both molecularly and biologically [49], raising controversies as to their clinical outcome [50].

1.4 ***Next Generation Sequencing***

NGS provides an orthogonal dimension to gene expression and IHC profiling. At least 40 cancer driver genes have been identified in breast tumors. The most frequently mutated or otherwise altered genes are TP53, PIK3CA, GATA3, MYC, CCND1, PTEN, FGFR1, RB1, ERBB2, and MAP3K1 [51, 52].

Generally, mutations are rare in breast cancers, and only the 3 most common mutations (TP53, PIK3CA, and GATA3) have incidences over 10% [31]. Many of these mutations are associated with distinct clinical and pathologic features. For instance, TP53 mutations are dominant in basal-like cancer [31, 53].

The overall rate of mutation is lowest in luminal A. The highest overall mutation rate was found in basal-like and HER2-enriched breast cancer [31]. Each tumor subset may harbour a characteristic set of mutational signatures.

NGS also identifies breast cancers with high Tumor Mutation Burden (TMB-H) [54]. These may be highly responsive to immune therapies such as PD-1 immune checkpoint blockade, making their identification of paramount importance [15].

1.5 ***EGFRs family members***

Like all epithelia, breast is under the influence of epidermal growth factors. HER1, HER2, HER3 and HER4 (called epidermal growth factor receptors ErbB1, ErbB2, ErbB3 and ErbB4) are transmembrane tyrosine kinase receptors that normally regulate cell growth and survival, as well as adhesion, migration, differentiation and other cellular responses [55, 56]. All members have an extracellular ligand-binding region, a single membrane-spanning region and an intracellular cytoplasmic tyrosine kinase domain [57, 58]. These proteins are encoded by genes expressed on chromosomes 7, 17, 12, and 21 respectively. The tyrosine Kinase domains are activated by both homo and hetero-dimerization, generally induced by ligand binding. However, HER2 has unique cell-signalling properties and undergoes ligand-independent auto-dimerization [59] and auto-phosphorylation [60] as well as ligand-dependent heterodimerization and phosphorylation.

Under normal conditions activation is controlled by the spatial and temporal expression of cellular ligands [61, 62]. The dysregulation of these mechanisms alters the expression of genes that contribute to the malignant phenotype [58, 63].

Of these dimerizations, three have demonstrated relevance to HER2+ BC:

1) HER1/HER2 hetero-dimerization allows HER1 to be recycled rather than degraded, promoting sustained signalling.

2) HER2/HER2 homo-dimerization leads to downstream oncogenic RAS/MAPK and indirect PI3K/AKT pathway activation.

3) HER2/HER3 heterodimerization promotes HER2 phosphorylation and activation of the HER3 kinase domain, leading to extremely potent stimulation of the downstream oncogenic PI3K/AKT pathway [24, 64].

HER2 is overexpressed or activated in a wide range of cancers, not just breast cancer. HER2-addicted cancers include bladder cancer, cervical cancer, colorectal cancer, endometrial cancer, esophageal cancer, liver cancer, lung cancer, ovarian cancer, and salivary adenocarcinoma [22].

1.5.1 ***HER2 Signalling and overexpression in breast cancer***

This paragraph is focused on HER2 breast cancers, that are the main subject of this thesis. Specifically, it underlines the evidence that supports HER2 as a major cancer driver in breast cancer, and why this may turn into an elective tumor vulnerability.

HER2 plays important functions during cell growth and differentiation. Its overexpression

sustains multiple cancer pathways, including self-sufficiency in growth signals, increased angiogenesis, accelerated cell division and invasion [61, 65]. This receptor has been thoroughly studied to understand his role in cancer biology as a therapeutic target. Many HER2-targeting agents have been designed and are now used in the clinic [66].

Previous studies indicate that 15%-20% of breast cancer patients carry HER2 gene amplification and overexpression [67], and about 50% of these patients express estrogen and/or progesterone receptors (ER/PR) [68, 69]. It is reported that HER2 receptor protein expression in HER2+ breast cancers increases about 40-100-fold on the tumor cell surface compared to non-HER2 breast cancers [67]. A typical HER2-positive breast cancer cell has 10 to 10.000 times the number of HER2 receptors on the cell surface compared to a HER2 negative (normal) cell, promoting un-controlled growth, over-active cell signalling and malignant tumor formation [70, 71].

Fig.2 shows that overexpression of HER2 leads to its constitutive activation, presumably because of increased receptor concentration on the plasma membrane. Many of these tumors contain phosphorylated-HER3, which couples HER2 to the PI3K-AKT pathway. HER2 signalling promotes cell proliferation thorough the RAS-MAPK pathway and inhibits cell death through the PI3K-AKT mammalian target of rapamycin (mTOR) pathway [60].

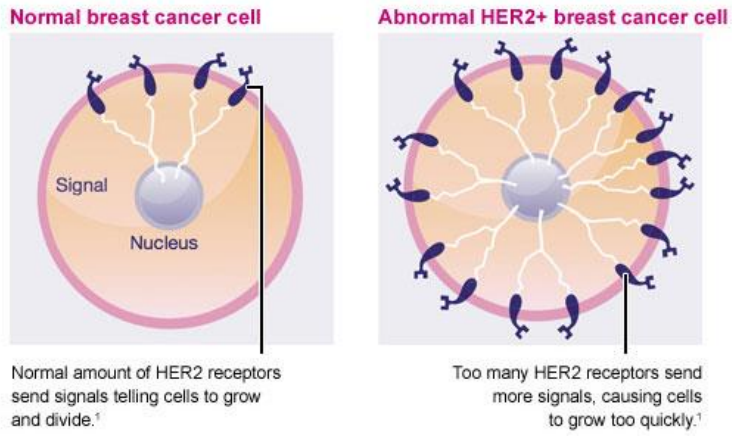

Fig.2 *HER2 gene copy number/expression in normal and HER2+ breast cancer cells*

While HER2 overexpression has been described in a variety of human malignant conditions, gene amplification is rare except in breast cancer. The very same feature exploited by the tumor to acquire a selective advantage (HER2 overexpression) has been exploited since the 1990's to selectively target this tumor dependency.

1.5.2 ***HER2 release in blood of breast cancer patients***

HER2 is an integral membrane protein. But the cell surface is not the only place where HER2 may be found. The HER2/neu protein consists of three domains: a 105 kDa extracellular domain (ECD), a transmembrane lipophilic segment and an intracellular domain with tyrosine kinase activity. The HER2 ECD may be released by proteolytic cleavage from the HER2 receptor and can be detected in serum [72, 73]. This mechanism is known as "ECD shedding". This proteolytic shedding leaves a constitutively active truncated receptor in the membrane ($p95^{HER2}$). This is 10–100-fold more oncogenic than the full-length receptor and promotes the growth and survival of cancer cells [74]. Shedding of the HER2 ECD is increased during metastasis: whereas 15% of primary breast cancer patients have elevated levels of serum HER2 ECD (sHER2), the levels reach 45% in patients with metastatic disease [75]. Elevated levels of sHER2 are also measured in patients with other malignancies like ovarian, lung, prostate and hepatic cancers [76, 77].
Over the past 20 years, several studies concluded that there is no correlation between tissue HER2 (tHER2) and sHER2 levels, and that measurements of sHER2 cannot replace FISH and IHC [78-81]. This conclusion was mainly drawn in patients at advanced disease stages [82]. High sHER2 levels were found to be positively associated with the tumor size, clinical stage, nodal status, histological grade, distant metastasis, and tHER2 status, and negatively associated with estrogen receptor expression [83]. These evidences suggest that monitoring sHER2 levels may complement the conventional FISH/IHC method to offer a real-time picture of the HER2 status in patients [84].

In 2000, the FDA recommended that sHER2 should be quantified with either of two validated immune enzymatic methods: an automated sHER2 assay platform (Immuno-1®, Siemens Healthcare Diagnostics), or a microtiter plate format (Oncogene Science, Siemens Healthcare Diagnostics). Both measure circulating sHER2 levels in serum by two monoclonal antibodies directed against distinct epitopes on the ECD. The threshold level for elevated sHER2, according to FDA guidelines, should be ≥15 ng/ml. An absolute change of ±20% or more from this threshold has been established as a significant change by the FDA. If elevated levels of sHER2 are found in patients suspected to bear cancer, other diseases should be excluded, since moderately high levels of sHER2 (up to 50 ng/ml) are also seen in association with liver diseases, pre-eclampsia, and chronic heart failure [76, 77]. For example, sHER2 levels were reported to be elevated in 40–60% of patients with non-malignant hepatic diseases [77]. The cut-off value above which sHER2 levels are considered to be elevated may not be the same in all clinical presentations. Ethnicity, for example, may

influence baseline levels of sHER2 [85]. Indeed, the somewhat arbitrary threshold value at 15 ng/ml may account for some of the variability in the comparison studies described below. Although elevated levels of sHER2 have been documented in many studies of breast cancer patients, they are most frequently observed in metastatic breast cancers [84, 86].

Additionally, the highest sHER2 concentrations correlate strongly with the presence of liver metastases [76] and correlated positively with parameters related to tumor aggressiveness, such as vascular invasion, metastatic status, and the absence of estrogen receptor, but not with invaded lymph nodes and progesterone receptor-negative tumors.

Several studies have also investigated the correlation between sHER2 level and survival parameters. In particular two recent studies showed that disease free survival (DFS) was shorter in patients with elevated sHER2 levels (≥15 ng/ml), and that sHER2 levels >15 ng/ml in tHER2+ breast cancers might predict the failure of anti-HER2 therapy [87]. They concluded that the median PFS was significantly longer in patients with low levels of sHER2. Patients whose sHER2 levels remain low or become low after treatment had significantly longer PFS times that those whose levels remained high or converted from low to high [81, 87-90]. These authors hypothesized that if sHER2 increases during therapy, fewer surface targets for trastuzumab remain on tumor cells, leading to therapy failure. Moreover, the truncated p95HER2 form that remains on the cancer cell membrane after cleavage does not bind therapeutic antibodies, but has a higher rate of constitutive tyrosine kinase activity and is 10–100-fold more oncogenic than the full-length HER2 polypeptide [75].

However, there is also evidence to the contrary. Witzel et al. [91], in the GeparQuattro trial, found a significant positive association between pathological complete remission (pCR, defined as no invasive and no in situ residuals in breast and nodes) and elevated sHER2 levels (above 15 ng/ml) during neoadjuvant trastuzumab therapy. A decrease (>20%) in sHER2 levels was associated with a higher response rate (defined as the percentage of patients whose cancer shrinks or disappears after treatment) with trastuzumab-based treatment was reported in other studies [92, 93]. At the same time, and in contrast to trastuzumab, the response to the tyrosine kinase inhibitor lapatinib does not affect the levels of sHER2, as demonstrated by Lee et al. [94]. They found that high levels of sHER2 predicted long PFS times with lapatinib, independently of tHER2 status.

These conflicting findings may be explained by differences in the methods used to asses response, the heterogeneity of patients populations selected, and differences in treatment [81]. In summary, there are no univocal interpretations for sHER2 and its role, possibly

because tumors and patients are very heterogeneous. This idea is revisited several times in the discussion of the experimental results presented herein.

1.6 ***HER2+ Breast Cancer: molecular diagnosis***

As per ASCO/CAP yr 2018 recommendations, Immunohistochemistry (IHC) and *In Situ* Hybridization (ISH; CISH/FISH) are the two main methods used in clinical practice for assessing the HER2 status[95]. This is done at diagnosis and in tumor tissue only. Tumors of the HER2 subtype must either carry HER2 DNA amplification (aHER2) according to a rather convoluted algorithm with a variable CNV threshold (from ≥4 to ≥6), or must overexpress HER2, e.g., ImmunoHistoChemistry (IHC) score must be 3+.

Currently, HER2-positive tumors are defined by >10% of cells with strong circumferential staining or HER2:CEP17 ratio ≥2. These detailed guidelines are regularly updated [47]. These amplification/overexpression criteria are met in 15-20% of breast cancers, that are therefore deemed targetable by small molecules and anti-HER2 antibodies. Treatment of breast cancer often consists of a combination of surgical removal, radiation therapy and medication (hormonal therapy, chemotherapy and/or targeted biological therapy). Treatment options are generally based on age, tumor size, histological grade, metastatic stage, the status of hormone receptors, and HER2 expression [96]. Women with recurrent or persistent breast cancer almost invariably undergo more than one treatment, requiring a multidisciplinary team approach. At early stages, they typically undergo surgical treatment, followed by radiation (if needed), and adjuvant systemic treatment to reduce the risk of relapse. Some patients receive preoperative systemic treatment (named neoadjuvant) to facilitate surgical approach as well as to assess response to the treatment [96].

In metastatic patients, different therapies are applied depending on the characteristics of the tumor and previous treatments [64]. Among systemic treatment options, one that has resulted in major advances in the last two decades is targeted therapy against HER2.

1.6.1 ***Treatment and Target Therapy***

The management of HER2–positive breast cancer has dramatically changed and has made significant progress over the last 2 decades. For decades cytotoxic chemotherapy in both early and advanced stages of breast cancer remained the Standard of Care (SOC) for neoadjuvant HER2-positive breast cancer treatment [154]. During recent years it was superseded by less invasive targeted therapies characterized by fewer side effects compared

to chemotherapy [97, 98]. The development of HER2 -targeted therapy has been one of the greatest advances in breast cancer treatment. Anti-HER2 drugs, particularly antibodies, prolong survival in HER2-positive metastatic disease, reduce recurrence in women with early HER2 positive breast cancer, and significantly increase the pathological complete response rate in the neoadjuvant setting [69].

During the past few years, several strategies have been developed to target HER2 including extracellular antibodies like trastuzumab (which targets domain IV of the receptor) and Pertuzumab (which binds to domain II and inhibits the heterodimerization of HER2 with other ErbB receptors); small tyrosine kinase inhibitors like lapatinib, tucatinib, or neratinib that inhibit the kinase activity; and finally, antibody-drug conjugates (ADCs) such as trastuzumab emtansine (T-DM1), which binds HER2 and introduces a potent cytotoxic agent into HER2- overexpressing cells [99, 100].

Trastuzumab (TTZ-Herceptin®), first entered clinical trials in the 1990s, but only in the early 2000s was it applied in the metastatic setting to demonstrate efficacy in the neoadjuvant setting through several small studies of early-stage HER2+ disease [101-103]. TTZ was then combined with Pertuzumab (PTZ), small molecule tyrosine kinase inhibitors (TKI) such as lapatinib and neratinib, and chemotherapy [104, 105].

Currently, in the metastatic setting, the standard of care for initial therapy in patients with HER2–positive metastatic breast cancer (MBC) is dual-HER2 antibody blockade with PTZ, TTZ, plus a taxane based on results from the phase 3 CLEOPATRA trial (ClincalTrials.gov identifier NCT00567190)[69]. This study demonstrates an absolute improvement in median overall survival (OS) of 16 months with the addition of PTZ: an unprecedented improvement in OS for patients with MBC [106].

For patients who have progressed on dual-HER2 therapy with TTZ/PTZ, the antibody-drug conjugate (ADC) trastuzumab emtansine or Kadcyla (T-DM1) is the preferred second-line therapy [107, 108], as revealed by the EMILIA (ClinicalTrials.gov identifier NCT00829166) and TH3RESA (ClinicalTrials.gov identifier NCT01419197) phase 3 trials, which demonstrated that T-DM1 had a significantly longer median progression-free survival (PFS), by 3 months compared with standard chemotherapy [99, 109, 110] and less toxicity [109, 111] (Fig. 3).

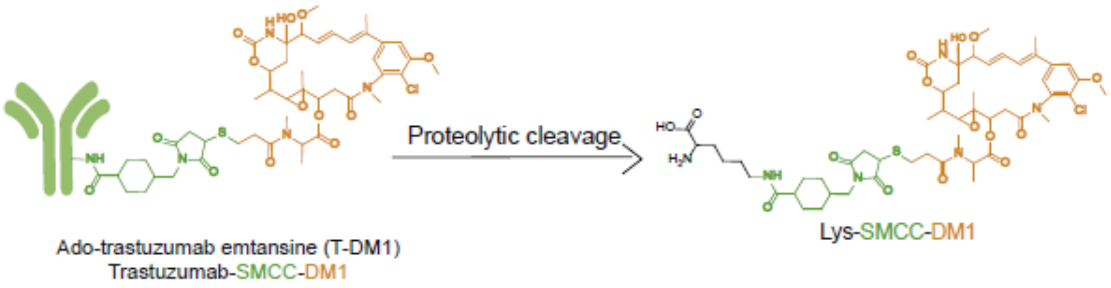

Fig. 3 *Structure of T-DM1*

The linker of T-DM1 is not cleavable and completely degraded in the lysosome to a linker-payload linker with lysine on one antibody
[112]

T-DM1 was also shown to be non-inferior to TTZ plus taxane and T-DM1 plus PTZ as first-line therapy [113]. For many years T-DM1 has represented the SoC in advanced HER2 BC following Trastuzumab/Pertuzumab treatment, although lesser than expected objective responses were observed [114, 115]. Recently, it was also approved in the adjuvant setting [116].

Biologically, TDM-1 takes advantage of a stable non-cleavable linker to bind the cytotoxic payload to TTZ in order to prevent release of the cytotoxic agent DM1 into the bloodstream and thereby reduce systemic toxicity. [117-121]. Once T-DM1 binds to HER2, a receptor-mediated endocytosis triggers the entry of the HER2 receptor-T-DM1 complex into the cells [122]. T-DM1 uses TTZ to specifically deliver the maytansinoid anti-microtubule agent DM1 to HER2 positive cells. Active DM1 is released in the cytoplasm as a result of proteolytic degradation of the antibody in the lysosome [123]. Inhibition of HER2 ECD shedding, block of the PI3K/AKT signalling pathway [120], antibody-dependent-cell-mediated-cytotoxicity (ADCC) [121], mitotic catastrophe [119], disruption of intracellular trafficking and apoptosis all contribute to the T-DM1 mechanism of action [124]. T-DM1 has a substantial effect on cells expressing high levels of HER2, but has limited activity on other cells with low or moderate expression [125, 126].
In this connection, several studies documented T-DM1 resistance and progression. Pharmacological resistance to T-DM1 has been associated with several direct or bypass alterations of the HER2 pathway (reviewed in [127]), but most of these were observed in preclinical models only [128-132].

The molecular mechanisms driving resistance to HER2–targeted therapy is probably complex. The following factors were shown to drive resistance: downregulation of HER2,

intra-tumoral heterogeneity of HER2 expression, HER2 receptor variants (e.g., p95HER2 isoform, splicing variant of HER2 lacking exon 16), constitutive downstream activation of the PI3K/AKT/mTOR pathway, escape from antibody-dependent cell cytotoxicity (ADCC), and upregulation of estrogen receptor (ER) [133].

Treatment options for patients who have HER2+ MBC after progression on PTZ, TTZ and T-DM1 are limited. Capecitabine plus lapatinib has shown modest benefit compared with capecitabine alone (median PFS, 8.4 vs 4.4 months; $P < 0.001$) [134]. To avoid this problem and reverse T-DM1 resistance, ADCs with an enzymatically cleavable peptide linker and a potent exatecan derivative topoisomerase I inhibitor (DXd), like Trastuzumab deruxtecan (DS-8201a) were developed (Table 2).

Antibody Drug Conjugate	HER2 Monoclonal Antibody	Payload	Linker Drug	Drug-to-Antibody Ratio	Developmental Phase	Preclinical Activity in HER2 Low tumors?
Trastuzumab emtansine (T-DM1)	Trastuzumab	DM1 (tubulin inhibitor)	Noncleavable	3	Phase 3, FDA approved	No
Trastuzumab deruxtecan (DS-8201)	Trastuzumab	Exatecan derivative (topoisomerase 1 inhibitor)	Cleavable	8	Phase 2, phase 3 ongoing, FDA accelerated approval	Yes
ARX788	Anti-HER2 specific monoclonal antibody	Auristatin analog 269 (tubulin inhibitor)	Noncleavable	1.9	Phase 1 ongoing	No
ZW49	ZW25 (bispecific HER2 [ECD2 and ECD4] monoclonal antibody)	Auristatin analog (tubulin inhibitor)	Cleavable	Unknown	Phase 1 ongoing	Yes
Trastuzumab duocarmazine (SYD985)	Trastuzumab	Duocarmycin (alkylator)	Cleavable	2.8	Phase 1/2, phase III ongoing (TULIP study)	Yes
RC48	Hertuzumab	Auristatin analog (tubulin inhibitor)	Cleavable	4	Phase 1/1, randomized phase 2 ongoing	No
MEDI4276	Bispecific HER2 (ECD2 and ECD4) monoclonal antibody	Tubulysin (microtubule inhibitor)	Cleavable	4	Phase 1	No

Table 2 *Summary of the various ADCs currently FDA approved for breast cancer or currently in clinical trials*

ECD2, extracellular domain 2; ECD4, extracellular domain 4;FDA, US Food and Drug Administration; TULIP, SYD985 Versus Physician's Choice in Participants With HER2–Positive Locally Advanced or Metastatic Breast Cancer (ClinicalTrials.gov identifier NCT03262935).
[135]

These have a bystander effect, e.g. they kill surrounding tumor cells despite they may have low HER2 levels. This occurs because T-DXD is able to release part of its payload into the extracellular environment, therefore affecting non-HER2 overexpressing cells [125]. Because of its bystander effect, this compound is employed in several malignancies including tumors with low levels of HER2 where it has been shown to overcome T-DM1 resistance [112, 136] (Fig.4).

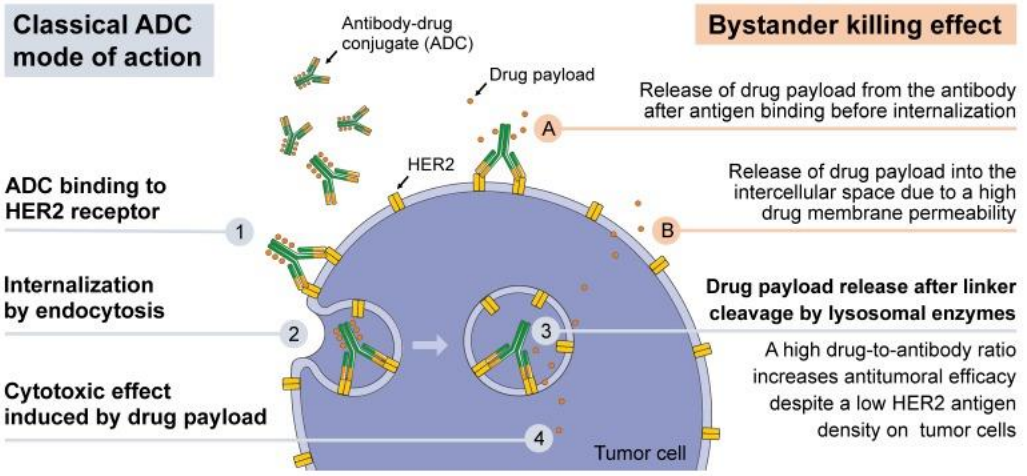

Fig. 4 *Mode of action of HER2 directed ADCs in HER2-low tumors*

Classical mode of action of ADCs with cleavable linkers: (1) After binding of the monoclonal anti-HER antibody component to HER2 expressed on the cell surface of tumor cells, (2) the ADC-HER2 complex is internalized by endocytosis. (3) After linker cleavage by lysosomal proteases, the drug payload is released and (4) can induce the cytotoxic effect leading to tumor cell death. A high drug-to-antibody ratio can increase antitumoral efficacy despite a low HER2 antigen density on tumor cells. Bystander killing effect: Using cleavable linkers, ADCs can be designed to promote drug release from the target cell to the extracellular space. Thereby, surrounding and bystander cells, which may or may not express the ADC target antigen, can be killed by taking up the cytotoxic drug. (A) This bystander killing can occur if the cytotoxic drug is released from the antibody after antigen binding before internalization. (B) Additionally, the drug payload can be released from the tumor cell into the intracellular space due to a high membrane-permeability of the ADC drug payload.
[137]

*1.7 **Unmet medical needs***

This thesis is focused in HER2+ advanced breast cancer (HER2+ ABC) that have gone through several lines of therapy and the changes that should be introduced to the SOC. The idea behind this study is that HER2 amplification and expression levels change with time. As extensively discussed above, breast cancer patients often receive several lines of HER2 targeted treatments associated with chemotherapy, until after several years and different lines of therapy they ultimately develop resistance. The strategy to intensify HER2 blockade has been very successful, with the introduction of promising novel agents, antibody-drug conjugates, and new-generation tyrosine kinase inhibitor (TKIs). Altogether, these have provided clinicians with enhanced chances to blunt the HER2 pathway across multiple lines of treatment. For all these reasons the molecular landscape of HER2+ ABC, HER2 levels, and associated treatments should be considered three elements in continuous reciprocal adaption/evolution, and an active area of research.

Although more resolutive therapies are at sight, advancements in biomarker discovery will remain critical in optimizing treatment selection. Liquid Biopsy is one of these areas of biomarker discovery, possibly one of the most promising.

CHAPTER 2

LIQUID BIOPSY

Overview

According to the latest WHO assessments, cancer is now considered the most frequent cause of mortality worldwide. Cancer healthcare represents a crucial challenge for the scientific community around the world. Primary prevention, early diagnosis and improvement of therapeutic efficacy are major unmet medical needs and major objectives of biomedical research, the big Pharma, and policy makers worldwide. Non-invasive cancer diagnosis and monitoring is a must.

Although the Food and Drug Administration has recently approved liquid biopsy for therapeutic assignment, this is standard of care in the USA only, whereas in EU tissue biopsy remains the reference standard for the molecular analysis of tumors in all clinical guidelines and recommendations.

Clearly, only tissue biopsies offer the possibility to examine the architecture, cellular structure, and the features of invasive lesions associated with the tumour, the proliferative activity of cancer cells, and the interactions between neoplastic cells and their microenvironment. However, tissue biopsy by itself has intrinsic limitations, particularly because a tissue-only approach is a significant barrier to easy and frequent monitoring of oncogenic alterations. Moreover, tissue biopsy may be risky for the patients, and the patient's compliance with this procedure is variable given its invasive nature and associated discomfort. Tissue biopsy may be difficult depending on the anatomical location of the lesion to be sampled [138, 139]. But the most crucial drawback is represented by the snapshot nature of the method [140].

As summed up in the work of TRACERx [141] in recent years we have learnt that any tumor consists of different sub-populations, and that tissue biopsy (even in its sophisticated multi-region sampling protocol) fails to portray the complex clonal equilibrium of the disease. The standard analysis based on 'clinical' or 'bulk' tissue biopsy inevitably underestimates genomic complexity an changes thereof [142-146], and may result in inaccurate information as to actionable markers, acquired pharmacological resistance, tumor burden, population complexity.

Tumor heterogeneity poses a serious challenge to determining the best course of a therapy. A single (in space and time) tissue biopsy underestimates the complexity of the tumor

genomic landscape and is unconfortable as well as risky for the patients and fails to provide the complete molecular picture of the tumor.

In contrast, Liquid biopsy has the potential to reflect the overall genomic landscape of the tumor, both spatially (across all metastatic sites) and longitudinally (across time). The ease of access to the blood and the nearly non-existent morbidity of this approach suggests that routine cancer surveillance using liquid biopsy is possible [147]. Although one may argue that Liquid Biopsy is less sensitive than tissue biopsy (analytes are much more diluted and admixed with normal components in blood than in tissues), liquid biopsy is the only technique that provides a 'weighted average' of molecular cancer alterations in a given patient. Liquid Biopsy may sample different body fluids such as blood, urine, cerebrospinal fluid, saliva, stool, and more [148], and detects tumor-derived cellular and sub-cellular materials e.g., cells or nucleic acids obtained from them [149-152].
In fact those body fluids contain a variety of tumor-derived materials such as tumor DNA, [153], intact circulating tumor cells (CTCs)[154], tumor-educated platelets, or extracellular vesicles and proteins [155, 156] (Fig.5). Other tumor-derived materials include circulating RNA such as cell-free messenger RNA and microRNA, which are noncoding RNA molecules of 19 to 24 nucleotides in length [157].

Over the last decade, numerous liquid biopsy studies have been carried out, expanding research horizons and deeply influencing our views on the fundamental nature and mechanisms of cancerization. Of the various tumor-derived materials in a liquid biopsy, CTCs and ctDNA are arguably the best studied, and are equally important because they provide information that is independent and complementary[158].

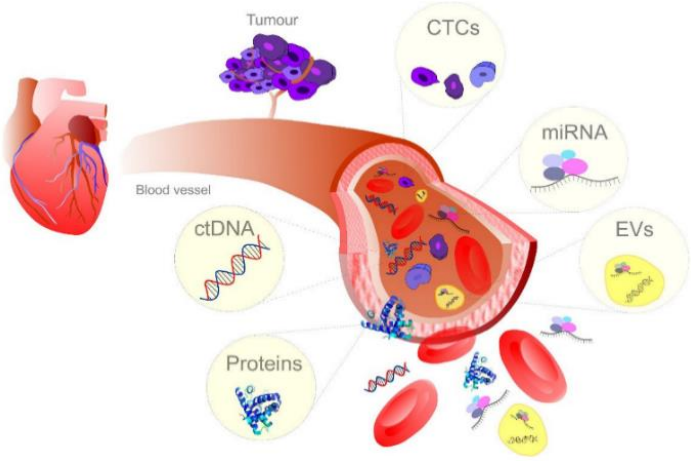

Fig. 5 *Blood-based cancer biomarkers in liquid biopsy*.

CTC, circulating tumor cells;
ctDNA, Circulating tumor cell-free DNA; EVs, extracellular vesicles.
[159]

For all the above reasons, Liquid Biopsy can be considered a real-time monitoring exercise (Fig.6) to track tumor heterogeneity between tumours of the same type in different patients (inter-tumour heterogeneity) as well as between cancer cells within a tumour (intra-tumour heterogeneity)[160], ultimately resulting in highlighting the acquisition of mutations that confer resistance to conventional therapies before progression [161, 162].
In this context, there is a clear clinical need to apply all the novel diagnostic and molecular tools taking advantage of the concept of liquid biopsies in oncology.

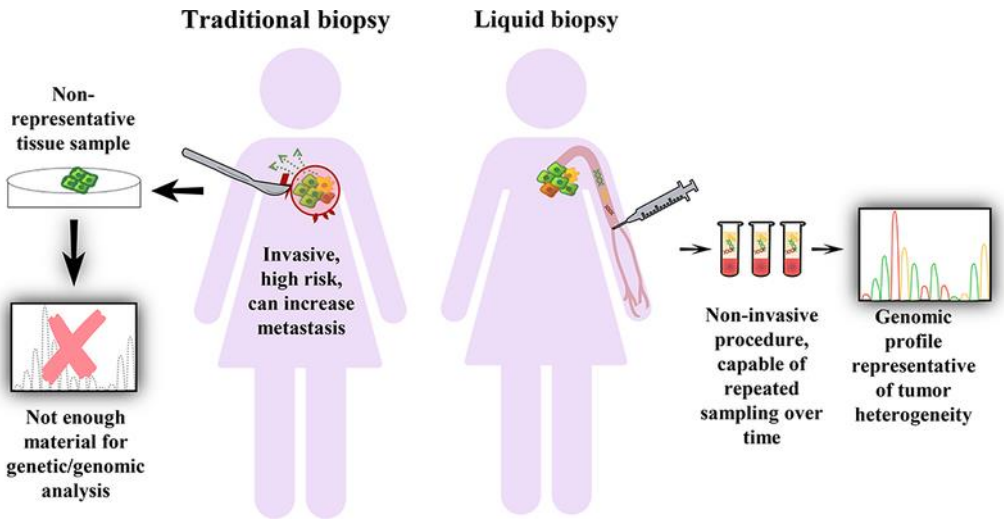

Fig. 6 *Traditional biopsy vs liquid biopsy*

The location of the tumor, tumor heterogeneity, and insufficient tumor tissue. Compared with traditional biopsy, liquid biopsy offers a non-invasive option for detecting and monitoring oncological disease. By extracting tumor DNA from blood plasma, risks and complications associated with traditional biopsy are eliminated. A more complete survey of the heterogenous genomic landscape of a tumor may also be obtained, and the process is easily repeatable over time in order to perform longitudinal and replicate analyses. [147]

2.1 The many facets of circulating-free DNA (cfDNA) and circulating tumor DNA (ctDNA)

DNA can be released from cells into the circulation through apoptosis, necrosis, and active cellular secretion [163], and possibly thorough other unknown mechanisms, as shown by the group of professor Gambari in collaboration with our group in Rome [164]. Particularly under specific pathological conditions (e.g. in an inflamed, poorly perfused tumour mass) the accumulation of cellular debris and DNA leads to massive release into blood [165]. Alternatively, there are active mechanisms of circulating tumor DNA (ctDNA) release, a spontaneous phenomenon and regulatory mechanism that determines a preferential release of newly synthesized DNA [166].

Even though the mechanisms leading to circulating free DNA (cfDNA) release in blood are not yet fully understood, cell death (e.g., apoptosis and necrosis) is claimed to be the main processes responsible for the release of fragments of cellular DNA. Fragment size ranges in length from a few hundred to a few thousand base-pairs. cfDNA fragments of about 145-180 bp are produced from cell apoptosis, while longer fragments (up to 10 kbp) are generated from cell necrosis [167]. Also of interest, cfDNA originating from solid tissues is usually shorter than the majority of cfDNA derived from hematopoietic cells [168, 169].

The existence of cfDNA was reported for the first time in 1948 by Mandel and Metais [170] but only in 1977 Leon et al. [171] discovered that cfDNA concentration is significantly higher in the serum of patients with cancer in comparison to healthy individuals. Then, in the 1990s Stroun [172, 173] provided evidence that cancer cells release their DNA into blood (ctDNA)[174], and introduced for the first time the concept of *liquid biopsy*.

There are a lot of factors influencing cfDNA levels, including exercise [175], surgery [176], age [177], trauma or injury [178], inflammation [179], and obesity [180]. Generally, the level of cfDNA in plasma is low and does not exceed 7 ng per ml in a healthy individual [181, 182], because phagocytes remove all of apoptotic and necrotic debris [183]. Greater cfDNA levels may be present in healthy individuals [184] due to traumas, myocardial infarction, stroke, or in autoimmune conditions [185].

Observational studies have determined that cfDNA has a variable half-life in the circulation ranging from 15 minutes to several hours [186-188], which makes ctDNA analysis a real-time snapshot of the disease burden at the very time of blood drawing.

ctDNA possibly circulates in blood before CTCs are evident [189]. Indeed, studies comparing ctDNA and CTC levels in the bladder, breast and colorectal cancer patients demonstrated that ctDNA is often present in patients with no detectable CTC levels [158, 174]. This issue is worth further investigation and there is some controversy.

*2.2 **Technical Approaches to cfDNA/ctDNA analysis***

Working with circulating tumor nucleic acids is extremely complex. First of all because of low concentrations, high variability (there is no consensus on ‘normal’ thresholds) and to the fact that concentration is strongly influenced by pre-analytical processing, including nucleic acid extraction [190].

Typically, median cfDNA concentrations are lower than 20 ng mL^{-1} even in plasma obtained from cancer patients, which represents a minimal difference when compared to healthy individuals who show lower median concentrations (lower than 7 ng mL^{-1} in plasma).

Thus, it would be prohibitive to discriminate cancer patients from healthy individuals solely based on cfDNA/ctDNA levels, the overlaps between the two populations being wide enough to make this distinction highly unreliable [181, 191-194]. It is then mandatory to identify ctDNA and not just cfDNA. ctDNA is cfDNA released from tumor cells, e.g., DNA that shows hallmarks of cancer, typically a mutation (e.g., KRAS G12C).

Having said this, cfDNA and ctDNA are similarly isolated (e.g., they are mixed with one another) in all tumor-derived materials, such as blood drawings or urine collection, etc.

To isolate cfDNA/ctDNA, whole blood is separated into either plasma or serum. After blood draw, it is essential to avoid cell lysis because this releases unfragmented cellular DNA, diluting the original ctDNA in even greater amounts of leukocyte DNA. Therefore, plasma is preferred over serum due to a lower risk of contamination with cellular DNA from clots [195]. To avoid cellular degradation, plasma should be isolated within two to six hours after collection [196, 197]. In our lab, we isolate ctDNA within 45 minutes, and we routinely observe no more than 3 ng/ml cfDNA in healthy subjects. Plasma is subsequently stored at –80°C or directly used for cfDNA extraction. Freeze-thaw cycles of both plasma and extracted cfDNA should be avoided as they lead to DNA degradation [198].

In case plasma cannot be isolated soon after blood is drawn, commercial tubes from many different brands, all containing preservatives, can be used to inhibit cell lysis and nuclease activity in the sample, allowing the sample to be stored at room temperature for days [197, 199].

Finally, cfDNA is extracted. There are many methods, but DNA binding to chromatographic microcolumns, magnetic beads or silica-based membranes are most used [200].

Recent advances in circulating tumor DNA (ctDNA) research underline the potential applications of liquid biopsies at every stage of patient management, but there is a need to further optimize the yield of ctDNA by improving extraction methods and the sensitivity of current DNA analysis assays, particularly at early disease stages when tumor load and ctDNA are low [161].

The Human Genome Project (HGP) is an international scientific research project with the goal of determining the base pairs that make up human DNA, and of identifying and mapping all of the genes of the human genome from both a physical and a functional standpoint. Launched in 1990, HGP was declared complete on April 14, 2003. The Consortium was able to entirely sequence the human genome with far reaching implications on genetic diseases on the one hand, and cancer on the other [201]. As to the latter, somatic alterations occur in

individual cells during our lifetime and are believed to be major drivers transforming normal cells into malignant cells.

Detecting nucleic acid alterations gives us the opportunity to improve our techniques of screening, early diagnosis, prediction of response to (targeted) cancer therapy [202-204], and detection of pharmacological resistance to treatment. Altogether, these may guide patient management [158, 205-207].

In 1999, Vogelstein and Kinzler [208] invented digital PCR (dPCR), a method providing an accurate identification and absolute quantification of mutated fragments allowing for the first time the quantification of the mutant allele fraction in the plasma of colorectal cancer patients at different stages [186, 209]. However, only in 2008 it was demonstrated that ctDNA is a highly specific biomarker of tumour dynamics and could indicate residual disease after the pharmacological treatment [186].

During the last years, the development of new deep next-generation sequencing-based techniques and refinement of ultrasensitive dPCR methods has simplified the analysis of the genome on a larger scale than previously possible. The increasing availability and reliability of those techniques are facilitating novel high-sensitivity applications for ctDNA, the generation of large clinical data sets and a better understanding of the origins of ctDNA. In 2012, deep sequencing of multiple genes in ctDNA was employed through panels of tagged amplicons for the identification and monitoring of multiple tumour-specific mutations in a single assay, directly in the plasma of cancer patients [210]. Hybrid-capture sequencing method, based on the hybridization of DNA target in solution through complementary DNA sequences bound to magnetic beads, was presented as a non-invasive method for the analysis of evolving genomic profile of mutations across the entire exome in cancer [211]. Shortly thereafter, whole-genome sequencing (WGS) of ctDNA in plasma was used for the detection of tumour-derived chromosomal aberrations [212], focal amplifications [213] and gene rearrangements [214].

Targeted, massive parallel sequencing of multiple genes offers a wider scope for the molecular interrogation of the tumor than dPCR. Nevertheless, it requires dedicated bioinformatics support and can suffer from specificity issues when trying to distinguish low-allele-frequency variants. There is also a lack of standardization of techniques that extends into every step of the liquid biopsy analysis, from the analyte, its quantification, to the assay itself (dPCR, amplification-refractory mutation system, BEAMing, or NGS).

2.3 ***Liquid Biopsy in Breast Cancer***

Although breast cancer is one of the few cancers with a well-established routine for population screening, several studies have investigated tumor-derived materials in the blood of patients as an additional, or alternative, method for early cancer detection. Two pioneers were Schwarzenbach et al. and Agostini et al. who demonstrated that the quantity of cfDNA in breast cancer patients were higher than in normal healthy controls and could have discriminatory value, but at the same time demonstrated that patients with benign breast alterations also have high cfDNA [215, 216]. Agostini et al. also observed that cfDNA fragments released by necrotic tumor cells are usually larger compared with the smaller fragments of cfDNA released during apoptosis in normal individuals [216]. Afterwards, Board et al, using amplification-refractory mutation system allele-specific PCR and Scorpion probes, were able to detect the presence of PIK3CA mutations in ctDNA in 80% of patients with metastatic breast cancer harbouring PIK3CA mutations but not in the ctDNA from patients with localized breast cancer [217].

Sometime after, Bettegowda et al. managed to detect the presence of ctDNA in 50% of patients with localized breast cancer using highly sensitive technologies such as BEAMing (beads, emulsification, amplification, and magnetics), PCR ligation, and massive parallel sequencing. They were able to detect the presence of ctDNA also in patients with localized breast cancer [158]. The different results of these studies reflect the varying sensitivities of different ctDNA assays and highlight the need for highly sensitive assays to detect the presence of ctDNA, particularly if ctDNA is to be incorporated into breast cancer screening. For example, in a meta-analysis of 69 studies on 5736 patients with breast cancer, Lee et al [218] found ctDNA mutation rates of TP53, PIK3CA, and ESR1 to be approximately 38%, 27%, and 32%, respectively, and concluded that they were too low to be used for breast cancer screening. Apparently, breast cancer screening by liquid biopsy remains impossible or premature. Nowadays there is also uncertainty as to the management of patients with extremely early-stage disease where there is detectable ctDNA but without a clinically or radiologically evident lesion. For example, the same genetic mutation, such as a TP53 mutation, can also be found in more than one cancer type and may not be specific to breast cancers [161]. In order to overcome this challenging limitation, Cohen et al [219] designed a test that takes into account not just the somatic mutations found in ctDNA but also the protein biomarkers found in plasma in order to accurately localize the primary site of the cancer. This groups demonstrated that breast cancer (and other cancers) can be detected in

blood by a proteogenomic approach combining somatic mutations found in ctDNA and protein biomarkers. The assay detects cancer on the day of surgery and also assigns the primary site, but it is still inapplicable, at the time of writing, to screen pre-symptomatic populations.

Therefore, liquid biopsy finds application in early BC. A particularly important setting is detection of minimal residual disease (MRD) [220-222]. Several authors demonstrated how detect early relapses using dPCR liquid biopsy, and observed a lead time between ctDNA detection and radiological evidence of relapse. By the TARDIS approach, McDonald et al. demonstrated high accuracy in detecting molecular response and MRD during neoadjuvant chemotherapy (NAC) [223]. They also demonstrated that patients with a decrease in ctDNA level during NAC respond to therapy. In a recent study with a similar aim, Zheng and colleagues [224] tracked tumour mutations in plasma, trying to understand the utility of ctDNA in predicting therapy outcome. Several clinical trials (e.g. [225]) were developed following similar aims.

Although a very valuable tool in the context of these pioneering efforts (asymptomatic subjects and early breast cancer), liquid biopsies are mostly used to predict outcome in advanced cancer. In this setting, the main obstacle that may be tackled by liquid biopsy is to resolve the heterogeneity of these tumours [226]. It is very well known that cells of the HER2+ subtype are admixed within histologically HER2- tumors, which can give rise to significant differences in response to treatment and biological evolution [227-229]. Several studies demonstrated that critical genomic changes in BC occur during cancer progression creating significant variability between primary tumor and metastases [226, 230]. In addition, systemic treatment creates dynamic molecular pressure, which can lead to greater biological heterogeneity [231].

The HER2 status is a clear example of cancer heterogeneity [228] since it has been demonstrated that HER2 expression can change from primary disease to metastases by 9% to 60% [232, 233]. This phenomenon can lead to inappropriate treatment selection if HER2 status is not reassessed throughout different disease stages. Liquid biopsy may permit a closer disease monitoring of the HER2 status, hence providing better clinical management. Although this is unknown for HER2, it has been proven for other breast cancer alterations. When ctDNA is serially monitored, changes in ctDNA levels or mutant allele frequencies after treatment correlate with parameters such as changes in tumor size and outcomes [234, 235]. This can be used to assess and monitor responses to treatment.

In a study by Dawson et al, ctDNA levels showed a closer correlation with changes in tumor

burden when compared with plasma CA 15-3 levels or CTCs. This study also provided the earliest measure of treatment response, with an average lead time of 5 months before medical imaging [174].

The possibility to profile ctDNA and find driver mutations such as ESR1 may be superior to sequencing the primary tumor, as they may mirror the molecular profile of the relapsed/metastatic tumor more accurately as demonstrated by Spoerke et al. [236]. They found concordance between ESR1 status in ctDNA and metastatic tissue.

Gerlinger et al, showed that portions taken from different parts of a primary tumor and its metastases displayed intertumoral and intra-tumoral differences [237]. Serial longitudinal tissue biopsies, which theoretically can map the phylogenetic progression of the tumor and overcome the problem of tumor heterogeneity are, however, impractical in the clinical setting, as tissue biopsy is invasive with potential complications, and therefore not amenable to repetition.

Among the myriad of technologies and testing procedures that characterize liquid biopsy currently, there is a need to determine the optimal testing strategy and to establish standardized protocols for its use in the clinical setting. With time, it will certainly become possible that liquid biopsies will acquire an even greater role in breast cancer clinic.

CHAPTER 3

BACKGROUND AND RATIONALE:

LiqBreasTrack, a real-life liquid biopsy study

In the previous chapters, I have outlined some recent milestones in our present understanding of breast cancer. We have now considerable knowledge on molecular classification, and have learnt how to leverage on this knowledge to assign therapy. I illustrated why I am interested in oncogenic HER2 signaling, a major paradigm in oncology, and provided a short summary on ADCs, with their unique two-fold mechanism of action: direct blunting of oncogenic signaling and payload delivery. Most relevant to the thesis I am about to defend, I introduced liquid biopsy and its many achievements across very different cancer conditions, particularly breast cancer.

On these premises, a few years ago, my group undertook a study that has the ambition of bringing together these basic ideas of precision oncology in the attempt to provide additional insight into breast cancer of the HER2 subtype. This subtype has all the 'ingredients' necessary for the precision oncology recipe. There is a single strong oncogenic addition, multiple layers and lines of antiHER2 clinical blockade, extensive use of therapeutic antibodies and ADCs and, despite all this, HER2 breast cancer remains a major unmet medical need. Essentially all patients with advanced metastatic disease develop pharmacological resistance at some time during their disease course. Moreover, we noted a major missing piece in the HER2 cancer puzzle: pharmacological resistance (including resistance to the ADC epitome T-DM1), has been associated with several direct or bypass alterations of the HER2 pathway [reviewed in [127]], but most of these were observed in preclinical models only [128-131, 238].

We hypothesized that LB might instead provide a unique opportunity to non-invasively capture resistance traits in the clinical setting. Then, we designed (clinically and molecularly) *LiqBreasTrack*, a small clinical-translational study that was carried out at the Regina Elena National Cancer Institute from November 2016 to February 2021 to assess tumor molecular alterations occurring in blood under T-DM1 pressure, and recapitulate adaptive tumor evolution in archival tissues (Fig.7).

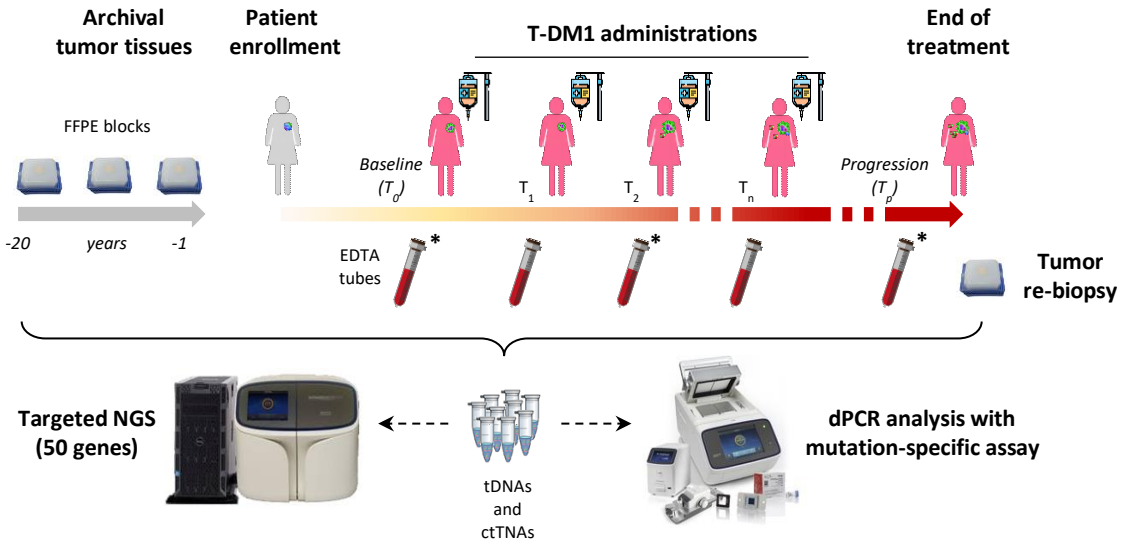

Fig. 7 LiqBreasTrack workflow

As shown in the figure, *LiqBreasTrack* was designed to aggressively capture circulating alterations, since blood drawing was performed at every T-DM1 infusion, and many tumor tissues were collected to recapitulate adaptive tumor evolution. Eligibility and T-DM1 administration were as per SoC.

Demographics and clinical pathological features are presented in Table 3. The study was approved by the competent Ethical Review Board (RS-857/16).

Patients signed a written informed consent including the option of re-biopsy.

Tumor tissues (n=28) and blood drawings (n=337) were tested by targeted NGS and dPCR. Progression-free survival (PFS) was calculated between the first T-DM1 administration and progressive disease or last follow-up. *LiqBreasTrack* was originally built around a double readout: targeted NGS and dPCR, as shown in the figure.

Characteristics	N (%)
Age, years (range)	56.8 (39.4-83.5)
ECOG Performance Status ≤2	22 (100)
IHC molecular markers	
Primary tumor tissues	22
ER+ and/or PgR+	15 (68.2)
ER- and/or PgR-	7 (31.8)
HER2 1+/SISH or FISH+	3 (13.6)
HER2 2+/SISH or FISH+	2 (9.1)
HER2 3+	17 (77.3)
Metastatic tumor tissues	9
ER+ and/or PgR+	6 (66.7)
ER- and/or PgR-	3 (33.3)
HER2 1+/SISH or FISH+	1 (11.1)
HER2 2+/SISH or FISH+	3 (33.3)
HER2 3+	5 (55.6)
Previous lines of therapy	
1	14 (63.6)
2	7 (31.8)
3	1 (4.5)
Pertuzumab as first line treatment	
Yes	9 (40.9)
No	13 (59.1)
Dominant Metastatic sites	
Liver	3 (13.6)
Lung	3 (13.6)
Bone	5 (22.7)
Soft tissues	7 (31.9)
Brain	4 (18.2)
Number of metastatic sites per patient	
1	8 (36.6)
2	9 (40.9)
≥ 3	5 (22.7)

Table 3 *Patients Characteristics*

Subsequently, we introduced and additional readout, e.g., circulating levels of the HER2 protein, as assessed by a sandwich ELISA, for reasons that will be detailed below. To our knowledge, *LiqBreasTrack* is the first study to investigate HER2 oncogenic addiction integrating longitudinal, dynamic proteogenomic readouts. *LiqBreasTrack* relies on molecular background built on cellular *in vitro* models. The following section summarizes all the materials (biological and clinical) that were used in this thesis.

CHAPTER 4

MATERIALS AND METHODS

4.1 ***Cell Lines***

Protein extracts were obtained from ERBB2 positive (BT474, SK-BR-3, KPL-4, T47D) and negative (MDA-MB-231) cell lines. Cells were propagated respectively in RPMI 1640 with 10% FBS. Cells were maintained in culture for 72h, centrifuged at 1000 rpm for 5 min and the medium was stored at -80°C for further analysis. Cells were counted by Trypan Blue exclusion and lysed in *CST lysis buffer* supplemented with 1mM PMSF and 0.1mM aprotinin protease inhibitor (approximately $10x10^6$ cells/ml) (all from Thermo Fisher Scientific, USA). For whole cell protein extracts, we followed a common protocol mostly used in many others labs. Briefly cells were cultured in T75 flasks and lysed in CST-Buffer. After an incubation during 30 min at 4°C, the samples were centrifuged 10 min at 13000 rpm ad the supernatant was aliquoted and stored at -80°C. Yields in whole cell lysates were assessed by the BCA Assay (*QuantumProtein kit*, #EMP014250 Euroclone) (mean= 3,89 ug/ul). The lysates were serially diluted in reagent diluent 1 to 2 with a range between 2 ug/ul and 0.16 ug/ul and the concentration of 0,5 ug/ul was used to design ELISA experiment. gDNA from the same cell lines was extracted using the *SpinNAker Universal Genomic DNA mini kit* (Euroclone) and Her2 amplification was confirmed using the same dPCR assay adopted for patients. At the same time, the supernatant was recovered after 144h of monitoring starting with a cellularity between $3\text{-}4*10^6$ cells and treated as a plasma sample, centrifuged 2 times respectively at 2000xg,20 min at 4°C and 10000xg, 10 min at 4 °C and diluted 1:100 in RD to perform the experiment.

4.2 ***Tissue Samples***

Frozen Tissues from untreated patients (n=8) with Her2 positive breast cancer were obtained from our Institutional BioBank following informed consent (IRB CEC/707/15), with a tumor fraction from 20% to 90%. Tissue DNA was extracted by the *QIAmp DNA FFPE Tissue Kit* (Qiagen, Hilden, German) according to the manufacturer's instructions. Before protein extraction, the fresh frozen tissue cylinders were weighed in order to compare and standardize the sampling procedures (means 25±30 mg). The cylinders were than homogenized in *CST Lysis buffer* (Cell Signaling Technologies) by a rotating blade homogenizer for approximately 10 seconds and incubated for 30 min on ice. The extracts

were centrifuged at 14,000 x rpmi x 10 min and the supernatant was stored at -80°C (same as cell lysate). The protein concentration of the extracts was determined by the BCA assay (*QuantumProtein kit*, Euroclone) (mean= 3,54 ug/ul).

4.3 ***Patients and study design***

Plasma samples from HER2 positive breast cancer patients (n=22) were collected during GIM21-project, following the *LiqBreasTrack* study procedures, processed and bio-banked as per international good laboratory practice.

This is a no profit, open, interventional, non-pharmacological, prospective study conducted at the Regina Elena National Cancer Institute as coordinator of the project to assess tumor molecular alterations occurring in blood under T-DM1 pressure, and recapitulate adaptive tumor evolution in archival tissues.

T-DM1 effects will be monitored by a combination of NGS and LB to capture molecular events (gene aberrations, mainly mutations) associated with (or causative of) relapse as well as primary/adaptive resistance to HER2 blockade.

Plasma from healthy donors (n=8) and other malignancies (n=8) were collected in order to establish the background of the assays adopted and verify their sensitivity and specificity.

Before enrolment, patients signed a written informed consent as per Bioethical Board authorization (RS-857/16). This includes to biobanking and full genomic testing. Since blood drawing can be performed on the occasion of T-DM1 treatment, longitudinal monitoring involves only minimal discomfort for patients.

4.3.1 ***Inclusion criteria***

The inclusion and exclusion criteria coincide with the criteria of T-DM1 administration in relapsing patients. This complies with appropriateness of administration, adherence to ethical and budget considerations, and immediate clinical applicability. Patients are considered eligible with an histologically confirmed diagnosis of metastatic breast cancer HER2+ pre-treated with no more than one line of anti-HER2 therapy for advanced breast cancer, including chemotherapy (trastuzumab alone and in association with Pertuzumab).

1. Male and female patients with a documented diagnosis defined by an immunohistochemistry (IHC) score of 3+, alternatively score 2+ and HER2 amplification ratio ≥ 2.0;

2. All patients eligible to treatment with T-DM1, according to SmPC previously treated with a taxane and trastuzumab. Patients who previously underwent first-line treatment with an association of TTZ with PTZ are also eligible. No more than one line of anti-HER2 treatment for advanced disease are allowed;

3. Patients with both measurable and non-measurable disease (according to modified RECIST 1.1 criteria) are eligible;

4. 18 years of age on day of signing informed consent;

5. a left ventricular ejection fraction of 50% or more (determined by echocardiography or multiple-gated acquisition [MUGA] scanning);

6. an Eastern Cooperative Oncology Group performance status of 0 or 1:

7. Adequate organ function (obtained within 14 days prior to treatment study) as evidenced by:

- Absolute neutrophil count (ANC) 1.5 X 109/L without myeloid growth factor support for 7 days preceding the lab assessment;
- Haemoglobin (Hgb) 9 g/dL (90 g/L); < 9 g/dL (< 90 g/L) is acceptable if hemoglobin is corrected to 9 g/dL (90 g/L);
- Platelet count 75 X 109/L without blood transfusions for 7 days preceding the lab assessment;
- Bilirubin 1.5 X upper limit of normal (ULN), except for patients with a documented history of Gilbert's disease;
- Alanine aminotransferase (ALT), and aspartate aminotransferase (AST) 2.5 X ULN (for patients with liver metastases 5 X ULN);

8. Life expectancy > 12 weeks;

9. Written informed consent obtained before any screening procedure and according to local guidelines.

4.3.2 ***Exclusion criteria***

1. Prior treatment with T-DM1.

2. Symptomatic central nervous system (CNS) metastases, or treatment for these metastases within the 2 months preceding enrolment.

3. Current participation in study therapy, or previous participation in a study involving the administration of an investigational agent within 4 weeks of administration of the first dose of treatment.

4. History of symptomatic congestive heart failure or serious cardiac arrhythmia requiring treatment; a history of myocardial infarction or unstable angina within 6 months before.
5. Female patients who are pregnant or lactating, who plan to get pregnant, or who have a positive serum pregnancy test prior to first dose of study treatment.
6. Prior malignancy (other than breast cancer) except for non-melanoma skin cancer and carcinoma in situ (of the cervix or bladder), unless diagnosed and definitively treated more than 5 years prior to enrolment.
7. Any other significant co-morbid conditions that in the opinion of the Investigator would impair study participation or cooperation.
8. Patients with psychiatric illness, social situation or geographical situation that would preclude informed consent or limit compliance with study requirements, as determined by the Investigator.
Additional inclusion/exclusion criteria will be as per international guidelines.

4.4 ***Schedule of Treatment***

Patients received trastuzumab emtansine (T-DM1) at 3.6 mg/kg intravenously every 21 days, as per Summary of Product Characteristics (SmPC).
Dose delays, reductions, and discontinuations owing to toxic effects are defined in the summary of product characteristic. The first dose reduction will be to 3.0 mg per kilogram and the second to 2.4 mg per kilogram. Dose escalation is not allowed after a dose reduction. If a toxic event does not resolve to a grade 1 level or to baseline status within 42 days after the most recent dose, the treatment is discontinued. Patients will continue to receive the treatment until disease progression or the development of unmanageable toxic effects.

4.5 ***Blood sampling, plasma processing and ctDNA extraction and quantification***

Whole blood (30ml) was drawn throughout the entire follow up of the patients on the occasion of T-DM1 administration in *BD Vacutainer K_2EDTA* tubes and processed within 1h.
Plasma was isolated by two successive rounds of centrifugation at 4°C (2000 x *g* for 20 min, and 13000 x *g* for 10 min), and stored at -80° in single-use 2 ml aliquots until circulating free total nucleic acids (cfTNAs) extraction. No freeze-thawing cycles were allowed. cfTNAs were extracted from 4ml of plasma by the *QIAmp Circulating Nucleic Acid kit*

(Qiagen) according to the manufacturer's instructions [239] in a final volume of 30 µL, and stored at -20°C until analysis. cfTNAs were fluorimetrically quantified using the *Qubit dsDNA HS assay kit* (Life Technologies, Carlsbad, CA, USA(Q33230)). 6.5µL (corresponding to approximately 0.86 ml of plasma) were assessed in the chip-based QuantStudio™ 3D Digital PCR System (Life Technologies).

4.6 ***Library preparation and sequencing***

Blood NGS libraries were prepared from 40 ng of cfTNAs whenever available (mean 23.7 ng, range 4.4 – 40 ng) and used as input to prepare libraries with the *Oncomine™ PanCancer Cell-Free Assay* (Life Technologies) as per manufacturer's instructions. The application of this technology is described in recent publications of our group [239, 240].

This panel generates an amplicon library from both ctDNA and ctRNA covering 52 genes, 12 copy number variations (CNVs) and 92 fusions. Libraries were equalized, pooled and then automatically loaded onto the *Ion 520* (tissue) or *540* chips (blood) by the *Ion Chef system* (Life Technologies). After sequencing on *Ion S5*, data were analyzed with the *Ion Reporter suite v5.16* (Life Technologies).

A custom filter chain including restriction on location (exonic), *p* value (<.05), variant effect (unknown, missense, nonsense, stoploss, frameshift insertions and deletions), variant type (SNV, small INDELS, MNV), filtered coverage (n=250) and VAF ≥2% was applied to filtering tissue NGS data. The pre-set *Oncomine Variants filter v5.16* (Life Technologies) was used to call alterations in blood samples without any adjustments.

4.7 ***digital PCR assay***

Matched tissue and blood samples from each patient and cfDNA from healthy donors were run in the same experiment using the chip-based *QuantStudio™ 3D Digital PCR System* (Life Technologies). Reactions were set up in a final volume of 16 µl including 8 µl of 2x Master Mix, 0.9 nM of each forward and reverse primers, 0.25 nM of TaqMan® MGB probe, 7.0 µl of template, and loaded onto dPCR chips.

Input DNA for tissue analysis was normalized to 20 ng. By contrast, input cfDNA was equalized by volume (7.2 ul) to accurately measure ctDNA copies/mL of plasma.

Thermal cycling was as follows: 10 min at 96.0°C, 39 cycles at 56.0°C for 2 min, 30 sec at 98.0°C, and a final elongation step of 2 min at 60°C. Threshold values of FAM and VIC fluorescence were automatically calculated by the *Thermo Fisher Cloud Analysis Suite* in

tissue samples, manually reviewed, and then applied to the corresponding ctDNA.

4.7.1 ***Assessment of the HER2 amplification status***

An existing, optimized dPCR assay was applied to calculate copy number ratios between the HER2 locus and the EFTUD2 reference gene[241-243]. Unlike diagnostic CISH/FISH, this assay is applicable regardless of the analyte (tissue or blood) and is quantitative on a continuous scale, providing a unified readout. HER2:EFTUD2 thresholds of 2.0 and 1.25 were applied to define amplification in tissue and blood, as per HER2:CEP17 ASCOCAP guidelines[47] and original assay descriptions[241-243], respectively. HER2 amplification was also called as CNV by the *Oncomine™ PanCancer Cell-Free Assay* through the pre-set *Oncomine Variant filter v 5.16* (Life Technologies).

4.8 ***Quantitative ELISA HER2 assay***

Human ErbB2/Her2 DuoSet ELISA (R&D-System #DY1129B) was used to quantitate sHER2. ELISA values were used as an 'overexpression surrogate'.
A Standard curve was obtained using a solution containing *Recombinant Human ErbB2/Her2 Fc Chimera* in Reagent Diluent at a known concentration to obtain a series of two-fold dilutions spanning 3,5 ug/ml down to 0,054 ug/ml. The curve was generated by reducing the data using a four-parameter logistic (4-PL) curve fit and the optical density of each sample was determined reading at 450nM.
The protocol was optimized identifying the best dilution of capture and detection antibodies respectively of 5ug/ml and 0,25 ug/ml.
To verify the lower limit of quantification (LLOQ) with our microplate reader, we prepared different standard dilutions to confirm and point the lowest nonzero validation sample concentration level which can be accurately and reproducibly quantitated.
At the same time precision and accuracy were determined by analysis of serial dilution of this standard protein prepared at lower limits of quantification and at least three additional concentration spanning the curve standard range.
Plasma from patients affected by tumors with different ontogenesis were analyzed in all experiment to verify the background and the specificity of the assay.
At the same time a random set of human plasma from healthy volunteers was analyzed during development and validation and all tested samples had an observed concentration less than LLOQ. The output (sHER2 and tissues) will be generated by a four-parameter logistic curve-fit (4-PL) via GraphPad Prism 8, pre-selecting for optimal blood dilutions. The results

will used in combination with an HER2 dPCR CNV assay to have a dedicated proteogenomic (2D) readout, exemplified in preliminary results.

4.9 ***Statistical analysis***

Descriptive statistics were used to summarize plasma and proteins correlations. Variant allele frequencies (VAFs) in blood samples and protein concentration were correlated by regression analysis. Two-sided p values <0.05 were considered statistically significant. The Variant Allelic Frequencies (VAF) were estimated by analyzing the 2D plots (WT/MUT) from dPCR experiments, automatically elaborated using the on-board *Thermo Fisher Cloud Analysis Suite*.

The VAF from dPCR and NGS were compared by excel to show the correlation between the methods. Absorbance versus concentration curve was fitted to a four-parameter logistic regression using *GraphPad Prism v8.3* (GraphPad Software, CA, USA). Progression-free survival (PFS) was calculated between the first T-DM1 administration and progressive disease or last follow-up.

CHAPTER 5

RESULTS and DISCUSSION

5.1 ***Progressive reversal of HER2 amplification in tissues and blood***

Tissues and plasma from the *LiqBreasTrack* study were tested by a dPCR assay shown by others to quantitatively detect HER2 amplification [241, 242]. Due to normal DNA present in blood and in tissues with an abundant stromal component, absolute copy numbers are underestimated by the assay [241, 244]. Nevertheless, dPCR was accurate and quantitative also in our hands, as shown by its remarkable concordance with NGS (see below). Testing all samples under identical conditions clearly documented progressive HER2 counter-selection. HER2 amplification was detected in 7/11 (64%) primary tumors but only 5/12 (42%) metastatic lesions collected during previous anti-HER2 treatments, and in 7/20 (35%) blood drawings collected before T-DM1 treatment, but only 2/20 (10% overall) blood drawings at progression (Fig.8a-b).

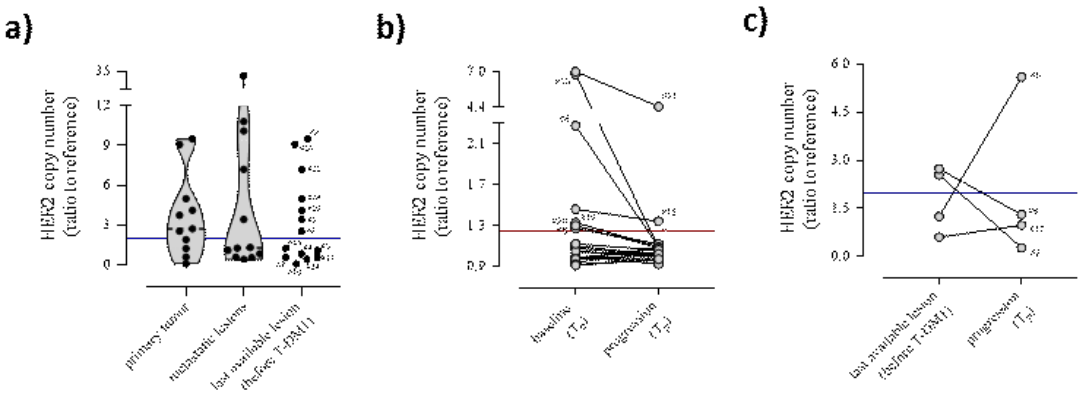

Fig.8 *Evaluation of HER2 status in different settings*

a) HER2 CNV in tissues samples; b) HER2 CNV in blood samples;
c) HER2 CNV in tissues biopsies before T-DM1 and at TP;

HER2 counterselection in blood was confirmed in 3/4 matched (from the same patient) tumor re-biopsies at progression, the only exception being a HER2-positive brain metastasis developing against a HER2-neutral blood background (Fig.8c).

Interestingly, median PFS did not significantly differ depending on the HER2 blood status (amplified vs neutral) at baseline (Fig.9). Persistence of T-DM1 efficacy despite (and beyond) HER2 neutralization was suggested by 2 observations.

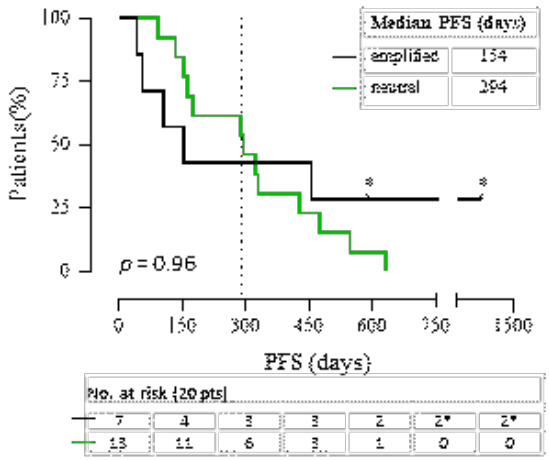

Fig.9 *HER2 status/PFS correlation*

PFS variation in amplified/neutral BC patients by Log-rank (Mantel-Cox) test

First, the highest baseline HER2 levels and the deepest HER2 losses at T_p were seen in the two patients (#13 and #21) who experienced early (before the third administration) T-DM1 failure. Second, median PFS did not differ between the HER2-amplified and HER2-neutral patient groups, and long-term responders were seen in both groups. Perhaps, like Trastuzumab Deruxtecan [245] T-DM1 remains active on tumors with attenuated HER2 signalling, e.g. a HER2-neutral, but druggable, status spans a much larger patient cohort and a much wider time window than appreciated so far.

5.2 ***Remodeling in oncogenic dependencies***

Breast cancer alterations other than HER2 amplification were identified and sorted out in 3 steps. First, orthogonal testing with targeted NGS and alteration-specific dPCR assays of tumor tissues (n=14) and plasma samples (n=20) concordantly detected 150 and 27 mutational hits, respectively. Second, dPCR testing detected 3 of the above hits in genomic DNAs from the peripheral blood mononuclear cells (PBMCs) of 2 distinct patients, demonstrating the occasional origin of some alterations from clonal hematopoiesis (not shown). Filtering these 3 hits out left 147 and 24 hits in tissues and plasma respectively, all deemed to represent genuine breast cancer alterations. Third, counting each alteration once (several hits recurred in different samples and patients) yielded a total of 136 and 15 unique tumor variants. A synopsis of patients, clinical-biological features, and a list of genes with detectable alterations is displayed in Fig.10a.

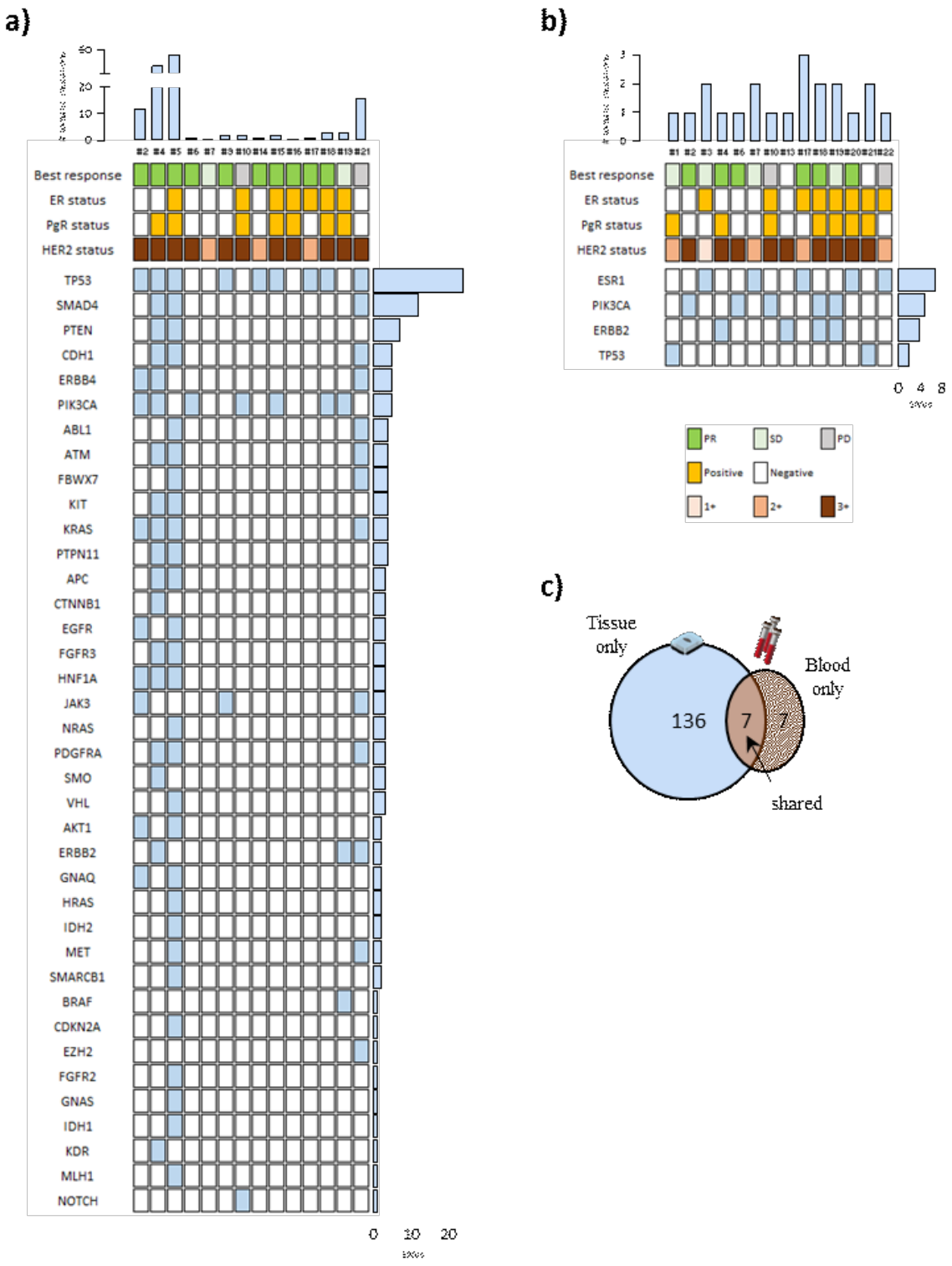

Fig.10 *Gene list and clinical-biological features.*

a) tumor variants identified; b) patients with tumor variant detected in blood;

c) Venn diagram indicating blood and tissues variant overlap

Interestingly, 14 of the 15 unique tumor variants seen in plasma were detected in a subset of 12 patients (Fig.10b) with at least one available matched tissue sample, making it possible

to calculate that only 7 variants were shared between tissue and blood in this representative subset, whereas the remaining 7 were observed in blood only (Venn diagram in Fig.10c). Altogether, HER2 neutralization and the appearance of new variants in blood suggest an extensive remodeling in oncogenic dependencies that would have been missed by tissue-only bulk sequencing.

*5.3 **LB dynamics hint at several distinct clonal selection mechanisms***

Since blood was drawn every 21 days, on the occasion of each T-DM1 administration, detailed clonal trajectories could be assessed. LB identified diverse molecular trajectories revealing 4 clonal selection mechanisms (Fig.11).

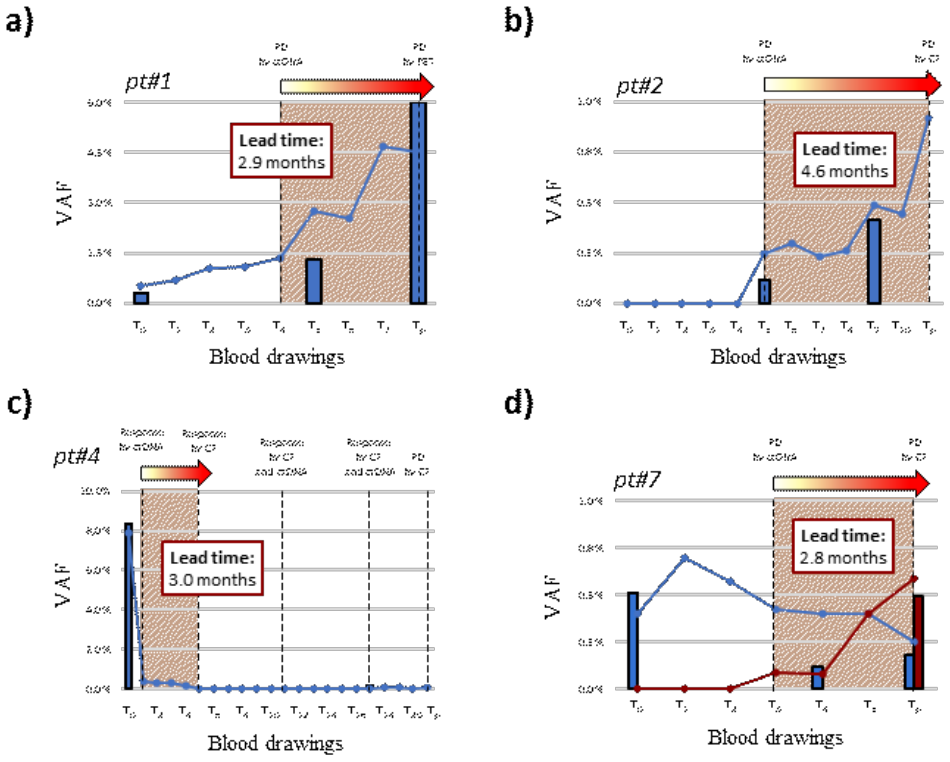

Fig.11 *Molecular trajectories revealed by LB*

a) expansion of pre-existing clonal resistance; b) *De Novo* appearance;
c) irreversible clonal decrease; d) divergent clonal evolution

Alterations undergoing at least two consecutives > 1.5-fold increases in their VAFs were assumed to mark clonal expansion. Depending on whether immediately evident or delayed (e.g., since the first blood drawing or afterwards), clonal expansions were consistent with primary and adaptive pharmacological resistance, respectively.

Presence at T_0, fast doubling times, and highest blood VAFs at T_p (Fig.11a) were consistent with the expansion of a pre-existing (e.g., primary) clonal resistance. *De novo* appearance (Fig.11b) likely marked adaptive (secondary) resistance. In contrast, sharp and irreversible decreases (defined as a reduction by > 50%) were invariably steep, e.g., they occurred abruptly, typically after a single T-DM1 administration and were consistent with clonal contraction/susceptibility (Fig.11c). Interestingly, this was seen with mutations (HER2 p.L755S and PIK3CA p.E545K) described to cause resistance to anti-HER2 drugs other than T-DM1[246, 247]. These were irreversibly wiped off within weeks despite they had been selected during years of previous therapies. Coexistence of more than one type of trajectories was also noted (Fig.11d), suggesting divergent clonal evolution.

Overall, swift clonal suppression provides a rationale for innovative pulse dosing/de-escalation schedules. In selected patients, these may elicit response with minimal treatment-associated toxicity.

5.4 ***LB anticipates progression at extracranial locations***

The *LiqBreasTrack* design takes advantage of a narrow (Fig.6) but sensitive (Fig.12, most samples tested at a Limit of Detection, LOD of 0.1% or less) targeted NGS panel to detect a few major cancer drivers.

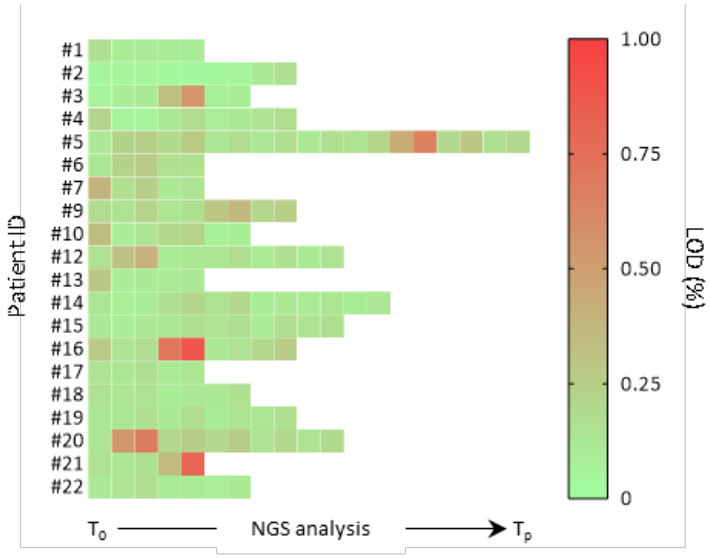

Fig.12 *NGS sensitivity*

Therefore, it was not expected to detect circulating alterations in most patients. Accordingly, 10/18 (54%) patients who were monitored until progression did not display alterations or

quantitative changes in their levels (6 and 4 patients, respectively). However, and interestingly, all 3 patients who progressed exclusively due to cerebral metastases (Fig.13) were included in this non-informative, LB-negative subset.

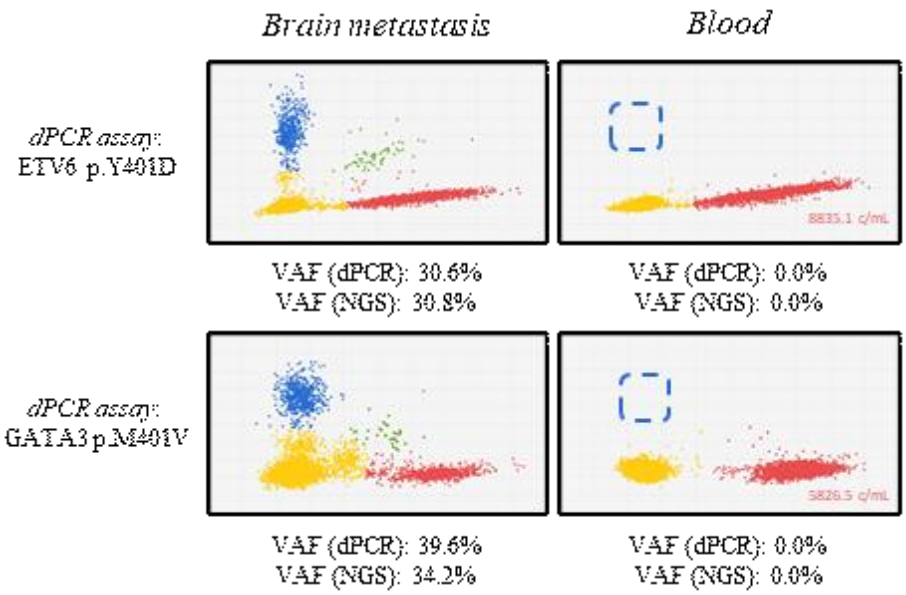

Fig.13 *LB in blood is not informative about brain metastasis*

This is not surprising since brain involvement is best monitored through the cerebrospinal fluid [248]. In the remaining 8 patients (44%), progression as per medical imaging was anticipated by 2.6 (range 0.7-4.6) months on average. Although shorter than in other settings, this anticipation may be clinically useful since the expected median PFS during T-DM1 treatment is about 6.4 months (range 4.8-7.7 months) in real life studies [114].

5.5 LB identifies circulating positive and negative PFS predictors

Most likely due to limited case accrual, none of the mutated genes and variants seen in blood at baseline significantly correlated with PFS (Fig.14).

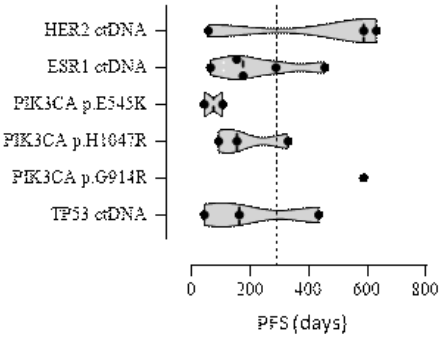

Fig.14 *Variant at baseline/PFS correlation*

We then hypothesized that dynamic (VAF at T_p *vs* T_0), LB-informed criteria might better identify variants associated with different outcomes. Therefore, we took into consideration trends and not the mere presence of a circulating alteration. Discordant alteration trends (up- and down-regulation both observed in different patients) were color-coded blue and disregarded while concordant alterations were instead considered, and sorted into positive and negative trends (red and green, respectively) (Table 4).

ctTNA	*pts ID*	*Trend over time*
ESR1 p.D538G	#7, #17, #22	↗, ↑, ↑
ESR1 p.Y537S	#17	↑
FGFR1 amp	#17	↑
MYC amp	#17, #22	↑, ↑
TP53 p.R273H	#1	↑
ESR1 p.Y537N	#17	↑
PIK3CA p.H1047R	#2, #10, #18	↗, ↑, ↗
ESR1 p.Y537C	#3, #7	↗, ↔
ESR1 p.S463P	#3, #20	↗, ↘
HER2 p.D769Y	#18	↔
TP53 p.G245S	#21	↘
PIK3CA p.E545K	#6, #21	↘, ↓
PIK3CA p.G914R	#19	↓
HER2 p.V777L	#13	↓
HER2 p.L755S	#4, #19	↓, ↓

Clonal expansion

Clonal contraction

Table 4 *Trend of detected alterations*

'Red' alterations (VAF at T_p>T_0; clonal expansion) included PIK3CA p.H1047R (3/3), ESR1 mutations increasing the kinase activity (p.D538G, and p.Y537S/N/C; 7/7), as well as MYC (2/2) and FGFR1 (1/1) amplifications. 'Green' alterations (VAF at T_p<T_0; clonal contraction) included HER2 p.L755S (2/2), HER2 p.V777L (1/1), and PIK3CA mutations other than p.H1047R (3/3). Since clonal expansion ultimately determines clinical progression, patients with both red and green ctTNAs were color-coded as red, whereas patients exclusively bearing green alterations were color-coded as green. This dynamic classification identified two groups of patients with significantly ($p < 0.05$) different PFS (Fig.15). This figure also shows that all but one PFS values in the red group cluster below

the overall median PFS, suggesting that negative predictors are particularly robust and possibly coincide with drivers of clinical resistance to T-DM1.

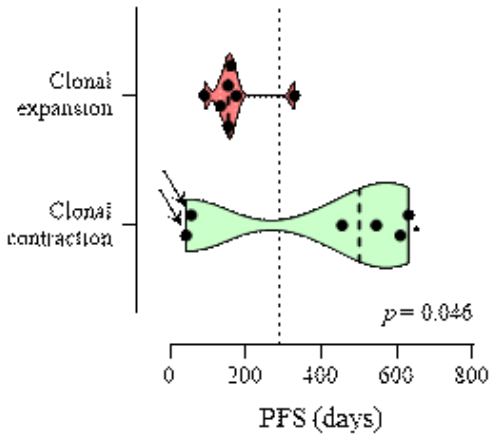

Fig.15 *LB identify variant that prove to be PFS predictors*

Conversely, scatter in the green group is due to two early progressors (arrows), both patients with brain metastases, in whom LB invariably gives rise to false negatives). Interrogation of the OncoKb knowledge base [249] revealed that 20/24 (83%) blood variants in 13/18 (72%) of the patients progressing on T-DM1 were actionable, mostly at level 3A (Table 5). This suggests bypass of the HER2 blockade through forced, and systematic, molecular subtype switch.

In summary, in this first part of my thesis I found that LB identifies drivers/predictors (at extracranial sites only) of T-DM1 escape as they gradually replace HER2. Predictors of progression may be cryptic (blood-only), and include actionable ESR1 and PIK3CA mutations as well as MYC and FGFR1 amplifications that may find application to assign further therapy lines. In contrast, other PIK3CA mutations and all tested HER2 mutations are wiped off by T-DM1 in weeks and may be associated with a more durable T-DM1 response.

pt	ctTNA	Druggable	OncoKB level *
#1	TP53 p.R273H	N	-
#2	PIK3CA p.H1047R	Y	3A
#3	ESR1 p.S463P	Y	3A
	ESR1 p.Y537C	Y	3A
#4	HER2 p.L755S	Y	3A
#6	PIK3CA p.E545K	Y	3A
#7	ESR1 p.Y537C	Y	3A
	ESR1 p.D538G	Y	3A
#10	PIK3CA p.H1047R	Y	3A
#13	HER2 p.V777L	Y	3A
#17	ESR1 p.D538G	Y	3A
	ESR1 p.Y537N	Y	3A
	ESR1 p.Y537S	Y	3A
	MYC amplification	N	-
	FGFR1 amplification	Y	4
#18	PIK3CA p.H1047R	Y	3A
	HER2 p.D769Y	Y	3A
#19	HER2 p.L755S	Y	3A
	PIK3CA p.G914R	Y	3A
#20	ESR1 p.S463P	Y	3A
#21	PIK3CA p.E545K	Y	3A
	TP53 p.G245S	N	-

Table 5 *OncoKb actionability*

Although dynamic prediction of response and resistance to therapy by liquid biopsy meets with success in an area where 'static' predictors always fail, we were in part unhappy with our results because at this stage they were providing only partial mechanistic insight. For instance, based on the drastic HER2 counter-selection seen by LB, it was expected that the 7 *LiqBreasTrack* patients retaining elevated HER2 copy numbers might have conceivably retained some 'actionable' HER2 amplification prior to T-DM1 application, and would respond best. However, much to our surprise this was not the case, as extensively described above. To address this, we hypothesized that HER2 amplification and (over)expression should be both considered, and factored in a single and more predictive biological metric.

As known, breast cancers are assigned to the HER2 molecular subtype by assessing gene over-expression and amplification in two steps. If strong homogeneous immunohistochemical tissue staining (IHC 3+) is lacking, Chromogenic/Fluorescence In Situ Hybridization (CISH/FISH) must detect a minimum of 4 to 6 HER2 copy numbers. Once assigned, the HER2 status provides absolute indication for a number of successive and

progressively more intense anti-HER2 therapy lines including small molecules, naked antibodies, and ADCs, resulting in long-lasting clinical benefit [104, 105].

However, whether or not the HER2 status should be considered immutable is highly questionable, since adaptive HER2 counter-selection during treatment was not only described in the present study, but has been previously noted by several groups [131, 250, 251]. Moreover, it remains questionable whether these dynamic changes (e.g., in HER2 copy numbers) affect therapeutic efficacy or, vice-versa, treatments should be tailored and adjusted on the basis of a dynamic longitudinal assessment of the HER2 status.

Puzzled by counterintuitive findings and interpretations, we reasoned that 'copy-pasting' the standard two-step tissue diagnostics into blood would better recapitulate the two-sided nature (amplification/overexpression) of HER2 addiction. To this end, HER2 protein and DNA copy numbers were quantitatively and simultaneously assessed by LB using a commercial 'sandwich' ELISA and dPCR, respectively, a testing scheme that was named HER2-2D. Before describing its application to the *LiqBreasTrack* cohort, in the next chapter we briefly outline its technical validation.

5.6 ***Validation of HER2-2D in cell lines, breast cancer tissues and blood***

To validate HER2-2D, dPCR and ELISA were carried out on genomic DNAs and polypeptide extracts of five cell lines (BT474, SKBR3, KPL4, T47D, MDA-MB-231) with different, representative levels of HER2 amplification, resulting in different levels of HER2 polypeptides (Fig. 16a). These largely reflect the different HER2 copy numbers detected by dPCR and as expected, HER2 DNA copies were >20 in HER2-amplified cells, and close to the unit in HER2-neutral cells (data not shown).

As also expected, ELISA (Fig. 16a) detected similar levels of HER2 overexpression in HER2-amplified cells, and either low or no detectable levels in HER2-neutral cells as follows: the 3+ HER2-amplified [BT474 (± 306,37 ng/ml), SKBR3 (± 225,47 ng/ml) and KPL4 (± 63,62 ng/ml)]; the low-level amplified T47D (± 0,13 ng/ml) and the HER2-neutral cell line MDA-MB-231 (0 ng/ml).

Lysates of HER2-neutral cells are deliberately loaded in higher amounts than those from HER2-amplified cells in Fig.16a. Secondly, the assay was applied on soluble extracts from fresh frozen tissue (n=8), previously tested by an IHC conventional diagnostic method. As per IHC, n=2 tissues showed strong staining (3+), whereas the remaining (n=6) showed weak-to moderate staining (1+ and 2+). Following identification of the optimal dilution of

cell extracts (Fig.16b), ELISA was able to consistently reveal high levels of HER2 proteins only in IHC3+ samples (Fig.16c), whereas IHC-negative/1+/2+ tissues were poorly resolved (Fig.16c) e.g. there are false-negatives. Inspection of cryostat sections revealed that false-negatives are mostly seen in tissue samples with low tumor cellularity, suggesting that HER2 is diluted into proteins contributed by normal tissue components.

Nevertheless, dPCR and ELISA, plotted in the HER2-2D format, demonstrated significant correlations (Fig.16d) between HER2 overexpression and HER2 amplification in tumor tissues, as expected. This correlation is maintained when the % of neoplastic cell is ≥ 20% (Fig.16d).

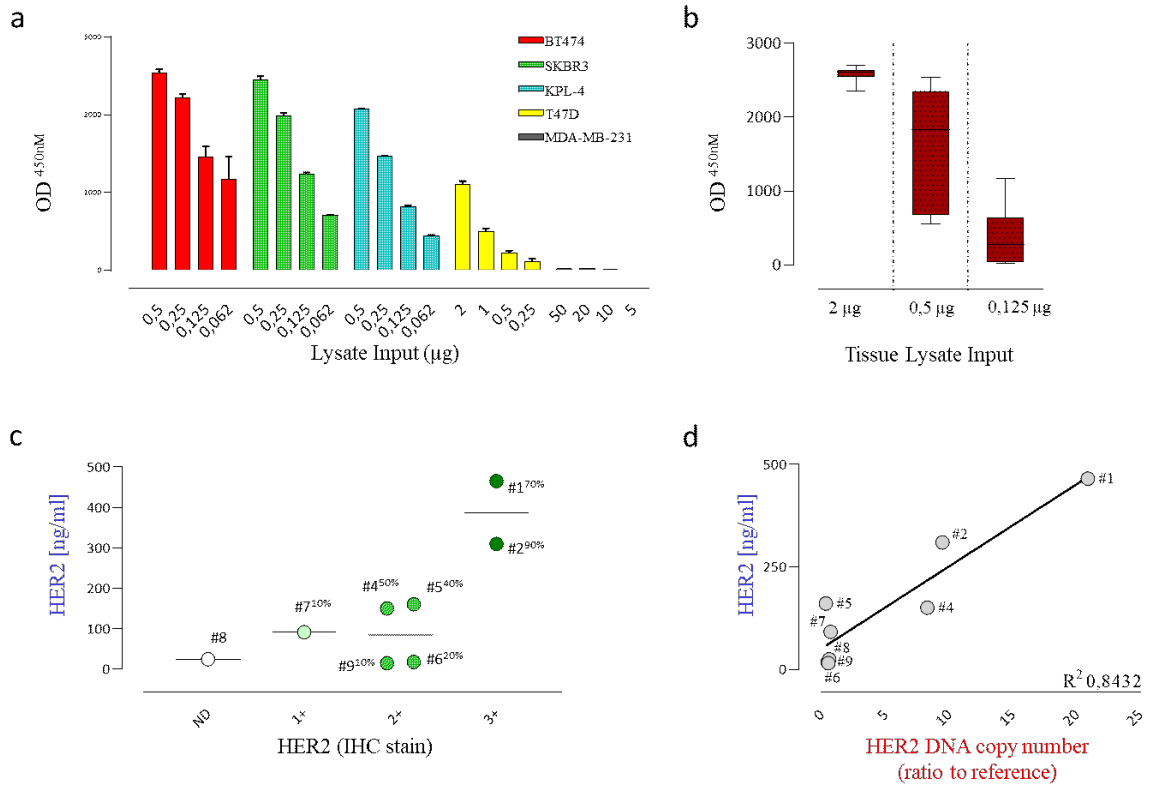

Fig.16 *HER2-2D scheme validation*

in a) cell lines; b-c) tissues samples; d) VAF/[HER2] linear regression

Following validation on cell and tissue extracts, plasma samples from healthy donors (n=4) and a small cohort of breast cancer patients (n=4) were tested by ELISA to identify the optimal testing dilution, e.g., to work in an optimal dynamic assay range and enhance the normal vs HER2-positive difference. The 1:100 dilution was selected, that corresponds to 1ul of plasma per microwell on a 96-well ELISA plate (Fig.17)

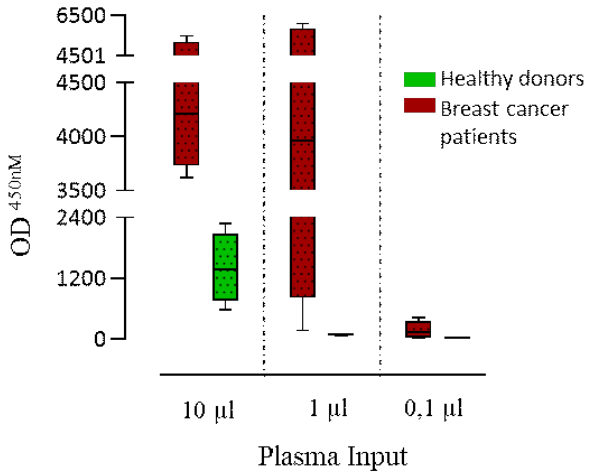

Fig.17 *HER2-2D scheme validation on plasma samples*

5.7 ***T-DM1 dissociates HER2 amplification from (over)expression***

Following technical validation on cell lines, tumor tissues, and representative human blood plasma samples, the optimized HER2-2D protocol was adopted employing 1uL of plasma, and ctDNA from 1.4 ml of plasma for ELISA and dPCR, respectively, in the 22 patients selected.

Blood drawings were tested at baseline (T_0, before the first T-DM1 administration) and at progression (T_p) for three readouts: (1) HER2 copy number estimated by the Pan Cancer NGS panel; (2) HER2 copies estimated by dPCR; (3) sHER2 ECD assessed by ELISA. Interestingly, dPCR displayed a remarkable concordance with NGS (Fig.18a; regression close to the unit). In contrast, HER2 amplification and overexpression did correlate, as expected, but to a much lesser extent, as synoptically demonstrated in Fig.18b-c.

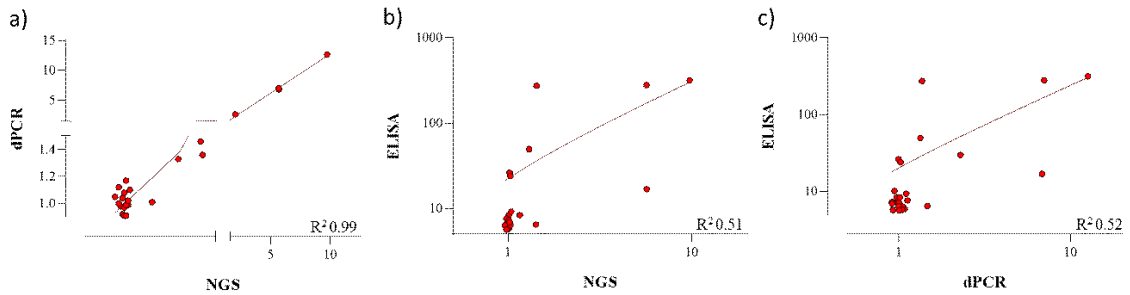

Fig.18 *Linear Regression Model*

a) NGS/dPCR; b) NGS/ELISA; c) dPCR/ELISA

It may be concluded that in blood HER2 copy numbers and HER2 overexpression are moderately but not precisely correlated.

Having validated our technical tools, we went on to test clinical blood samples by dPCR and ELISA. HER2 copy numbers and sHER2 levels in normal blood donors (n=8), and patients bearing tumors known to very rarely host HER2 alterations (n=8) were well below the thresholds of 1.25 (copy numbers) and 15 ng/ml, the former validated above, and the latter corresponding to the Food and Drug Administration (FDA)-approved 15 ng/mL value [252] that coincides with HER2 overexpression (Fig.19a).

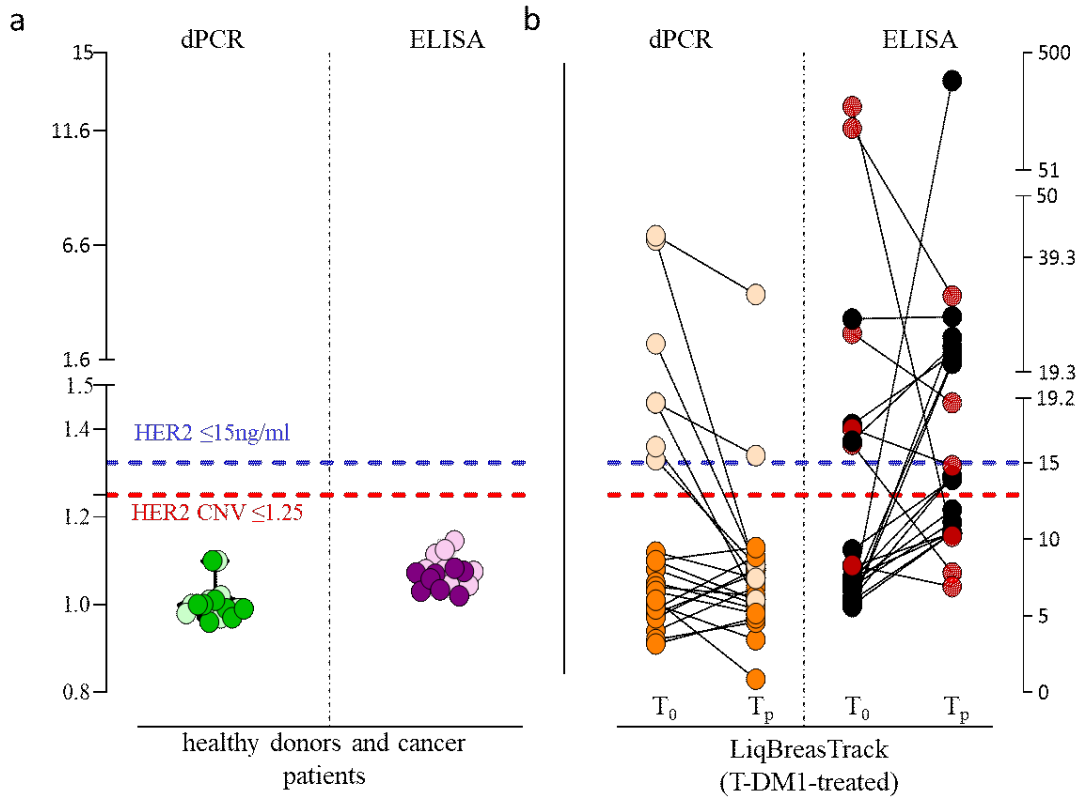

Fig.19 *dPCR/ELISA Multiple Regression Model*

Next, we compared blood drawn before T-DM1 treatment (T_0) and at progression (T_p), and found that copy numbers decrease, as extensively described above, in all the 6 (27.27%) patients presenting with above-threshold aHER2, whereas sHER2 levels determined by ELISA were up-regulated in most patients (n=16), although decreases were also seen in 6 patients (Fig.19b).

The three events shaping the HER2 status during T-DM1 treatment (HER2 loss, sHER2 gain and sHER2 loss) are synoptically displayed by HER2-2D plots (compare Fig.20a and 20b). Infact, as shown in Fig.20, all 6 double positives were depleted from the upper right

quadrant, and the upper left quadrant became populated with 9 single positives (see blue dots Fig.20b). Although two outliers (pts #21 and #20) selectively acquired/retained high aHER2/sHER2, the two conditions remained mutually exclusive at progression (Fig.20b). Therefore, T-DM1 dissociates HER2 expression from amplification, at least as judged by liquid biopsy in 18/22 tested patients, including outliers.

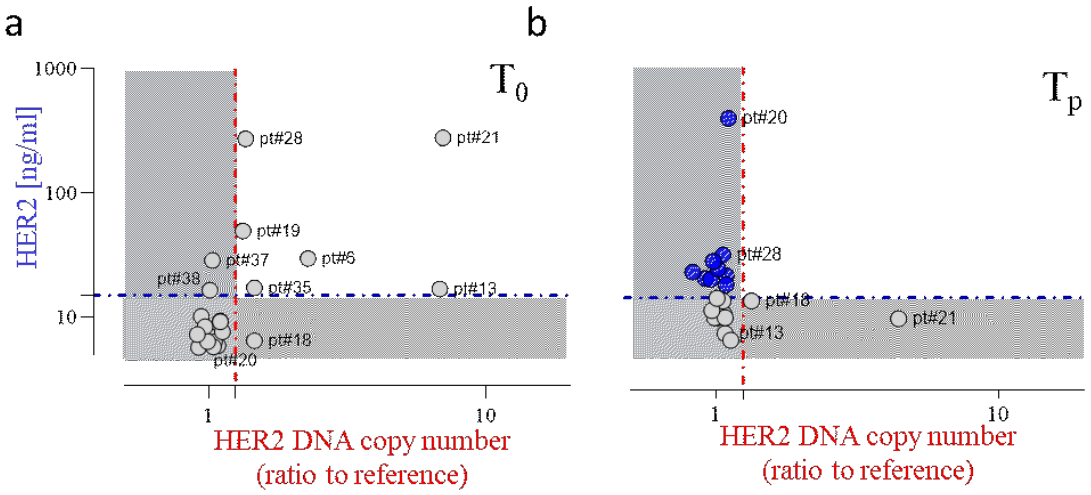

Fig.20 *HER2-2D scheme applied at the baseline and at progression*.

Puzzled by the discordant aHER2/sHER2 trends, we asked whether they reflect the tumor HER2 status. A strong homogeneous 3+ HER2 IHC staining was seen in 4/5 available HER2-neutral tissue re-biopsies, all of which were obtained at progression, coincident with sHER2 gain (data not shown). Although ethical constraints limit re-biopsy to single accessible lesions from selected patients, these results indicate that T-DM1 blunts HER2 amplification but not (over)expression in most tissues, and HER2-2D recapitulates this dissociation in blood.

5.8 ***Elective susceptibility to T-DM1 of patients bearing tumors that regain moderate HER2 expression***

It is difficult to formally prove that sHER2 derives largely or exclusively from tumor tissues. Systematic re-biopsy at multiple metastatic sites is unfeasible and unethical, and studies limited to subsets of patients with accessible tumor lesions (e.g., skin and possibly superficial lymph nodes) would remain biased in time and space and poorly informative. However, since HER2 is one of the most tumor-restricted surface molecules [253], its presence and rapid (weeks to months) changes in blood are compatible with HER2 being shed by tumor

cells into the bloodstream, where HER2 amounts likely represent a weighted average of the contribution of all tumor sites. Based on the assumption that HER2-2D combines metrics that likely measure a practical surrogate of HER2 tumor addiction, we inferred that T-DM1 sorts out two alternative metrics and selects tumors with 4 distinct features/phenotypic subsets: HER2-gain/loss and HER2-high/low (color-coded in Fig. 20a and 20b). It was hypothesized that those populations might respond differently to T-DM1. The HER2-low and HER2-high subsets did not appreciably differ in PFS (n=9 patients high-above 15 ng/ml vs n= 13 patients low-below 15 ng/ml; Fig. 21a), as confirmed by the Kaplan-Meier curve in Fig. 21b (Mann-Whitney and Mantel-Cox (log-rank) p=0,99, non-significant).

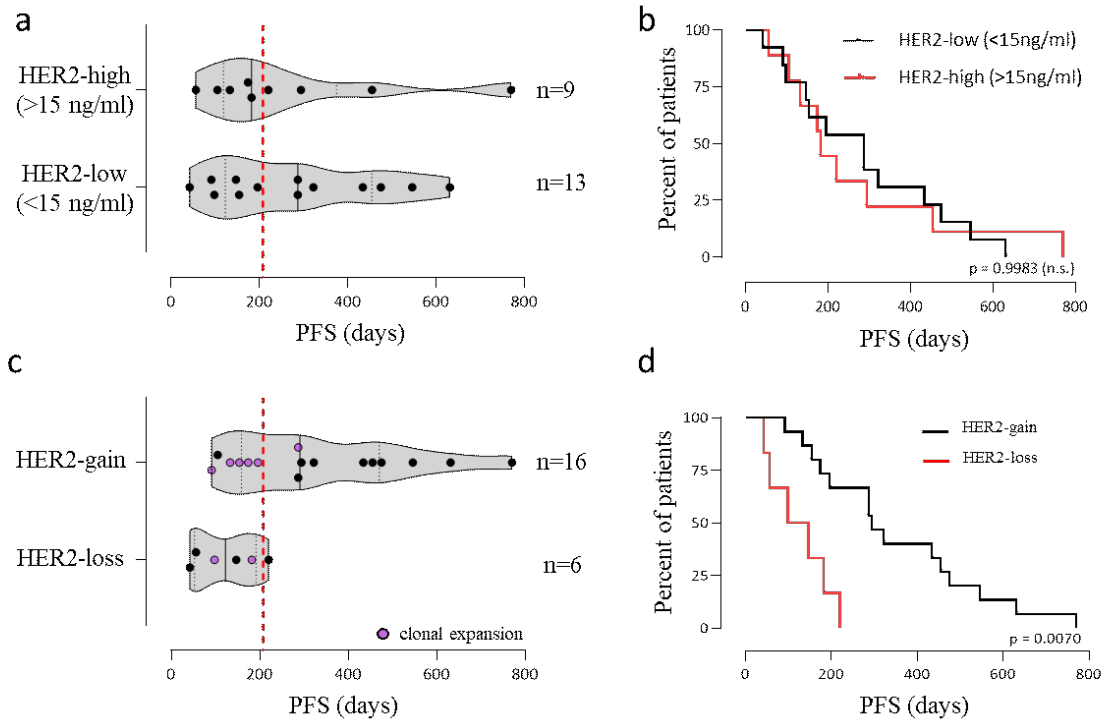

Fig.21 *PFS/ELISA readout correlation*
a-c) PFS variation in clustering patients in null-low HER2; b-d) PFS variation in clustering patients according to HER2 gain or loss

The biological/clinical significance, if any, of HER2-2D clustering on the two sides of the FDA threshold line was no longer investigated. Then, pre- and post-T-DM1 absolute value were disregarded, and we turned our attention to trends (gain and loss) rather than absolute sHER2 ECD levels. Remarkably, sHER2-gain was notably associated with a much longer PFS and a far lesser Kaplan-Meier slope than sHER2-loss (Mann-Whitney and Mantel-Cox (log-rank) test: p=0,007) (median PFS 291 vs 122 days; 95% CI 240-459 vs 50-198) (Fig. 21 c and d).

In conclusion, it is clear that a fixed-threshold metric (e.g., HER2-2D high/low) fails to distinguish outcomes because it is based on a single determination (at T_p), whereas heavily metastatic patients differ in tumor burden, number of metastatic foci, and absolute T_0 sHER2 levels (Fig.19b). In this complex setting, only a dynamic (T_0 vs T_p) patient-dependent threshold (e.g., sHER2 gain/loss) may infer a weighted average (in blood) of the overall changes in the tumor HER2 status.

The easiest interpretation of these data (Fig. 22) is that breast cancers ascribed to the HER2 subtype at diagnosis, and then HER2-counter-selected by sequential therapies, are ultimately caught by T-DM1 at an evolution stage whereby T-DM1 forcefully blunts HER2 amplification precluding the canonical mechanism of abundant HER2 transcription from multiple gene copies.

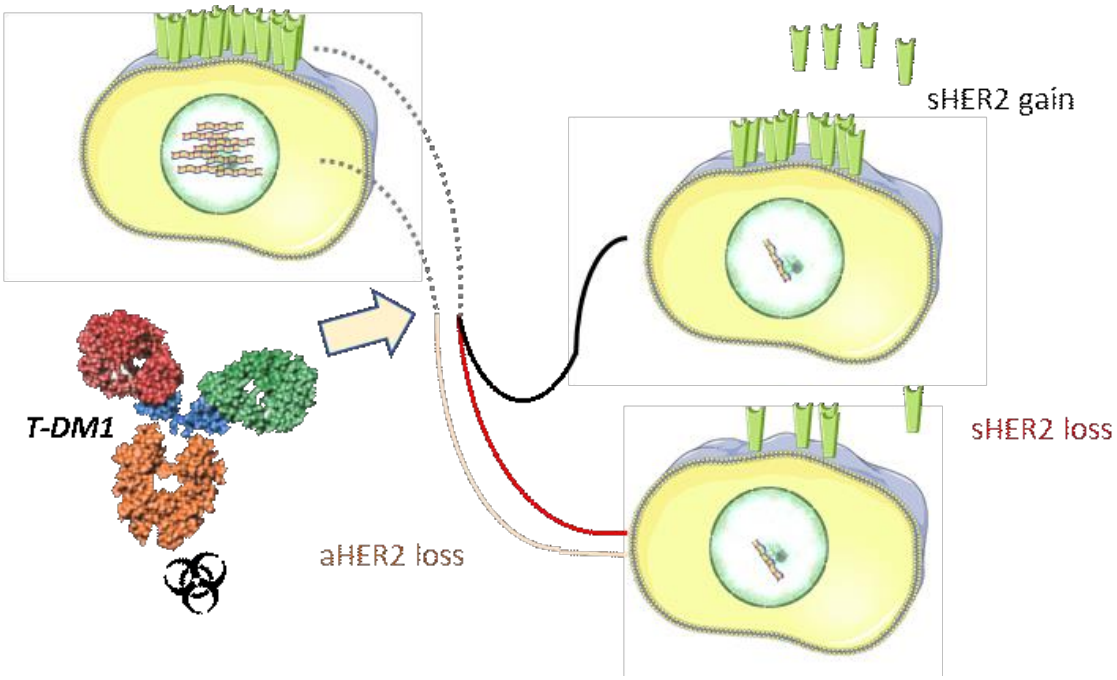

Fig.22 *A model for HER2 adaptive selection during T-DM1 treatment*

Since HER2 neutralization by T-DM1 appears to be irreversible [254], tumors are left with limited options: (a) either they switch to other hard-wired cancer drivers [254](Figs.10-15), and in this case they get rid of HER2 addiction and rapidly progress through molecular subtype switch, or (b) they are unable to emancipate from HER2 addiction, and in this case they have no choice but regaining at least some HER2 through a non-conventional, soft-wired up-regulation in protein expression not requiring gene amplification. If this is the case, tumors have then to strike a balance between two opposing selective forces: T-DM1 pressure

preventing full-fledged over-expression, and HER2 oncogenic dependence, precluding complete HER2 shutoff. It is this low expression window that may be therapeutically exploited by T-DM1.

Next, circulating alterations other than HER2 amplification were considered that had been previously shown to associate with clonal expansion and rapid progression [255] (Fig.21c). Accordingly, these were mostly seen in cases clustering below median PFS (purple dots in Fig. 21c). Excluding these cases resulted in expanded PFS differences between the sHER2-gain and sHER2-loss subsets (Mann-Whitney p=0.007), further supporting positive HER2 selection in T-DM1-sensitive tumors.

In summary, it is suggested that sHER2 gain identifies a 'stubborn' HER2-addiction making breast cancers intrinsically less aggressive and/or more vulnerable to T-DM1. It is tempting to speculate that ADCs are particularly apt to split copy number maintenance from protein overexpression, because they suppress HER2 signaling and at the same time directly dispatch a cytotoxic payload, pushing HER2-faithful tumor cells for quite some time into a dead end whereby copy numbers and gene expression are dissociated. Wiping off HER2-amplified genomic variants leaves 'stubborn' tumors no choice but regaining at least some HER2 (Fig.22) through a non-conventional, soft-wired up-regulation in protein expression not requiring gene amplification. HER2-gain may help T-DM1 to keep the tumor in check for quite some time until, similar to sHER2-loss, tumor variants arise hosting alternative, hard-wired genomic cancer drivers that ultimately override HER2 oncogenic addiction. It will be of interest to confirm this prediction by HER2-2D LB testing of patients treated with T-DXD and other next generation anti-HER2 agents.

CHAPTER 6

CONCLUSIONS

Breast cancer has led the way to the introduction of prognostic and predictive biomarkers for cancer patients. Over 40 years ago, ER was first introduced for predicting response to endocrine therapy. Twenty years later, HER2 became available for identifying patients likely to benefit from trastuzumab and later to other forms of anti-HER2 therapy.

The ASCO/CAP guidelines on HER2 interpretation in breast cancer, which were released first in 2007 and subsequently updated in 2013 and 2018, provide clear instructions for HER2 status designation. The updated 2018 ASCO/CAP guideline recommended concomitant IHC and ISH analysis to achieve the most accurate determination of HER2 status with no equivocal results. However, as shown herein, the HER2 status is not immutable.

LB-based approaches may improve this assignment identifying new prognostic and predictive biomarkers to assign target therapy and refine HER2 subtype assignment in a 'dynamic' way.

The highlights of this thesis are: a) T-DM1 rapidly pushes most or all tumors toward a presumably irreversible HER2 neutral state that had not been achieved by year-long previous therapies; (b) in at least some cases, HER2-low tumors emerging from this extensive HER2 blockade acquire features (e.g. hormone addiction) reminiscent of the naïve population of HER2-low tumors; (c) replacement of dominant HER2 addition (HER2 neutralization) occurs in most but not all patients, and comes at the cost (for the tumor) of developing alternative vulnerabilities; (d) most actionable alterations seen in blood had not been previously detected in tumor tissues, e.g. blood-only alterations are the optimal actionable biomarkers during clinical progression; (e) to evade T-DM1, tumors pay a high price, and the highest price is paid by tumors that are unable to emancipate from HER2 addiction. These retain/regain HER2 and may be controlled for longer times by T-DM1, and possibly other anti-HER2 treatments and novel ADCs; (f) possibly, a static, one-time-only, tissue-only HER2 CDx by IHC/ISH should be dismissed. Novel CDx schemes should be adopted. To be useful, they must capture HER2 dynamic changes, enable dynamic reallocation of patients to different subtypes, and assign non-standard treatment in a potentially practice-changing setting.

In this doctoral dissertation I have shown how HER2-2D can be considered a novel way to look at the long-vexing question of changes in HER2 addiction during the natural/clinical history of breast cancers, in contrast with the invasive, sequential, one-point, threshold-dependent, and based on a discontinuous (yes/no) scale IHC/FISH approach.

Additional published studies in collaboration with the laboratory of Prof. Francesco Michelotti at the University of Sapienza, Rome, not described herein in the interest of time, demonstrate that novel photonic bio-sensors may be of interest as an alternative, sensitive, economical, label-free alternative to ELISA in HER2-2D [256-258]. A novel HER2 biosensor may be of interest for future applications in the Health Technology Assessment area.

At least one puzzling unresolved aspect remains that may be addressed in future studies. Elevated levels of sHER2 were suggested by some to positively correlate with tumor aggressiveness and poor survival. The negative prognostic value of sHER2 may be enhanced when combined with measurement of other biomarkers like CA 15-3. At first sight these data may appear in conflict with the favourable outcome of moderate sHER2 gain in *LiqBreasTrack*. However, this contradiction is to a large extent only apparent. Our data clearly show that the moderate increase favourable to PFS occurs after T-DM1 application, e.g., after T-DM1 has drastically blunted HER2 amplification and blood levels. Therefore, anti-HER2 treatments decrease HER2 when tumors are sensitive, but when tumors become resistant HER2 is regained, e.g., high and low HER2 may be both detrimental, but at distinct disease stages. This observation is novel and highlights a basic difference in oncogenic addition in responding vs unresponsive patients. Re-expression of HER2 is therefore an adaptive mechanism that follows a first phase of HER2 suppression. There is no contradiction between high levels of HER2 and poor outcome under 'weak' HER2 blockade on the one hand, and moderate levels of HER2 regain and favourable outcome under strong ADC-mediated blockade. These are two sides of the same coin.

This conclusion may be easily challenged by deploying liquid biopsy more extensively, in real-life cohorts of breast cancer patients throughout disease stages and a variety of therapeutic HER2 blockade strategies, a task that I am eager to pursue in the near future.

In summary, I defend my thesis by claiming that experimental evidence has been provided to the Board of Experts and the University of Ferrara that LB is an essential tool for molecular oncology. Its use will certainly improve the oncologist's ability to identify crucial disease nodes for empirical, evidence-based therapy adjustment and patient management.

PUBLICATIONS DURING MY PhD PERIOD

1. Article: ***Liquid biopsy identifies actionable dynamic predictors of resistance to Trastuzumab Emtansine (T-DM1) in advanced HER2-positivebreast cancer.***
   Mol Cancer **20,** 151 (2021). https://doi.org/10.1186/s12943-021-01438-z
   Matteo Allegretti*; Alessandra Fabi*; Elena Giordani; Cristiana Ercolani; Paolo Romania; Cecilia Nisticò; Simona Gasparro; Vittoria Barberi; Maria Ciolina; Edoardo Pescarmona; Diana Giannarelli; Gennaro Ciliberto; Francesco Cognetti; Patrizio Giacomini

2. Conference paper EOSAM 2021: ***Optical multiplexed bioassays on photonic crystals for breast cancer biomarker detection***
   Tommaso Pileri, Alberto Sinibaldi, Agostino Occhicone, Elena Giordani, Matteo Allegretti, Peter Munzert, Frank Sonntag, Norbert Danz, Patrizio Giacomini and Francesco Michelotti

3. Article: ***Cross-sectional analysis of circulating tumor DNA in primary colorectal cancer at surgery and during post-surgery follow-up by liquid biopsy.***
   J Exp Clin Cancer Res. 2020 Apr 20;39(1):69. doi: 10.1186/s13046-020-01569-z.
   Allegretti M, Cottone G, Carboni F, Cotroneo E, Casini B, Giordani E, Amoreo CA, Buglioni S, Diodoro M, Pescarmona E, Zazza S, Federici O, Zeuli M, Conti L, Cigliana G, Fiorentino F, Valle M, Giacomini P, Spinella F.

4. Article: ***A Distinctive microRNA (miRNA) Signature in the Blood of Colorectal Cancer (CRC) Patients at*** Surgery. Cancers (Basel). 2020 Aug 25;12(9):E2410. doi: 10.3390
   Gasparello J, Papi C, Allegretti M, Giordani E, Carboni F, Zazza S, Pescarmona E, Romania P, Giacomini P, Scapoli C, Gambari R, Finotti A.

5. Article: ***Bioassay engineering: a combined label-free and fluorescence approach to optimize HER2 detection in complex biological media***.

Anal Bioanal Chem. 2020 May;412(14):3509-3517. doi: 10.1007/s00216-020-02643-3. Epub 2020 Apr 16. PMID: 32300843.
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6. Review: ***Circulating microRNAs and liquid biopsy: murine xenograft models for technical validation of clinical protocols,*** Giugno 2019
Journal of Cancer Metastasis and Treatment DOI: 10.20517/2394-4722.2019.17
Jessica Gasparello, Matteo Allegretti, Chiara Papi, Elena Giordani, Patrizio Giacomini, Roberto Gambari, Alessia Finotti

7. Conference Paper: ***Label-free and fluorescence photonic crystal biochips for early cancer biomarker detection***,March 2019 DOI: 10.1117/12.2514310
Alberto Sinibaldi,Norbert Danz,Elisabetta Sepe,Peter Munzert,Agostino Occhicone,Matteo Allegretti,Elena Giordani,Patrizio Giacomini,Francesco Michelotti

8. Abstract P4-01-19***: Liquid biopsy and re-biopsy: Tracking mutational trajectories in HER2 + breast cancer patients undergoing T-DM1 treatment***
February 2019, Cancer Research 79(4 Supplement): P4-01-19-P4-01-19. DOI: 10.1158/1538-7445.SABCS18-P4-01-19
Matteo Allegretti, Elena Giordani, Beatrice Casini, Patrizio Giacomini

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