Seven shades of tamoxifen resistance

Molecular mechanisms of drug resistance in breast cancer
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Molecular mechanisms of drug resistance in breast cancer

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Academic Dissertation

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Tamoxifen treatment of estrogen receptor-positive breast cancer reduces breast cancer mortality. However, resistance to tamoxifen develops frequently. A plethora of resistance mechanisms have been described but their biological importance, clinical significance, and possibilities for diagnostic or therapeutic intervention are poorly understood. Fusion genes, for example, have the potential as therapeutic targets or diagnostic tools as they are highly cancer-specific.

In order to determine the mechanisms underlying endocrine therapy resistance and to identify new opportunities to defy resistance in breast cancer, we created seven tamoxifen-resistant breast cancer cell lines. We characterized the resistant cell lines by exome-sequencing to identify possible mutations or genomic rearrangements involved in drug resistance. RNA-sequencing was applied to shed light on the nature of fusion genes in the parental cell line as well as their contribution to acquired drug resistance. RNA-sequencing also exposed gene expression and pathway changes, which were followed up in detail in one of the resistant cell lines. In addition to drug sensitivity and resistance testing combined with high-content imaging, network analysis determined the drug response profiles. We further uncovered potential drug targets of tamoxifen resistance.

This intensive molecular profiling revealed that each tamoxifen-resistant cell line developed its own resistance mechanism and acquired individual drug vulnerabilities. However, we were able to detect a common increased sensitivity towards an ERK1/2-inhibitor. On the other hand, we discovered co-resistance to paclitaxel, which is mostly driven by the slower growth rate of the resistant cells. Analysis of differentially expressed genes identified pathways which were associated with cell cycle, protein modification, and metabolism, es-
especially with the cholesterol pathway. After further investigation we identified that the prevention of lysosomal membrane permeabilization was associated with drug resistance in the T-47D tamoxifen-resistant cell lines. Targeting this mechanism remains challenging. We further revealed the complex nature of fusion genes, which include the high prevalence of alternative splicing and the lack of recurrence across different breast cancer cell lines. Additionally, we identified fusion genes only present in the resistant cell lines. However, these were mainly cell clone-specific or recurrent read-through fusions. Exome-sequencing revealed no known or common mutations or copy number changes related to resistance development.

With the diversity of fusion genes, mutations, copy number changes, differentially expressed genes, pathway changes, and drug responses in tamoxifen-resistant cells, it is safe to say that tamoxifen resistance cannot simply be explained by one common mechanism. Therefore, it is likely that countering the resistance will require different therapeutic approaches.
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* Equal contribution
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<th>Full Form</th>
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>Abl1</td>
<td>Abelson murine leukemia viral oncogene homolog 1</td>
</tr>
<tr>
<td>AEBS</td>
<td>Antiestrogen binding sites</td>
</tr>
<tr>
<td>AF-1</td>
<td>Activation function domain 1</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BCR</td>
<td>Breakpoint cluster region protein</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BRCA1/2</td>
<td>Breast cancer 1/2 early onset</td>
</tr>
<tr>
<td>CAR</td>
<td>Chimeric antigen receptor</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myelogenous leukemia</td>
</tr>
<tr>
<td>CNV</td>
<td>Copy number variation</td>
</tr>
<tr>
<td>CP</td>
<td>Ceruloplasmin</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per million</td>
</tr>
<tr>
<td>CTG</td>
<td>CellTiter-Glo</td>
</tr>
<tr>
<td>CTSD</td>
<td>Cathepsin D</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
</tr>
<tr>
<td>DSRT</td>
<td>Drug sensitivity and resistance testing</td>
</tr>
<tr>
<td>DSS</td>
<td>Drug sensitivity score</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERα/ESR1</td>
<td>Estrogen receptor α</td>
</tr>
<tr>
<td>ERβ/ESR2</td>
<td>Estrogen receptor β</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen response element</td>
</tr>
<tr>
<td>ETS</td>
<td>E26 transformation-specific</td>
</tr>
<tr>
<td>EV</td>
<td>Extracellular vesicle</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FDA</td>
<td>food and drug administration</td>
</tr>
<tr>
<td>GS-X</td>
<td>glutathione S-conjugate export</td>
</tr>
<tr>
<td>HER2</td>
<td>human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF-1</td>
<td>hypoxia-inducible factor 1</td>
</tr>
<tr>
<td>ICI</td>
<td>Imperial Clinical Industries</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin growth-factor</td>
</tr>
<tr>
<td>LBD/AF-2</td>
<td>ligand-binding domain/activation function domain 2</td>
</tr>
<tr>
<td>LLOMe</td>
<td>L-leucyl-L-leucine methyl ester</td>
</tr>
<tr>
<td>LMP</td>
<td>lysosomal membrane permeabilization</td>
</tr>
<tr>
<td>IncRNA</td>
<td>long non-coding RNA</td>
</tr>
<tr>
<td>MCL-1</td>
<td>Induced myeloid leukemia cell differentiation protein</td>
</tr>
<tr>
<td>MET</td>
<td>Met proto-oncogene tyrosine kinase</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>MRP1</td>
<td>multidrug resistance-associated protein 1</td>
</tr>
<tr>
<td>NCoR</td>
<td>nuclear receptor co-repressor</td>
</tr>
<tr>
<td>NF-kB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NSCLC</td>
<td>non-small-cell lung cancer</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SERM</td>
<td>selective estrogen receptor modulator</td>
</tr>
<tr>
<td>SOD1</td>
<td>superoxide dismutase 1</td>
</tr>
<tr>
<td>SRC</td>
<td>steroid receptor co-activator</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TMPRSS2</td>
<td>transmembrane protease, serine 2</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
INTRODUCTION
Immediately, with the discovery of usefulness of cytotoxic agents in cancer therapy, the phenomenon of cancer drug resistance was observed in the 1940s (Goodman et al. 1946). To study drug resistance, researchers applied lessons learned from the selection process of antibiotic-resistant bacteria and cultivated patient-derived cell lines exposed to the drug of interest and developed drug-resistant cancer cell lines (McDermott et al. 2014). Samples were also taken from drug-resistant tumors and investigated in animal models, for example, as xenografts (Houghton, Houghton, and Green 1982; Bertotti et al. 2011; S. Li et al. 2013). Results from cell line or animal studies were not always easily translatable to the clinic. Hence, even after 70 years of research, drug resistance is still a problem not only in the case of chemotherapeutics but also of targeted therapy. Even though cancer is a genetic disease, it became obvious that this might not always be the case in terms of drug resistance. Drug resistance can arise from genetic, epigenetic, as well as environmental factors and those factors can act simultaneously. Another contributor to drug resistance is the molecular diversity of cancer in which breast cancer, with its multiple subtypes, is a poster child. In breast cancer, commercial diagnostic tests, such as Oncotype DX (Carlson 2006), MammaPrint (Glas et al. 2006), or EndoPredict (Filipits et al. 2011) may provide prognostic information but also guide therapy. Given that we can now detect circulating tumor cells as well as circulating free DNA using so-called liquid biopsies from the blood (Friedlander, Premasekharan, and Paris 2014), it would be very beneficial to identify drug resistance as soon as it occurs. Maybe even by implementing such a test. However, we still need to learn more about drug resistance mechanisms and vulnerabilities associated with these to be able to treat drug-resistant cancers.
The Breast

The breast, or mammary gland, is not fully developed at birth. In its post-natal development, it undergoes a course of transformations such as cell proliferation, differentiation, and morphogenesis. Various hormones and growth factors regulate these transformations. During puberty estrogens, together with progesterone, are responsible for the expansion of the gland and the development of the ductal epithelium, which give rise to its glandular tissue structure (Brisken et al. 1998; Mallepell et al. 2006). The fully developed adult mammary gland is composed of different cell types. The inner lumen of the ductal and lobular structures are lined with a continuous layer of epithelial cells that are surrounded by a layer of contractile myoepithelial cells. The myoepithelial cells are further enclosed by a basement membrane which separates the epithelial from the stroma. The stroma consists of adipocytes, lymphocytes, neurons, fibroblasts, and endothelial cells (Anderson, Clarke, and Howell 2000). The hormone-sensing cells such as estrogen- and progesterone receptor positive cells are located within the luminal layer ranging from 7-20% of cells. Interestingly, those ER-positive cells do not divide in normal tissue but are in close proximity to them (Clarke et al. 1997).

Overall, the female mammary gland experiences a lot of structural and functional changes that are characterized by proliferation, differentiation, and apoptosis of cells due to hormonal changes during the menstrual cycle, pregnancy, lactation, and menopause (Macias and Hinck 2012).


2 Estrogen Receptor - it's complicated

The activities of estrogens are not only restricted to the development and maintenance of sexual and reproductive functions, they are additionally involved in biological processes of the musculoskeletal, immune, cardiovascular and central nervous system in men and women (Gustafsson 2003). There are three types of estrogens: estrone (E1), estriol (E3) and the predominant 17β-estradiol (E2), which binds to the nuclear transcription factor receptors ERα and ERβ. The receptors are encoded by ESR1 and ESR2 genes and have three functional domains. The hormone-independent transcriptional activation function domain (AF-1) at the N-terminus harbors the biggest functional differences between the two receptors. The DNA-binding domain (DBD) shares about 55% homology and the ligand-binding domain (LBD/AF-2) at the C-terminus is 95% homologous between the two receptors (Kuiper et al. 1996). Nevertheless, primarily the activation of ERα target genes is responsible for promoting cancer cell proliferation and a decrease in cancer cell apoptosis (Iwao et al. 2000; Roger et al. 2001). There are several paths by which estrogens and ER convey signaling (Figure 1). The ligand-dependent arm, as the name implies, involves the binding of estrogen to the LBD/AF-2 of ER, forming an estrogen-ER complex, which promotes different gene activation scenarios. The complex can directly bind to estrogen response elements (EREs) in the promoter region of a target gene (Figure 1A). The ERE is defined by two inverted DNA-sequences separated by three random nucleotides (GGTCAnnnTGACC) (Klinge 2001). However, it is now clear that the complex also binds to enhancer regions in the genome (Carroll et al. 2006).
Alternatively, the estrogen-ER complex can interact with other transcription factors such as activator protein 1 (AP1), specificity protein 1 (SP1), NF-κB, or cAMP response element-binding protein (CREB) to name just a few amongst many others (Galien and Garcia 1997; Kushner et al. 2000; Lazennec, Thomas, and Katzenellenbogen 2001; W. Porter et al. 1997). This co-activation facilitates the transcriptional regulation of genes that do not harbor EREs (Figure 1B). In order to fully facilitate transcriptional regulation the estrogen-ER complex needs co-regulators that can modify the chromatin structure or interact with the transcriptional machinery. Plenty of those co-reg-
ulators have been identified (Métivier et al. 2003). The most intensely studied co-regulators belong to the p160 family proteins (or SRCs). They can either directly or indirectly recruit co-activators and so remodel chromatin and modify histone activity (Koh et al. 2001), for example, by recruiting histone acetyltransferase (HAT), which is known to play a role in the initiation of transcription (Brownell and Allis 1996; Kraus and Kadonaga 1998). *Cathepsin D (CTSD)* and *trefoil factor 1 (TFF-1*, also known as *pS2*) have been among the first identified target genes of the estrogen-ER and the p160 co-regulators complex *in vivo* (Shang et al. 2000). As if this isn't already complex enough, the estrogen-ER complex has been shown to activate multiple signaling cascades through direct interactions with various proteins including tyrosine kinase Src, phosphatidylinositol 3-kinase (PI3K), and insulin-like growth factor 1 receptor (IGF-1R) (Castoria et al. 2001; Kahlert et al. 2000; Simoncini et al. 2000). This so-called non-genomic mode of action leads to an immediate response by the estrogen-ER complex (Figure 1C).

Finally, there is a ligand-independent activation of ER signaling with various effects on cellular processes (Cenni and Picard 1999). The best-studied signals inducing ER activities are growth factors, for example, EGF and even IGF-1, which induce signaling cascades that lead to the phosphorylation and thereby activation of ER in an estrogen-independent manner (Kato et al. 1995, Figure 1D).

The different mechanisms of action demonstrate the complex nature of ER signaling. However, it needs to be stated that most of the functional studies have been conducted in cancer cell lines and little is known about the interactions of ER in healthy human mammary tissue.
3 Breast Cancer

Despite losing its first place in cancer mortality among young European women to lung cancer, breast cancer is still with around 460’000 cases, the most often diagnosed cancer among European women, and remains a major public health concern (Malvezzi et al. 2015; Ervik et al. 2016). Fortunately, breast cancer is often treatable if it represents one of the subtypes that have treatment options available. If breast cancer is diagnosed late or belongs to a subtype with limited treatment possibilities it is still deadly. Another major drawback is the development of therapy resistance, which is followed by tumor progression and development of metastasis.

3.1 Breast Cancer Subtypes

Breast cancer is extremely heterogeneous. According to the World Health Organization, the invasive ductal carcinoma, not otherwise specified (ductal NOS) represents 40-75% of breast cancer cases. These cases, as the NOS addition suggests, represent a heterogeneous group that does not exhibit sufficient histological properties in comparison to the invasive lobular carcinoma (ILC, 5-15%) or tubular carcinoma (<2%) (Lakhani et al. 2012). Breast cancer is routinely clinically characterized by tumor type, size, grade, lymph node status as well as four immunohistochemistry markers ER, PR, Ki67, and HER2. Alternatively, the more sensitive fluorescent in situ hybridization (FISH) is used to detect HER2. However, a combination of IHC and FISH is recommended (Lakhani et al. 2012; Wolff et al. 2018). Intensive molecular profiling has shed light on this cancer's biology, which can also guide clinical decisions.
More than 15 years ago, Perou, Sørlie, and colleagues divided breast cancer into five different subtypes based on gene expression profiles (Perou et al. 2000; Sørlie et al. 2001). The most dominant subtypes are the luminal A and luminal B types and as their name suggests, they mainly express genes of the lumen of the mammary duct (Table 1). Both subtypes are ER-positive. Additionally, they can be either positive or negative for the progesterone receptor (PR). These subgroups are usually responsive to endocrine- and chemotherapy (Early Breast Cancer Trialists’ Collaborative Group (EBCTCG) 2005). The luminal B type is also HER2-positive and has high levels of Ki67 compared to luminal A and has, therefore, a higher proliferation rate. Importantly, the HER2-positivity makes it responsive to therapeutic approaches against HER2, such as herceptin. Other subtypes do not express ER and PR, including the HER2-positive subtype (Table 1), which is non-responsive to endocrine therapy (Loibl and Gianni 2017). Finally, the basal-like (or triple negative) subtype has a similar expression profile to the myoepithelial cells. The treatment options are restricted to surgery, radiation and/or chemotherapy, as this type does not express ER, PR, and/or HER2 (Fulford et al. 2007; Holliday and Speirs 2011). A controversial subtype is the normal breast-like type. It has gene expression similar to normal breast tissue and high expression of genes that reassemble the characteristic for adipose tissue (Sørlie et al. 2001; Fan et al. 2006). However, the normal breast-like type has been regarded as an artifact due to high contamination of “true” normal breast tissue in the sample (Parker et al. 2009).
Table 1: Breast cancer subtypes (based on http://www.pathophys.org/breast-cancer/ by Eric Wong)

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Characteristics</th>
<th>Prevalence</th>
<th>Medical therapy</th>
</tr>
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<tbody>
<tr>
<td>Luminal A</td>
<td>ER ++  PR +</td>
<td>40 %</td>
<td>Endocrine therapy</td>
</tr>
<tr>
<td></td>
<td>HER2 -  Low Ki67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal B</td>
<td>ER +  PR +/-</td>
<td>20 %</td>
<td>Chemotherapy, Endocrine therapy,</td>
</tr>
<tr>
<td></td>
<td>HER2 +/-  high Ki67</td>
<td></td>
<td>anti-HER2 targeted therapy</td>
</tr>
<tr>
<td>HER2-positive</td>
<td>ER -  PR -</td>
<td>10-15 %</td>
<td>Chemotherapy with anti-HER2 targeted</td>
</tr>
<tr>
<td></td>
<td>HER2 ++  high Ki67</td>
<td></td>
<td>therapy</td>
</tr>
<tr>
<td>Triple negative</td>
<td>ER -  PR -</td>
<td>15-20 %</td>
<td>Chemotherapy</td>
</tr>
<tr>
<td></td>
<td>HER2 -  high Ki67</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This first classification (Perou et al. 2000; Sørlie et al. 2001) was followed by several more suggestions. By additionally utilizing copy number alterations, a study by Curtis et al. divided the existing classification in even 10 different subgroups (Curtis et al. 2012), whereas others suggested an extra claudin-low group (Herschkowitz et al. 2007), or identified six TNBC subgroups of which one is androgen receptor-positive (Lehmann et al. 2011). For patients with the latter subtype there are currently several clinical trials with anti-androgen-therapy ongoing (clinicaltrials.gov: NCT01889238; NCT02689427; NCT03383679).

The examples of the different subgroups (Table 1) show that even within the subtypes tumor-specific dissimilarities occur, which likely contribute to tumor development and progression. It also
highlights that a “one-size-fits-all” therapy is not applicable in breast cancer and a more personalized treatment is needed.

4 Fusion genes

As mentioned above, breast cancer is a heterogeneous disease with frequent genetic alterations. These alterations can activate cancer-promoting genes, so-called oncogenes, and inactivate tumor suppressor genes thereby shifting the balance towards cancer progression. Germ-line mutations, for example in BRCA1/2, TP53 or CHEK2, account for about 25% of breast cancers (Olopade et al. 2008). More than 30 genes such as somatic mutations in PIK3CA, GATA3, and TP53, as well as gene amplifications like the HER2 amplification, have been recurrently identified and are responsible for driving growth in a subset of breast cancers (Cancer Genome Atlas Network 2012; Desmedt, Yates, and Kulka 2016).

However, a potentially relevant but still overlooked class of cancer mutations are fusion genes. A fusion gene is a chimeric DNA molecule where either by chromosomal translocation, inversion, deletion, insertion, or tandem duplication a part of a gene shifts to a new genomic locus (Figure 2).

Figure 2: Main fusion gene types. A chromosomal translocation, B inversion, C deletion and D duplication (modified from Guy Leonard, 2012, Wikimedia Commons)
This event might just inactivate a gene but can also result in a fusion protein, which can drive neoplastic cell growth and cancer progression (Edwards 2010). Mechanisms such as the proximity between different chromosomes during the interphase or incorrect recombination due to shared sequence motifs at the chromosomal breakpoints have been suggested to give rise to fusion genes (Mitelman, Johansson, and Mertens 2007).

The fusion of $BCR$ and $ABL1$, for example, leads to an abnormal tyrosine kinase in 90% of chronic myelogenous leukemia (CML) cases. This fusion is formed by a reciprocal translocation of chromosome 9 and 22 (Rowley 1973). The subsequent fusion protein, encoded by the smaller so-called Philadelphia chromosome, is based on its molecular weight associated with different leukemia types. The 210kDa protein ($p^{210}_{BCR-ABL1}$) is detectable in CML, the 185/190kDa protein ($p^{190}_{BCR-ABL1}$) is linked to B-cell acute lymphoblastic leukemia (B-ALL) and the heavier 230kDA ($p^{230}_{BCR-ABL1}$) has been found in a subtype of CML, which was once called chronic neutrophilic leukemia (Clark et al. 1987; Hermans et al. 1987; Wada et al. 1995). The Philadelphia chromosome was already discovered in 1960 by Peter Nowell and David Hungerford (Nowell and Hungerford 1960) and several years later imatinib, a drug that inhibits the fusion protein's enzyme activity, was developed (Thiesing et al. 2000).

Several decades after the discovery of the Philadelphia chromosome, $TMPRSS2-ETS$ was the first recurrent fusion gene discovered in solid tumors, namely prostate cancer (Tomlins et al. 2005). It has been suggested that $TMPRSS2-ETS$ can function as an additional diagnostic marker, which is even detectable from urine samples (Cornu et al. 2013; Rostad et al. 2009). Furthermore, in a
subgroup of non-small cell lung carcinoma patients, the *EML4-ALK* fusion was detected of which the protein domains of the ALK fusion partner can be targeted with crizotinib (Shaw et al. 2011).

In breast cancer, the *ETV6-NTRK3* fusion, for example, has been identified as the cause for about 90% of secretory breast cancers. This fusion facilitates a ligand-independent activation of a signaling cascade and thereby transforming mammary epithelial cells into tumor-forming cells (Tognon et al. 2002). Larotrectinib, a drug targeting NTRK1/2/3, is available and clinical trials with patients harboring NTRK-fusions are ongoing and showing promising results (Drilon et al. 2018).

As these few examples show, fusion genes have the potential to be used as therapeutic targets or diagnostic tools as they are highly cancer-specific. Additionally, they can be utilized as an indicators of drug treatment success or failure as they are detectable in urine (Cornu et al. 2013; Rostad et al. 2009) and blood (R. J. Leary et al. 2010; McBride et al. 2010).

5 **Tamoxifen**

Tamoxifen belongs to the drug class of selective estrogen receptor modulators (SERMs). Due to its anti-estrogen behavior in breast tissue, high efficiency and low price it is the preferred treatment of ER-positive breast cancer in pre- and postmenopausal woman (Jordan 2008; Senkus et al. 2015).

5.1 **A summary of tamoxifen's history**

Tamoxifen failed to be a contraceptive, but got repurposed as an anti-cancer drug and became helpful for millions of women with breast cancer (Jordan 2003). In 1967 the non-steroidal compound
ICI46,474, the drug which became tamoxifen was synthesized by Dora Richards and reported by Arthur Walpole and Michael Harper from ICI Pharmaceuticals Division (today AstraZeneca). This team of reproductive endocrinologists discovered that the drug was an estrogen agonist in mouse, but functions in the immature rat as an agonist/antagonist (Harper and Walpole 1967). Further clinical studies showed that tamoxifen could be used as a treatment for advanced breast cancer because it caused fewer side effects and was as effective as contemporary treatments when given for 1 year (Cole, Jones, and Todd 1971; Ward 1973). As the focus of the company was not cancer research, it took Walpole a bit of persuasion to market the drug as an advanced breast cancer treatment in the UK. In addition, there were patent issues in the US (Jordan 2003).

Further, a lot of interpersonal connections helped to advance the research on tamoxifen, which showed that 4-hydroxytamoxifen, the active metabolite of tamoxifen, binds with high affinity to the estrogen receptor (Jordan et al. 1977). These findings and further research into the duration of tamoxifen treatment (Baum et al. 1983; Jordan and Allen 1980) led to the more direct use of tamoxifen in ER-positive breast cancer, making tamoxifen the first targeted breast cancer therapy and one of the widest used anticancer drug (Jordan 2003).

5.2 Mode of Action
As complex as the molecular mechanism of ER is, so is the mechanism of tamoxifen. The active metabolite 4-hydroxytamoxifen binds to the LBD/AF-2 domain of ER and changes the protein structure of the receptor, thereby preventing the binding of certain co-regulators such as p160 (Norris et al. 1998; Shiau et al. 1998). However, this conformation change enables the binding of nuclear receptor co-repressors (NCoRs), which inhibit the gene transcription (Huang,
Norris, and McDonnell 2002; Lavinsky et al. 1998). The ER-dependent mode of action already takes place at low concentrations of tamoxifen (< 0.1µM) (Coezy, Borgna, and Rochefort 1982; Murphy and Sutherland 1985). However, in patients' plasma tamoxifen concentrations of 1 µM and even higher within the tumor have been measured (MacCallum et al. 2000). Therefore, other mechanisms by which tamoxifen induces cell death have been suggested.

Tamoxifen has been shown to inhibit protein kinase C (PKC), an enzyme that modulates cell growth regulating signals, among others (Horgan et al. 1986; Lavie et al. 1998; O’Brian, Housey, and Weinstein 1988). Tamoxifen also induces transforming growth factor-β (TGF-β), which is a hormonally regulated growth inhibitor (Butta et al. 1992; Knabbe et al. 1987). Other signaling mediators influenced by tamoxifen include calmodulin (Gulino et al. 1986; O’Brian, Housey, and Weinstein 1988), the mitogen insulin-like growth factor I (IGF-I) and the proto-oncogene c-myc (Kang, Cortina, and Perry 1996). Also, other proteins involved in apoptosis such as Bcl-2, Bax or different caspases are impacted by tamoxifen (Thiantanawat, Long, and Brodie 2003; G. J. Zhang et al. 1999). Additionally, tamoxifen has been shown to bind to so-called microsomal antiestrogen binding sites (AEBS), which are associated with cholesterol metabolism (Kedjouar et al. 2004). This mechanism, together with the induction of oxidative stress by tamoxifen, has been shown to induce apoptosis as well (Bekele et al. 2016; de Medina et al. 2011).

It now becomes more and more clear that tamoxifen has an array of ways to induce cell death in breast cancer patients, and could even provide benefits for ER-negative tumors.

6 Drug resistance in cancer
Despite a patient's overall ability to absorb and metabolize a drug
as well as the drug's accessibility to the tumor, there are factors within a tumor that cause drug resistance (Pluen et al. 2001). Scientists differentiate between de-novo/primary and acquired resistance (Giaccone and Pinedo 1996). Primary resistance is defined as follows: a tumor consists of cells with diverse genetic backgrounds and some of those cells are resistant to the given therapy to begin with. In contrast, in acquired resistance, all cells within a tumor, despite their genetic and epigenetic diversity, are initially responsive to the therapy, but during the treatment, some cells undergo changes that lead to drug resistance (Figure 3).

De-novo resistance

Acquired resistance

*Figure 3: Comparison of de-novo and acquired resistance. Pills indicate the start of drug treatment and color drug resistant cells (modified from Anna Azvolinsky 2017)*

### 6.1 Resistance mechanisms

Drug resistance can occur for just handful of drugs, whose functions are similar to each other or to a plethora of drugs with no functional relation. In the second case, scientists talk about multidrug resistance (Gottesman et al. 1994).
6.1.1 Drug uptake, efflux, and metabolism

Some drugs need to enter cancer cells via transporters. Resistance to those drugs can occur if the transporters are downregulated or mutated resulting in structural changes (Gottesman 2002). For instance, expression changes in the folate transporters lead to resistance of folate analogs, such as methotrexate (Gorlick et al. 1997). Also, the uptake of cytarabine (AraC), a nucleoside drug, is impaired due to alterations in the nucleoside transporter (Galmarini et al. 2002; Gati et al. 1998).

Another way to confer drug resistance is to increase the drug efflux, which is mainly facilitated by the membrane-embedded transporters of the ABC (ATP-binding cassette) superfamily (C. J. Chen et al. 1990). The efflux mechanism is important to clean out toxins within a healthy cell. However, an overexpression of ABC-transporters has been identified in multiple cancers (Goldstein et al. 1989; Nooter et al. 1995). The activity of the ABC-transporter ABCB1 or P-glycoprotein, encoded by the \textit{MDR1} gene, was the first one to be identified to be responsible for multidrug resistance. When a drug binds to the transporter it activates an ATP-binding domain and further hydrolysis of ATP, which in turn changes the conformation of ABCB1 and the drug gets secreted. This cycle is repeated after the transporter restores its original state by hydrolyzing another ATP (Sauna and Ambudkar 2001).

Some drugs are administered as inactive prodrugs and need to be activated in order to induce cell death. However, cancer cells have found a way to decrease this activation. For example, the gene encoding for thymidine phosphorylase, an enzyme which converts the prodrug capecitabine into the active form of 5-fluorouracil (Miwa et al. 1998), can be downregulated by DNA methylation leading to drug resistance (Kosuri et al. 2010). Further, drugs can get inactivated if
they get conjugated to glutathione, a mechanism which has been found to inactivate platinum-based drugs (Meijer et al. 1992). In addition, the ABC-transporter MRP-1, sometimes referred as GS-X pump, has been found to clear out glutathione-conjugated drugs (Ishikawa and Ali-Osman 1993). Recently it has been shown that in pancreatic cancer, even bacteria play a role in drug resistance. Bacteria with a longer version of the cytidine deaminase (CDD) gene were able to inactivate gemcitabine and the effect was reversed when antibiotics were added (Geller et al. 2017).

6.1.2 Drug target alterations

Mutations and expression alterations also occur as means of drug resistance in targeted therapy, where the drugs affect oncogenes that drive tumor growth. These targeted therapies have the general advantage of causing fewer side effects due to their cancer specificity. Nevertheless, drug resistance is a major issue with these therapies as well. One of the best examples of an oncogene treated with targeted therapy is the activated EGFR-receptor, which is inhibited by gefitinib or erlotinib (Shepherd et al. 2005; Lynch et al. 2004). However, resistance frequently occurs within a year and in 50% of the cases, it is due to an EGFR-T790M mutation (Bell et al. 2005).

Fusion genes, more specifically the proteins they encode, are also highly cancer-specific and therefore offer good targets for cancer therapy. The activities of the BCR-ABL1 fusion protein are successfully blocked by imatinib (Thiesing et al. 2000) until a missense mutation in the kinase domain at position T315 occurs and imatinib cannot bind anymore (Gorre et al. 2001).

The efficacy of targeted therapy can be lowered by an increase in drug target expression, as more molecules must be inhibited by the drug. A good example is the genomically amplified androgen re-
ceptor in about 30% of prostate cancers leading to resistance towards bicalutamide, an androgen receptor antagonist (Palmberg et al. 1997).

Alternative splicing is another possibility by which cancer cells can evade their fate of therapy-induced cell death. This escape has been shown for the newly FDA approved cancer immunotherapy with chimeric antigen receptor (CAR) T-cells. In this therapy approach, the patient's own T-cells get genetically modified so that they target CD19 expressing leukemia cells (D. L. Porter et al. 2011). Sotillo et al. discovered that, among other mutations, exon 2 which encodes the binding domain for the CAR therapy was missing in the expressed CD19 gene thereby preventing binding of the anti-CD19 CAR T-cells (Sotillo et al. 2015).

Glycosylation is a form of post-translational protein modification where glycans get attached to lipids and proteins. Glycosylation has been linked to modulate the response of cancer cells to drug treatments. Deglycosylation of HER2+ breast cancer cells improves the response to chemotherapeutics and herceptin (Peiris et al. 2017) and branching N-glycans on VEGF2-receptor are associated with resistance towards anti-VEGF treatment (Croci et al. 2014). Even though the link between glycosylation and cancer formation and progression is well established (reviewed in Pinho and Reis 2015), research about the interaction of glycosylation and drug responses is in its early stages.

6.1.3 Cell cycle alteration and DNA damage repair
Chemotherapy leads to cell death due to DNA damage induced during the S-phase (DNA synthesis) of the cell cycle. If a cell is in the resting- (G0) or growth-phase (G1), it is, therefore, resistant
to chemotherapy (Stewart et al. 2007). One of the cell cycle regulating proteins is cyclin E, which associates with Cdk2 (cyclin-dependent kinase 2) to regulate the transition into the S-phase, where it gets degraded (Ohtsubo et al. 1995). If this mechanism is out of balance, it can lead to reduced cell size, growth factor-independent proliferation, as well as lower cell growth rates (D. C. Porter et al. 2001; Ohtsubo et al. 1995; Dulić et al. 1993). Additionally, a halt in the cell cycle gives cells time to repair therapy-induced cell damage and therefore cancer cells can evade their destiny of cell death. The DNA repair mechanisms nucleotide excision repair and homologous recombination reverse the effects of DNA crosslinks of platinum-based drugs (Pennington et al. 2014; Selvakumaran et al. 2003).

### 6.1.4 Avoiding apoptosis

The avoidance of apoptosis is a cancer hallmark (Hanahan and Weinberg 2000). Primary tumors present mechanisms that can lead to failure of cancer therapy, especially if the drugs fail to induce other forms of cell death, such as necroptosis (Krysko et al. 2017). For example, it has been shown that an overexpression of the anti-apoptotic protein Bcl-2 is present when resistance towards chemotherapeutic drugs occurs (Ellis et al. 1998; Sartorius and Krammer 2002; Miyashita and Reed 1993; Dole et al. 1994) and expression changes in other anti- and pro-apoptotic family members were observed as well (Kitada et al. 1998; G.-Q. Wang et al. 2001; Ni Chonghaile et al. 2011). However, whereas overexpression of MCL-1 can be connected to poor patient survival after chemotherapy, there have been studies that could not establish the link between overexpression of Bcl-2 and poor treatment outcome (Kitada et al. 1998; Bosari et al. 1995). Nevertheless, targeting mechanisms that lead to inhibition of apoptosis
are showing promising results. For example, preclinical data demonstrate the effectiveness of navitoclax, a drug that promotes pro-apoptotic and hinders anti-apoptotic signaling, when given as a single agent or in combination with chemotherapy (van Delft et al. 2006; Oltersdorf et al. 2005). However, overexpression of MCL-1 overwrites the cytotoxicity of this drug (Konopleva et al. 2006). Therapy that induces apoptosis by activating caspase-8 in combination with chemotherapy has also shown promising results (Soria et al. 2010). Even though not all changes in apoptotic signals are connected to drug resistance, research shows that some are, meaning that avoiding apoptosis is not only a cancer hallmark but also a hallmark of drug resistance.

6.1.5 Resistance mediated by autophagy

Autophagy has an important role in intracellular homeostasis as it is responsible for the recycling of unnecessary, damaged or aged cellular components by lysosomal degradation. The removal of a cell's damaged organelles, toxic proteins or oncogenic substrates reflects the tumor suppressive character of autophagy, whereas intracellular recycling provides food and energy for a cell and is tumor-promoting (White 2012). Even though autophagy has been shown to promote chemosensitization (Eum and Lee 2011; Zou et al. 2011) the latter effect may facilitate drug resistance as it enables a tumor to respond to environmental stress by increasing the breakdown of unnecessary proteins and organelles. Targeting the p210BCR-ABL expressing CML cells with imatinib can also induce autophagy. By inhibiting the autophagous process with chloroquine an increase of imatinib-induced cell death has been observed (Bellodi et al. 2009). Similar effects have been observed with the EGFR inhibitors gefitinib and erlotinib in human lung cancer cells (Han et al. 2011), in glioblastoma multiforme where chloroquine was administered as an additional op-
tional adjuvant therapy (Briceño, Calderon, and Sotelo 2007), and in colorectal cancer that was treated with the standard chemotherapeutic 5-fluorouracil and 3-methyladenine, which is another autophagy inhibitor (Jie Li et al. 2010; Sasaki et al. 2012).

6.1.6 **Adaption of signaling**
Cancer cells can also activate pro-survival signaling. For example, the EGFR signaling cascade is activated to prevent chemotherapeutic induced toxicity (Sumitomo et al. 2004; Van Schaeybroeck et al. 2006, 2005). Even when EGFR is targeted cells can reprogram their kinome and bypass the oncogene by activating HER3 (Sergina et al. 2007; Wheeler et al. 2008). In addition, amplification of \( \text{MET} \) has been observed to be the reason for drug resistance in 20% of EGFR-driven lung cancers. \( \text{MET} \) bypasses the drug blocked EGFR by inducing PI3K signaling via HER3 (Engelman et al. 2007).

6.1.7 **Epithelial-mesenchymal transition (EMT)**
During EMT, epithelial cells lose their polarization and cell-cell adhesion and gain the more migratory phenotype of mesenchymal cells. Several factors such as cytokines, chemokines, integrins, cadherins, and metalloproteinases are involved in this phenotype change, which has been undoubtedly linked to drug resistance (Singh and Settleman 2010). To name a few examples, overexpression of Nanog, a transcription factor linked to EMT, leads to cisplatin resistance in ovarian cancer cells (Liu et al. 2016). An upregulation of TGF-β signaling induces EMT in colon and triple-negative breast cancer cells, which leads to doxorubicin resistance (W.-C. Chen et al. 2013; Jinpeng Li et al. 2015). Also, EMT has been observed in patients with non-small-cell lung carcinoma (NSCLC) treated with EGFR-inhibitors (Uramoto et al. 2011; Sequist et al. 2011).
6.1.8 **Tumor microenvironment**

Not only can the tumor microenvironment contribute to tumor progression, invasion, and metastasis, it can also promote drug resistance. The tumor microenvironment comprises the extracellular matrix (ECM), blood and/or lymph vessels, cells associated to the immune system as well as inflammation, and cancer-associated fibroblasts (Bissell and Radisky 2001; Tlsty and Coussens 2006).

The heterodimeric integrin receptors are major connectors of cells to the ECM. These interactions between integrins and ECM can render different signaling pathways, such as the NF-κB and PI3K pathways, active (Danen 2005). High levels of β1-integrins have been associated with herceptin resistance in HER2-positive breast cancer (Lesniak et al. 2009). In a cell line model of oral carcinoma, the adhesion of β1-integrin even leads to cancer proliferation induced by chemotherapeutic treatment with cisplatin (Eberle et al. 2011).

Furthermore, soluble factors released by the tumor itself or its microenvironment have been shown to influence the outcome of drug treatment. Interleukin-6 (IL-6), for example, confers multidrug resistance in breast cancer cells lines (Conze et al. 2001) or cisplatin resistance in ovarian cancer (Cohen et al. 2013). Other soluble factors such as fibroblast growth factor (FGF), neuregulin (NRG1), hepatocyte growth factor (HGF) and epidermal growth factor (EGF) to name just a few, have also been identified to play a role in drug resistance (Wilson et al. 2012).

Some of these soluble factors are released by another important component of the tumor microenvironment, the cancer-associated fibroblasts (CAFs). HGF secretion by CAFs leads to resistance towards RAF inhibition in BRAF-mutant melanoma (Straussman et al. 2012). In addition, CAFs secrete platelet-derived growth factor C
and by this means induce resistance towards the inhibition of VEGF-mediated angiogenesis (Crawford et al. 2009). Matrix metalloproteinase secretion leads to cetuximab resistance in head and neck squamous cell carcinoma cell lines when co-cultured with CAFs (Johansson et al. 2012). Further, CAFs promote gemcitabine resistance in pancreatic cancer by stimulating proliferation and survival pathways (Hwang et al. 2008).

The tumor vasculature is abnormal and defective compared to the healthy tissue, leading to improper penetration of drugs, but also to tissue areas that are nutrient-deprived and hypoxic (Tong et al. 2004). Hypoxic conditions induce HIF-1 transcriptions factor (consisting of HIF-1α and HIF-1β, G. L. Wang et al. 1995), whose target gene is $MDR1$ among others (see 5.1.1 Drug uptake, efflux, and metabolism). Therefore, it does not come as a surprise that HIF-1α overexpression has been observed in plenty of drug-resistant cancer cell lines (Comerford et al. 2002; Nardinocchi et al. 2009; Zhu et al. 2005) as well as human colon and bladder cancer samples (Ding et al. 2010; Sun et al. 2016). In addition, other resistance mechanisms such as avoiding apoptosis (Chapter 5.1.4) and induction of autophagy (Chapter 5.1.5) can involve HIF-1 (Rohwer and Cramer 2011).

6.1.9 Extracellular vesicles

Extracellular vesicles (EVs) facilitate the communication between cells and can thereby influence the recipient cell's function. EVs can simultaneously deliver all the different biomolecule categories ranging from proteins to lipids, nucleic acids, and sugars (Yáñez-Mó et al. 2015). Therefore, it is not surprising that EVs take part in the communication between the tumor and its surrounding environment (Milane et al. 2015) and are able to mediate drug resistance between
stromal and tumor cells. Examples such as the 5-fluorouracil resistance in gastric cancer cells (Ji et al. 2015) or bortezomib resistance in multiple myeloma cells (J. Wang et al. 2014) have been reported.

In addition, EVs are able to mediate the transfer of resistance from a drug-resistant cell to a drug-sensitive cell. The best example is the transfer of ABCB1 (P-glycoprotein) and even its protein-coding mRNA _MDR1_ (see 5.1.1 Drug uptake, efflux, and metabolism) in prostate (Corcoran et al. 2012), leukemia (Bebawy et al. 2009), ovarian (F. Zhang et al. 2014), and breast cancer models (Pasquier et al. 2012; Dong et al. 2014). Further, in leukemia, the MRP-1 transporter (see 5.1.1 Drug uptake, efflux, and metabolism) has been transferred from resistant to drug-sensitive cells (Lu et al. 2013). Not only can EVs transfer drug efflux transporters, they can also sequestrate drugs. The ABCG2 transporter, for example, localizes to vesicles and the drug is then taken up into the EVs and released from the cell (Ifergan, Scheffer, and Assaraf 2005).

Other molecules that are transferred by EVs and have been associated with promoting drug resistance and sensitivity are non-coding RNAs such as miRNAs (19–25 nucleotides long) and lncRNAs (not translated long RNA-transcripts). miRNAs from docetaxel-resistant breast cancer cells target pathways related to drug therapy failure and convey docetaxel resistance in a previous drug-sensitive cell line (W.-X. Chen et al. 2014). The same has been observed with gemcitabine-resistant pancreatic cancer (Mikamori et al. 2017) or cisplatin-resistant ovarian cancer (R. C. Pink et al. 2015) as well as lung cancer cell lines (Wu et al. 2017). Even though drug resistance mediated by long non-coding RNAs is a newly emerging research field, several examples of how vesicles transfer lncRNAs exist. LincRNA-ROR induces sorafenib or doxorubicin resistance and linc-VLDLR induces the ABCG2 drug transporter (see above) in hepatocellular car-

It is obvious that drug resistance is a big complex research field as there is not just one drug resistance mechanism. It is clear that cancer cells can activate multiple mechanisms at the same time to avoid drug-induced cancer cell death. Furthermore, one drug can be inactivated by different resistance mechanisms as seen with cisplatin, gemcitabine, imatinib, and 5-fluorouracil (see above). In addition, researchers continuously discover new mechanisms.

6.2 Resistance to Tamoxifen

Even 15 years post-treatment, tamoxifen has been shown to reduce the death due to breast cancer by 30%, compared to patients that did not receive tamoxifen over a five-year period. However, the cancer recurrence rate of 15% five years after diagnosis of early-stage ER-positive breast cancer has risen to 25% after ten years (Early Breast Cancer Trialists’ Collaborative Group (EBCTCG) 2005). Almost all patients with metastatic breast cancer treated additionally with tamoxifen eventually relapse. Various examples of tamoxifen resistance have been proposed (Table 2), and more are to be discovered.

<table>
<thead>
<tr>
<th>Factors related to</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>Loss of ER; ESR1 mutations; ER regulator modifications</td>
</tr>
<tr>
<td>Growth and survival</td>
<td>Cell cycle regulation; deregulation of apoptosis; autophagy; cancer cell dormancy</td>
</tr>
<tr>
<td>Modification of signaling pathways</td>
<td>EGFR/HER2 signaling; AR signaling; PI3K/mTOR signaling</td>
</tr>
</tbody>
</table>
Factors related to | Examples
---|---
Drug metabolism | Decreased uptake; increased drug efflux/sequestration; metabolism of tamoxifen
Tumor microenvironment | CAFs; tumor stroma; extra cellular matrix; exosomes; immunomodulation

The most obvious mechanisms are related to the estrogen receptor, tamoxifen's primary target. Mutations in *ESR1* have been reported: for example, a K303R gain-of-function mutation was detected in a hinge region before the LB domain in hyperplastic lesions (Fuqua et al. 2000). Cell line studies showed that if overexpressed and a crosstalk with growth receptor pathways exists, this mutation can lead to estrogen hypersensitivity and thus cells are less sensitive to anti-estrogen treatment (Barone et al. 2010). However, several studies failed to detect this mutation in primary and metastatic tumors (Davies et al. 2005; Cancer Genome Atlas Network 2012; D. R. Robinson et al. 2013; S. Li et al. 2013; Toy et al. 2013; Merenbakh-Lamin et al. 2013; Jeselsohn et al. 2014). Less controversial is the detection of mutations in the LBD of *ESR1*, which have been reported in studies investigating metastatic ER-positive breast cancers. The LBD *ESR1* mutations were detected in 14%-54% of metastatic ER+ tumor patients who received hormonal therapy including tamoxifen. The amino acid residues Y537 and D538 were most frequently mutated. Further cell line studies showed that these mutations lead to a ligand-independent activity as well as enhanced ligand-stimulated activity, resulting in decreased tamoxifen sensitivity (S. Li et al. 2013; D. R. Robinson et al. 2013; Toy et al. 2013; Merenbakh-Lamin et al. 2013; Jeselsohn et al. 2014). In addition, *ESR1* amplification, as well as fusion genes, have been detected in patients. However, their role in tamoxifen resistance is still unclear (Jeselsohn et al. 2015).
Loss of ERα expression has been observed under tamoxifen treatment (Johnston et al. 1995). Events such as CpG island methylation and histone deacetylation (Parl 2003), hypoxia (Stoner et al. 2002), EGFR and HER2 overexpression (Stoica et al. 2000; Creighton et al. 2006) as well as the interaction of p53 with ERα (Angeloni et al. 2004) have been proposed to be responsible for ERα downregulation. However, roughly only 20% of patients with acquired tamoxifen resistance lose ERα expression (Johnston et al. 1995; Kuukasjärvi et al. 1996) indicating other means of tamoxifen resistance are at play. Further, different expression levels of ERα co-regulatory proteins have been linked to tamoxifen resistance. Well-studied examples include AIB1 (SRC3), SRC-1, NcoR and SMRT (Osborne et al. 2003; Redmond et al. 2009; Girault et al. 2003; L. Zhang et al. 2013).

As mentioned above, administered tamoxifen is a prodrug that needs to be metabolized in the liver to more potent metabolites, mainly endoxifen and 4-hydroxytamoxifen. One of the enzymes participating in the conversion is cytochrome P450 2D6 (CYP2D6) (Klein et al. 2013). Polymorphisms in this enzyme have been linked to a higher breast cancer recurrence rate during tamoxifen only treatment (Province et al. 2014). In addition, it has been observed that endoxifen is a substrate of ABC-transporter ABCB1 (Teft, Mansell, and Kim 2011) and a subsequent upregulation of the MDR1 gene could lead to tamoxifen resistance.

Probably the main mechanism by which tumor cells circumvent tamoxifen-induced death is the activation of alternative pathways and factors. Especially HER2, EGFR, and IGFR play an important role, but also the PI3K/AKT/mTOR signaling pathway, which is essential for metabolism, cell survival and growth (García-Becerrra et al. 2012). Results from the phase II TAMRAD trial or the phase III trial BOLERO2 indicate that patients could benefit from additional
mTOR1 inhibition with everolimus. Nevertheless, these studies also report a higher proportion of discontinuations due to adverse side effects (Piccart et al. 2014; Treilleux et al. 2015). Further, the complexity of the PI3K/AKT/mTOR pathway harbors the potential of developing drug resistance to the single inhibitors (Bihani et al. 2015; Choi et al. 2016). As the targeting of this pathway is currently an actively studied field, time will tell whether this approach will lead to a blockage of tumor progression.

As growth factors are known to induce EMT (Kalluri and Weinberg 2009), the upregulation of their receptors can be indirectly linked to drug resistance. An upregulation of HER2 inhibits E-cadherin leading to a reduced adherence of cells and then to EMT (D’souza and Taylor-Papadimitriou 1994; Ingthorsson et al. 2016). Further, other EMT promoting factors such as β-catenin, SLUG and SNAIL, Notch and Wnt signaling have been associated with tamoxifen resistance (Hiscox et al. 2006; Ye et al. 2010; Dhasarathy, Kajita, and Wade 2007; Bui et al. 2017; Loh et al. 2013).

Immune cells within the tumor microenvironment have been known to release growth factors that can lead to remodeling of ECM (see above). Moreover, predictive gene-profiling of patients who were to receive tamoxifen treatment linked an inflammation-related gene cluster to therapy failure (Loi et al. 2008). In addition, cancer-associated fibroblasts, as well as their release of ILβ1, are associated with tamoxifen resistance (Martinez-Outschoorn et al. 2011; Pontiggia et al. 2012; Jiménez-Garduño et al. 2017).

Proteins involved in cell cycle regulation have also been detected to impact tamoxifen resistance. Overexpression of ERα's direct target cyclin D1, which promotes the progression through G1 to S phase, has been linked to tamoxifen resistance in cell lines (Wilcksen et al. 1997) and correlates with poor treatment outcome (Stendahl et al.
Also, cyclin E has been associated with tamoxifen resistance (Dhillon and Mudryj 2002; Hui et al. 2002). The role of apoptosis avoidance in tamoxifen resistance is highly debatable, as changes in pro and antiapoptotic genes can be merely due to activation of other resistance pathways (Butt, Sutherland, and Musgrove 2007). However, increased expression of GRP78, a protein that balances prodeath apoptosis and prosurvival autophagy, has been reported to promote tamoxifen resistance (Cook et al. 2012). Further, hydroxychloroquine, an autophagy inhibitor has been shown to restore tamoxifen sensitivity (Cook et al. 2014).

In addition, it has been shown that miRNAs play a role in tamoxifen resistance development and can function as a predictive marker (Xin et al. 2009; Lyng et al. 2012; Huber-Keener et al. 2012). Interestingly, miR-221/222, a well studied miRNA conveying tamoxifen resistance (reviewed in Alamolhodaei et al. 2016), can be transferred via exosomes to other cells and transform a tamoxifen sensitive cell to a resistant one (Wei et al. 2014). The same phenomenon has been observed for lncRNA UCA1 (Xu et al. 2016, 1).

As these examples show, it becomes more and more evident that a cancer cell has a plethora of resistance mechanism towards tamoxifen at hand to prevent cell death. There is a need to define biomarkers for each resistance mechanism and multiple therapeutic options to combat them.
AIM OF THE STUDY

Several therapies, such as surgery, radiation, chemo-, targeted, and hormonal therapy, are used to treat breast cancer. Survival rates have been improving as a result of therapies and earlier diagnoses. However, cancer can develop drug resistances often leading to therapy failure. Development of drug resistance and tumor progression are believed to be caused by a variety of mechanisms. The heterogeneity of resistance mechanisms most likely reflects the molecular subtypes and intrinsic molecular properties of individual breast cancers.

Therefore, the aims of my PhD work was to utilize breast cancer cell line models to explore molecular pathogeneses and resistance mechanisms of breast cancer:

• Identify fusion genes in breast cancer, which could potentially open new therapeutic approaches.

• Establish the molecular basis of tamoxifen resistance in breast cancer by integrating exome-sequencing and drug profiling using newly generated isogenic drug-resistant variants of ER-positive breast cancers.

• Characterize the transcriptome of the tamoxifen-resistant cell lines and define pathways as well as biomarkers of drug resistance and identify ways to overcome resistance mechanisms.
MATERIALS AND METHODS

In this chapter, materials and methods used in this thesis are briefly portrayed. A detailed description can be found in the original publications, referred to by Roman numbers (I, II and III).

1 Cell culture and generation of tamoxifen-resistant cell lines (I, II, III)

BT-474 (HTB-20), MCF-7 (HTB-22), T-47D (HTB-133) and ZR-75-1 (CRL-1500) cells were obtained from American Type Culture Collection (Table 3). KPL-4 was a gift from Dr. Junichi Kurebayashi, D (Tabuchi et al. 2009).

Table 3: Cell lines from human mammary gland

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
<th>Used in</th>
</tr>
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<tbody>
<tr>
<td>BT-474</td>
<td>Ductal carcinoma</td>
<td>I, II, III</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Adenocarcinoma derived by pleural effusion</td>
<td>I, II, III</td>
</tr>
<tr>
<td>KPL-4</td>
<td>Inflammatory breast carcinoma derived by pleural effusion</td>
<td>I</td>
</tr>
<tr>
<td>T-47D</td>
<td>Ductal carcinoma derived by pleural effusion</td>
<td>II, III</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>Ductal carcinoma derived from ascites</td>
<td>II, III</td>
</tr>
</tbody>
</table>

BT-474, MCF-7 and KPL-4 cells were grown in DMEM (PAN Biotech/Euro Clone) supplemented with 10% FCS (Gibco), 1% penicillin/streptomycin (Gibco) and 0,1% bovine insulin (Sigma) except KPL-4. ZR-75-1 and T-47D were grown in RPMI-1640 (PAN Biotech) supplemented with 10% FCS (Gibco, Life Technologies, Carlsbad, CA), 1% penicillin/streptomycin (Gibco) and 0,1% bovine insulin (Sigma) for T-47D cell line.

Due to continuous exposure to 1 µM 4-OH-tamoxifen (Sigma) over a time frame of 8-12 months seven tamoxifen-resistant cell lines (MCF-
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7 Tam1, T-47D Tam1 & Tam2, ZR-75-1 Tam1 & Tam2, BT-474 Tam1 & Tam2) were derived from the parental cell lines (Figure 4). The culture media needed to be replaced every 2–3 days. Cells were passaged when about 80% confluent and incubated at 37 °C with 5% CO₂. As the cell lines were grown in the presence of estrogen (FCS and phenol), our studies mimic tamoxifen resistance in premenopausal woman.

![Figure 4: Schematic overview of tamoxifen-resistant cell lines. Tamoxifen resistant cell lines (color) were generated from parental cell line (no color) by exposure to 1µM 4-OH-tamoxifen (pills) for 8-12 months.](image)

2 Characterization of tamoxifen-resistant cell lines (II, III)

2.1 Growth analysis (II)

To measure the viability of the tamoxifen-resistant cells in study II, CellTiter-Glo Cell Viability Assay (Promega) was performed with increasing tamoxifen concentrations (0-1.8 µM). After 120 h of incubation luminescence was measured with the PHERAstar plate reader (Agilent Technologies). The active DNA synthesis and cell proliferation were measured with the Click-iT® EdU Alexa Fluor® 488 Flow
Cytometry Assay Kit (Life Technologies). The EdU Alexa Fluor® 488 incubation time was 4 h for T-47D and MCF-7 and 28 h for the BT-474 and ZR-75-1 cells. The permeabilization of the ZR-75-1 cells had to be adjusted to 0.1% TritonX-100-PBS. After Ribonuclease A treatment, the DNA content was detected using the FxCycle™ Far Red (Life Technologies). The stained cells in suspension were analyzed using Accuri C6 flow cytometer and its software (BD Biosciences).

2.2 **Responsiveness to estrogens and downstream signaling (II)**

The response to estrogen was measured by depriving all the cell lines of estrogen. Therefore, phenol red-free medium (PAN Biotech) was supplemented with 2.5% dextran–charcoal-treated FCS (Sigma-Aldrich). After 72 h, 17β-estradiol (Sigma, 10^{-8} M in ethanol) was added for 4, 8 or 24 h. RNA was isolated and converted into cDNA. Quantitative-PCR with the DyNAmo ColorFlash SYBRGreen PCR kit (Thermo Scientific) on the LightCycler 480 system (Roche) was performed, to detect changes in *pS2*, *PGR*, and *GREB1*, the direct target genes of ERα. Primer sequences can be found in Additional file 1 of the original publication (Kangaspeska et al. 2016).

2.3 **Measurements and drug induced changes in protein levels (II, III)**

Changes in ERα in all the cell lines (Study II) and cathepsin D in the T-47D cells (Study III) were investigated by Western blotting as described earlier (Östling et al. 2011). B-actin/-tubulin were used as reference proteins. The effects of VX-11E (ERK inhibitor) and selumetinib (MEK inhibitor) on several proteins were assessed with increasing concentrations of VX-11E (50 nM, 100 nM, and 250 nM) and with 100 nM VX-11E combined with 1 μM selumetinib. Information about all antibodies used for Western blotting is in Table 4.
Table 4: Primary antibodies used for Western blotting (WB) and immunofluorescence (IF)

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Product number and vendor</th>
<th>Application</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>ab16660, Abcam</td>
<td>WB</td>
<td>II</td>
</tr>
<tr>
<td>EGFR</td>
<td>CST4267, Cell Signaling Technologies (CST)</td>
<td>WB</td>
<td>II</td>
</tr>
<tr>
<td>pEGFR</td>
<td>CST3777, CST</td>
<td>WB</td>
<td>II</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>CST9207, CST</td>
<td>WB</td>
<td>II</td>
</tr>
<tr>
<td>pERK1/2</td>
<td>CST4370, CST</td>
<td>WB</td>
<td>II</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>610800, BD Transduction Laboratories</td>
<td>WB</td>
<td>III</td>
</tr>
<tr>
<td>β-actin</td>
<td>A1978, Sigma</td>
<td>WB</td>
<td>II, III</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>ab6046, Abcam</td>
<td>WB</td>
<td>III</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>ab110449, Abcam</td>
<td></td>
<td>III</td>
</tr>
<tr>
<td>Lamp 1</td>
<td>H4A3, Developmental studies hybridoma bank (DSHB)</td>
<td>IF</td>
<td>III</td>
</tr>
<tr>
<td>Lamp 2</td>
<td>H4B4, DSHB</td>
<td>IF</td>
<td>III</td>
</tr>
<tr>
<td>Galectin-3</td>
<td>556904, BD Biosciences</td>
<td>IF</td>
<td>III</td>
</tr>
<tr>
<td>Filipin</td>
<td>F9765, Sigma</td>
<td>IF</td>
<td>III</td>
</tr>
</tbody>
</table>

2.4 Immunofluorescence staining (III)

The parental and resistant T-47D cells were seeded on coverslips with and without 1 µM 4-OH tamoxifen. Cells were fixed and stained either directly with filipin for free cholesterol, LipidTOXGreen neutral lipid stain, or primary antibodies (Table 4). Nuclei were detected with DRAQ5 (Biostatus) or Hoechst. Images were acquired with a Nikon 90i microscope (Nikon) using the 40x objective and processed with ImageJ (Schneider, Rasband, and Eliceiri 2012) and the Adobe Photoshop software.
2.5 **Lysosomal membrane permeabilization (LMP) assay (III)**

Galectin-3 translocation in the parental and resistant T-47D cells was measured to investigate the lysosomal membrane integrity as described earlier (Aits, Jäättelä, and Nylandsted 2015). The cells were grown for 72 h +/- 1 µM 4-OH-tamoxifen and then incubated with 1 mM LLMOe to induce LMP. Cells were fixed and stained with galectin-3, ceruloplasmin as cell segmentation marker (Table 4) and Hoechst (405nm) to detect the nuclei. Confocal images were taken with the PE Opera Phenix HCS system (PerkinElmer) with the 40x water immersion objective (NA 1.1). Images were analyzed with the Columbus Image Data Storage and Analysis System (PerkinElmer). LMP was assessed by calculating the average spots per cell and the percentage of galectin-3 positive cells within an image.

2.6 **Measurement of triglycerides and cholesterol esters (III)**

Triglycerides and cholesterol esters were extracted from the parental and resistant T-47D cells and measured by thin layer chromatography (TLC) as previously described (Bligh and Dyer 1959). The lipid bands were quantified using ImageJ (Schneider, Rasband, and Eliceiri 2012).

3 **Paired-end RNA-sequencing (I, III)**

For study I, data from our previously produced paired-end RNA libraries of the BT-474, MCF-7, and KPL-4 cell lines were used (Edgren et al. 2011).

For study III, after RNA isolation with miRNeasy kit (Qiagen),
RNA quality control with the Agilent Bioanalyzer using the RNApico chip (Agilent) and determination of RNA amount with Qubit RNA-kit (Life Technologies), a strand-specific paired-end RNA-sequencing library was prepared with ScriptSeq™ Complete kit for human/mouse/rat (Illumina). The mean fragment size was ca 300-400 nucleotides, as fragments smaller than 200 bp were removed by SPRI beads (Agencourt AMPure XP). The paired-end library was sequenced with the Illumina HiSeq 2000 (Illumina) instrument. A detailed description can be found in Kumar et al. (Kumar et al. 2017).

4 RNA-sequencing analysis (I, III)

4.1 Fusion gene detection, characterization, and validation (I, III)

Fusion genes were detected in study I and III with the FusionCatcher algorithm using raw, unprocessed read files (Edgren et al. 2011; Nicorici et al. 2014). Ensembl version 61 (Study I) and Ensembl version 80 (Study III) were used for sequence alignment to the human genome. A database containing exon-exon fusion points and the junction sequences, as well as information about both fusion partners, was generated. A fusion partner was allowed to have multiple partner genes.

Fusion genes were further characterized and validated in study I. Protein domains were annotated using SMART (Schultz et al. 1998; Letunic, Doerks, and Bork 2012) and the 1M oligo Agilent aCGH data (Edgren et al. 2011) was used to determine copy number changes and gene coverage. Fusion genes were validated by Sanger-sequencing. Shortly, isolated RNA was reverse transcribed into cDNA. With fusion gene-specific primers, the region of interest was ampli-
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fied and the gel-purified PCR-product was cloned into pCR2.1-TOPO vector (Invitrogen). The vector was transferred into bacteria and colony PCR was used to confirm a successful transfection. After plasmid purification, the product was Sanger-sequenced and the obtained sequences were compared with the predicted fusion sequence, using the multiple sequence alignment tool (Corpet 1988). The chromosomal breakpoints of the fusion genes were determined by Sanger-sequencing of the genomic DNA of the BT-474 cell line. Primers for amplification of the region around the fusion breakpoint were designed based on already existing exome-sequencing data. The PCR product was directly Sanger-sequenced and the exact chromosomal breakpoints were located using BLAT (Kent 2002) with the “Near-exact matches” option. The primers of this study can be found in Table S4 of the original publication (Kangaspeska et al. 2012).

4.2 Integration of transcriptomic data and differential gene expression (III)

Public patient transcriptome data (McBryan et al. 2015) was downloaded from GEO database (Barrett et al. 2013). RNA-sequencing data analysis, which contained read pre-processing, read alignment to the human reference genome (Ensembl GRCh38) guided by the Ensembl reference gene models and features (Ensembl v80), and count estimation are described in detail in Kumar et al. (Kumar et al. 2017). The estimated expression counts of cell line and patient data were combined, normalized using the trimmed mean of M-values (TMM) method (M. D. Robinson and Oshlack 2010), converted to counts per million (CPM) estimates using edgeR (M. D. Robinson, McCarthy, and Smyth 2010), corrected for the batch effect associated with the study origin using limma (Smyth and Speed 2003), and assigned
to Ensembl information using biomaRt (Smedley et al. 2015) with default settings. Lists of differentially expressed genes, defined as log2 ratio of $\geq 1$ and CPM difference of $\geq 10$ when comparing parental versus resistant cell line was generated and uploaded to Enricher (E. Y. Chen et al. 2013; Kuleshov et al. 2016) to determine pathways involved in tamoxifen resistance development. The data was visualized using principle component analysis, Venn diagrams as well as heat maps.

5 Genomic profiling by exome-sequencing (II)

We utilized exome-sequencing to detect point mutations or copy number changes that arose during the development of tamoxifen resistance by comparing parental with the isogenic resistant cell line. The exomes were captured from genomic DNA with NimbleGen SeqCap EZ Human Exome v2.0 kit (Roche NimbleGen) and paired-end sequenced on the Illumina HiSeq platform. The detection of the point mutations has been previously described (Pemovska et al. 2013). The parental cell lines were used as controls and Ensembl v66 was used as annotation database. We excluded known false positive point mutations and considered mutations with $p < 0.05$ and resistant variant frequency $>30\%$ with high confidence as present. Copy number variants (CNVs) were detected with a variant calling pipeline (Su-lonen et al. 2011). Copy number values were calculated as RPKM (reads per kilobase length per million mapped reads) for exome target regions and the log2 copy number ratios determined by using the parental cell line as a reference. The values were then assigned to the human genome based on Ensembl database v67 using the extreme method option in GISTIC2 (Mermel et al. 2011). Thresholds to identify CNVs were set at -0.4 (heterozygous deletion), -1.2 (homozy-
gous deletion), +0,5 (gain) and +1,3 (amplification).

6 **Drug sensitivity and resistance testing (DSRT) (II, III)**

6.1 **Cell viability measurement (II, III)**

The DSRT platform has been described in detail before (Pemovska et al. 2013). For study II we used the FIMM FO2Baq library, which contains 279 investigational and approved cancer drugs, on all the parental and resistant cell lines. For study III we used 33 drugs targeting the lipid and cholesterol metabolism, the lysosomal membrane, and oxidation processes, in addition to drugs that have been part of study II (Kangaspeska et al. 2016). We tested the 33 drugs with the T-47D parental and resistant cell lines. In both studies, negative (0,1 % DMSO only) as well as positive (100 μmol/l benzethonium chloride) controls were included. Five different concentrations of one drug covered a 10000-fold concentration range. The optimal amount of cells was grown in the cell's normal media, and supplemented with 1 μM 4-OH-tamoxifen. The incubation time was 72 h at 37 °C. CellTiter-Glo Cell Viability Assay (Promega) was used to measure the viability with the PHERAstar plate reader.

6.2 **High-content phenotypic drug profiling (III)**

In study III we additionally fixed plates from the DSRT with the 33 drugs with 4% PFA in PBS for image-based phenotyping. We stained the plates with LipidTOXGreen neutral lipid stain (ThermoFisher Scientific) and Hoechst for nuclei detection. Confocal images were acquired with the PE Opera Phenix HCS system (PerkinElmer) with the 40x water immersion objective (NA
1.1. Laser power and exposure time were unchanged during the imaging of the different plates with the same staining.

7 DSRT data analysis (II, III)

7.1 Analysis of cell viability (II, III)
The raw ATP-measurements were analyzed with our in-house bioinformatic pipeline Breeze, which gives dose response curves, IC50 values as well as a Drug Sensitivity Score (DSS) for each individual drug treatment (Yadav et al. 2014). DSS scores (Study III) and DSS differences between parental and resistant cell lines (Study II) were visualized as a heat map. Rank product analysis (Breitling et al. 2004) was used to identify drugs that significantly changed their efficacy while tamoxifen resistance is obtained (Study II).

7.2 Construction of drug sensitivity and co-resistance networks (II)
In study II, drug sensitivity and resistance networks were constructed based on drugs that showed a DSS difference of at least five when comparing resistant with parental cell line DSS. Drug targets were extracted from the ChEMBL database (https://www.ebi.ac.uk/chembl) and assigned a Kinase Inhibitor BioActivity (KIBA)-score (Tang et al. 2014). Based on the KIBA-score, point mutations, copy number changes and canonical pathway for Estrogen Receptor Signaling (www.qiagen.com) we used the Ingenuity Pathway Analysis application (Ingenuity® Systems, Qiagen) (Krämer et al. 2014) to construct sensitivity and co-resistance networks.
7.3 **Analysis of high-content phenotypic drug profiling (III)**

Images were utilized to measure LipidTOXGreen signal and calculate a DSS based on cell counts. Images were flatfield corrected using CIDRE method (Smith et al. 2015), stitched to a single multi-channel image per well, as well as segmented and analyzed with CellProfiler 2.2.0 (Kamentsky et al. 2011). The LipidTOXGreen signal was measured for each individual cell and a mean of the intensities per well calculated. Dose-response curves were drawn for the cell counts and used to calculate a DSS for each drug in Breeze.
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1 Fusion genes
In study I we reanalyzed our already published RNA-sequencing data (Edgren et al. 2011) by changing parameters such as the annotation version of Ensembl to map the reads to the human genome, allowing a fusion partner to be fused to more than one gene and having as little as two reads to indicate the presence of a fusion gene. The re-analysis predicted 16 additional fusion genes, which we validated and characterized. Additionally, in study III we evaluated the changes of fusion genes after tamoxifen resistance had developed.

1.1 Fusion gene characteristics in breast cancer
Out of the 16 newly predicted fusion genes, we were able to Sanger-sequence 14 (Study I, Table 1 and S1). Our previously published and validated 27 breast cancer fusion genes (Edgren et al. 2011), and the results of others from solid tumors, show that fusion genes are not just an event prevalent to hematological cancers. Indeed, in 2015, 9189 fusion genes have been found in solid tumors compared to 759 fusion genes in hematological diseases. However, only around 2 % were found to be recurrent in solid tumors, compared to about 20 % in hematological diseases (Mertens et al. 2015). The fusion genes we identified were not recurrent. Nevertheless, one of the fusion partners, for example, MED1 was found to be fused to different genes (STXBP4, USP32, and ACSF2) in our study (Study I, Figure 3). Other studies, for example, identified genes of the MAST- and NOTCH-gene families to be fused to different partners in 4 or 6 % of breast cancer patients, respectively (D. R. Robinson et al. 2011). The clinical phase I trials with MK-0752, a γ-secretase inhibitor, which prevents
NOTCH activation, have shown promising results in advanced breast cancer (Schott et al. 2013) and solid tumor patients (Krop et al. 2012). However, those studies were not selective for the NOTCH-fusion genes and an evaluation of the fusion genes as predictive markers or even therapy targets would be necessary. Even though we did not detect NOTCH gene fusions, results like these show that a disease-driving fusion partner could be targeted by therapy. Indeed, FDA-approved drugs are already in use to treat NSCLC harboring ALK fusions and myeloproliferative neoplasms with PDGFR fusions (Mertens et al. 2015).

Alternative splicing events such as cis- (intrachromosomal) and trans-splicing (interchromosomal) or genomic rearrangements are underlying fusion gene formation. The majority of fusion genes are formed by genomic rearrangements due to genomic instability and are frequently found at high-level amplifications (Study I, Table 1 and Figure 2, Stephens et al. 2009; Edgren et al. 2011; Kalyana-Sundaram et al. 2012). Most of the studied fusion genes are at a genomic breakpoint. We were able to sequence the exact chromosomal breakpoints of three fusion genes (Study I, Figure 1b). The THRA-AC090627.1 and MED1-ACSF2 fusion shared sequence microhomology of 1 bp at the breakpoint (Study I, Figure 1b). It is therefore suggested that microhomology-mediated break-induced replication (Lawson et al. 2011) is the mechanism by which these two genes were joined.

We further identified the EEF1DP3-FRY fusion in the KPL-4 cell line as a read-through event (Study I, Table S1). A read-through fusion, also known as cis-transcription-induced gene fusion (cis-TIGF), occurs when a large pre-mRNA, which contains two neighboring genes, is transcribed and, due to splicing events, the two genes are fused together (Akiva et al. 2006). Around 5% of genes have been pro-
posed to form read-through fusions (Parra et al. 2006). However, the majority of read-through fusions have little or no cellular impact and may even be artifacts (Yu et al. 2014; He et al. 2018).

1.2 The role of fusion genes in tamoxifen resistance

In addition to the reanalysis of fusion genes in study I, we investigated the fusion genes in the tamoxifen-resistant, as well as their isogenic parental cell lines (Study III). At first sight it appeared that the cells acquire fusion genes during the resistance development as some fusion genes were not found in the parental cell lines (Study III, Additional file 6). After thorough investigation, all fusion genes annotated in the Fusion Catcher results file as “known_fusion” or “cell_lines” have been identified in the same parental cell line before (D. Kim and Salzberg 2011; Edgren et al. 2011; Kangaspeska et al. 2012; Kalyana-Sundaram et al. 2012; Klijn et al. 2015). The elimination of all previously reported fusion genes and read-through fusions, left only two acquired fusion genes (SMARCC1-EMCN in MCF-7 Tam1 and RAD9A-FUT10 in ZR-75-1 Tam1), which have not been detected in the parental cell line before. However, none are recurrent, suggesting that they do not play a major role in the development of tamoxifen resistance. We were able to detect two shared read-through fusions ABCC11-ABCC12 (BT-474 Tam1, T-47D Tam1 and ZR-75-1 Tam2) and TRIM3-HPX (T-47D Tam1 and ZR-75-1 Tam2, Study III, Additional file 6). The TRIM3-HPX has been detected in prostate adenocarcinoma (Nacu et al. 2011), indicating that it is neither breast cancer- and nor tamoxifen resistance-specific. ABCC11 and ABCC12 are highly homologous, which implies that this read-through event is an artifact due to the mapping of reads to similar genomes.
We were able to detect the \textit{ESR1-CCDC170} fusion in the MCF-7, as well as in the tamoxifen-resistant MCF-7 Tam1, but not in the ZR-75-1 as previously reported (Veeraraghavan et al. 2014) or in its isogenic tamoxifen-resistant clones (Study III, Additional file 6). The \textit{ESR1-CCDC170} fusion has been identified in about 4% of ER-positive breast tumors and tandem duplication has been proposed as the underlying mechanism for the fusion formation (Veeraraghavan et al. 2014; Holst 2016). \textit{ESR1} is downstream of \textit{CCDC170} and a fusion of the two genes should result in a \textit{CCDC170-ESR1} read-through fusion, which we detected in MCF-7, but not in MCF-7 Tam1 (Study III, Additional file 6). Whether the fusion contributes to endocrine resistance is highly questionable. \textit{ESR1} contributes only with its promoter region to form a fusion gene with \textit{CCDC170}'s coding regions and a still fully functional \textit{ESR1} gene encodes for ER\(\alpha\). Indeed, differential allele-specific expression (DASE) studies indicate that \textit{CCDC170} alone is responsible for the breast cancer susceptibility (Jiang et al. 2017), which has been earlier associated to the \textit{CCDC170/C6orf97-ESR1} locus (6q25.1) by genome-wide association studies (GWAS) (Turnbull et al. 2010; Dunning et al. 2016).

Nevertheless, true \textit{ESR1} fusion genes have been detected in ER-positive breast cancers. These include, \textit{ESR1-YAP1}, which lacks the ligand-binding domain (LBD/AF-2) and has therefore been shown to drive endocrine resistance by promoting estrogen-independent cell growth (S. Li et al. 2013).

2 \textit{Characterization of tamoxifen-resistant cell lines}

We generated seven tamoxifen-resistant cell lines out of four parental cell lines, by exposing them to 1 \(\mu\)M 4-OH-tamoxifen over a period of 8-12 months (Figure 4, Study II, Figure 1A). The selected
cell lines reflect the molecular subtypes which would benefit from endocrine-therapy. MCF-7, T-47D and ZR-75-1 were classified as luminal A and the HER2-positive BT-474 cells as the luminal B-subtype (Subik et al. 2010). The BT-474 cells functioned primarily as a control as HER2 overexpression is a known resistance mechanism to tamoxifen (García-Becerra et al. 2012). Before we obtained stable growing tamoxifen-resistant cells, each of the clones underwent at least one round of mass mortality, leaving just a few regrowing cells. Once regrown, we assessed their tolerance towards tamoxifen and found that the resistant cells were more tolerant, confirming that tamoxifen resistance has developed (Study II, Additional file 3A).

We further evaluated their growth, estrogen-responsiveness, genomic and transcriptional changes as well as their drug profiles.

2.1 Tamoxifen-resistant cells alter their cell cycle

Overall, we noticed during cell culturing that the resistant cells grew slower than the parental cells and increased their doubling time dramatically. Further, FACS analysis revealed that all the resistant cells accumulate in the G0/G1 phase (Study II, Additional file 3B). Studies with another anti-estrogen resistant as well as a tamoxifen-resistant model have also reported an increase in the doubling time and consequently G0/G1 accumulation of the cells (Fog, Christensen, and Lykkesfeldt 2005; H. S. Kim et al. 2015). Interestingly, the MCF-7 Tam1 cells accumulate less in G0/G1 (59 %), than the parental cells under tamoxifen treatment (68 %). When ZR-75-1 Tam1 cells are exposed to media without tamoxifen they dramatically halt in G0/G1 compared to tamoxifen treatment (86 % vs. 68 %), implying a tamoxifen addiction, which has been observed previously (van den Berg et al. 1989). Withdrawal response to tamoxifen has also been reported
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in breast cancer patients and can even lead to cancer remission (Howell et al. 1992; Canney et al. 1987). On the other hand, non-adherence to tamoxifen therapy is associated with a higher mortality (McCowan et al. 2008). These results show that there would be a need to define markers that could identify tamoxifen-addicted tumors and help to stratify patients for therapy.

Interestingly, RNA-seqencing and subsequent pathway analysis of differentially expressed genes showed that genes involved in cell cycle were especially enriched in MCF-7 Tam1 & ZR-75-1 Tam1 (Study III, Table 2, Additional file 8). The results indicate that altering the cell cycle properties is one way to overcome tamoxifen-induced cell death.

2.2 Changes in ERα protein-levels and estrogen-responsiveness

Western blotting revealed that ERα was decreased in the ZR-75-1- and T-47D-derived resistance models (Study II, Additional file 4C), which is in line with previous research (van den Berg et al. 1989; Graham et al. 1992). Acquired tamoxifen resistance has been associated with the loss of ERα expression (Oh et al. 2010) and epigenetic mechanisms such as DNA methylation and histone deacetylation have been proposed to underlay these changes (Sharma et al. 2005; Y. Zhou et al. 2007). By contrast, in MCF-7 Tam1 resistant cells, the levels of ERα remained almost unchanged, and in BT-474 resistant clones, an increase was observed (Study II, Additional file 4C). Therefore, depending on the molecular background, tamoxifen either renders ERα stable (Lykkesfeldt, Madsen, and Briand 1994; J. J. Pink and Jordan 1996; A. F. Leary et al. 2010) or promotes its destabilization via proteasome-mediated degradation (Marsaud et al. 2003). At the same time, we observed a decrease in ERα target gene transcrip-
tion \((pS2, PGR, GREB1)\) as well as impaired estradiol-responsivity in all resistant cell lines (Study II, Additional file 4A and 4B), suggesting that even if the ER\(\alpha\) is stabilized, its function may be impaired. Further, the loss of progesterone receptor frequently occurs upon acquiring anti-estrogen resistance (Lykkesfeldt, Madsen, and Briand 1994; Drury et al. 2011; Arnedos et al. 2014). Our results suggest that different resistant clones may exhibit either steroid-dependent or -independent growth and differ in their ER-mediated responses.

2.3 Genetic alterations in the genomes of tamoxifen-resistant cell lines

It has been reported that tamoxifen resistance is driven by genetic adaptations such as point mutations or copy number changes (Karnik et al. 1994; Borg et al. 1994; Dowsett et al. 2001; Moelans et al. 2010; Burandt et al. 2013; Lundgren et al. 2008; S. Li et al. 2013, 1; D. R. Robinson et al. 2013; Toy et al. 2013; Merenbakh-Lamin et al. 2013; Jeselsohn et al. 2015). We, therefore, performed exome-sequencing on all the cell lines and determined resistance-specific point mutations and copy number variations by comparing each tamoxifen-resistant cell line to its parental cell line. From around 250 to 350 acquired non-synonymous mutations per resistant cell, we compared the high confident ones and found that no acquired point mutation was shared across all the resistant cell lines (Study II, Additional file 8 and 9). Nevertheless, between the clones derived from the same parental cell line, we found some shared mutations, which involved \(TNS1, PTH2R,\) and \(NHLRC2\) in BT-474 Tam1 and Tam2 and \(TIMM23\) and \(RP11-368\ J21.2.1\) in ZR-75-1 Tam1 and Tam2 (Study II, Additional file 8 and 9). Copy number changes were predominantly large heterozygous deletions, affecting many of the loci across the genome, including chromosomes 7 and X. The ZR-75-1 Tam1 resis-
tant cell clone, on the other hand, acquired only copy number gains. Whereas T-47D Tam1 and Tam2 had some shared copy number alterations (mainly 7q), almost none were shared between the ZR-75-1 and BT-474 derived resistant clones (Study II, Additional file 8 and 10). Contrary to previous reports (Karnik et al. 1994; S. Li et al. 2013; Toy et al. 2013; D. R. Robinson et al. 2013; Merenbakh-Lamin et al. 2013; Jeselsohn et al. 2015), we observed no acquired loss or mutations of \textit{PTEN}, \textit{PIK3CA} or \textit{ESR1} in any of the drug-resistant variants. However, unlike with \textit{ESR1} mutations, the causal relationship between \textit{PTEN} and \textit{PIK3CA} mutations and tamoxifen resistance has been questioned (López-Knowles et al. 2010; Beelen et al. 2014). Thus, our results, and those from others (Block et al. 2012), suggest that accumulation of numerous genomic aberrations can trigger resistance development. In fact, antiestrogen resistance can be seen even in the absence of any evident mutations (Brünner et al. 1997), also without any in \textit{ESR1} (Martin et al. 2017). An absence of resistance driving mutations has been observed in leukemic cells as well (Pisco et al. 2013). The results imply that the emerged mutations and copy number changes are rather passengers due to the selection pressure than true drivers of tamoxifen resistance (Pisco and Huang 2015).

2.4 \textbf{Transcriptional changes in tamoxifen-resistant cell lines and their resemblance to patients}

Due to a lack of apparent genomic changes that could explain tamoxifen resistance, we investigated in study III the transcriptome of the tamoxifen-resistant cell lines, to detect gene expression as well as pathway changes. Further, we were able to compare our findings to sequential tumor samples from patients that developed endocrine resistance (McBryan et al. 2015).
In line with the exome-sequencing results (Study II), we did not identify a single common differentially expressed gene across all the resistant cell lines (Study III, Figure 1B). Even within the clones derived from the same parental cell line, a maximum of 38% of genes were commonly deregulated (Study III, Additional file 5 S1A). A handful of common point mutations, shared copy number alterations (Study II, Additional file 8 and 10) and diversified gene expression changes (Study III, Figure 1A), indicate that each of the resistant clones chooses its own way to acquire tamoxifen resistance.

Interestingly, the only study so far that has investigated global expression changes of sequential tumors from tamoxifen-treated patients identified less than 3% of differentially expressed genes (McBryan et al. 2015). Our reanalysis of their data set, where we compared primary with the corresponding metastatic tumor, confirmed the results (Study III, Figure 1C). Further, principle component analysis with the expressed genes of patients and cell lines classified as luminal A subtype (Subik et al. 2010) showed that the patient samples cluster together with the luminal A cell lines (Study III, Figure 1D). In addition, we were able to identify SERPINA1, PLXDC2, NAV1, DOCK10, and LRP1 as differentially expressed when looking within the luminal A subtype. Almost nothing is known about these genes and their relationship to tamoxifen resistance. Only SERPINA1, which is upregulated in the patient samples as well, has been shown to be upregulated by ligand-independent binding of ERα (Chan et al. 2015). Indeed, when ERα protein expression is down (Study II, Additional file 4C), we see a decrease of SERPINA1 in T-47D Tam1, T-47D Tam2, ZR-75-1 Tam1 and ZR-75-1 Tam2 and vice versa for the MCF-7 Tam1. In the patient samples, we see a slight increase in ESR1 expression (Study III, Additional file 7). Another interesting finding was the enormous increase in ceruloplasmin (CP)
expression in the resistant T-47D cell lines (240-290 fold-increase), in MCF-7 Tam1 (26 fold-increase) and the three metastatic patient samples (12 to 57 fold-increase; Study III, Additional file 7). Ceruloplasmin has been suggested to be a marker for metastatic breast cancer (Schapira and Schapira 1983) and it has been seen to increase in the blood during tamoxifen treatment (Rössner and Wallgren 1984; Kailajärvi et al. 2000). Unfortunately, those studies did not investigate the survival or recurrence rate of the patients with increased ceruloplasmin-levels. In addition, it has been reported that high levels of ceruloplasmin correlate with lower remission rate (Ozyilkhan et al. 1992), questioning the predictive value of ceruloplasmin as a marker for metastatic disease.

In addition to the analysis of expression changes of single genes, we used the differentially expressed genes and performed a pathway analysis (Study III, Table 2, Additional file 8). Also, the results of the pathway analysis supported the notion that every cell line finds its own way to cope with the tamoxifen pressure. As discussed earlier (2.1. Tamoxifen-resistant cells alter their cell cycle), MCF-7 Tam1 & ZR-75-1 Tam1 mainly changed the expression of genes involved in cell cycle. In the other cell lines, altered genes were mainly involved in protein modification (BT-474 Tam2), or extracellular matrix organization (ZR-75-1 Tam2 & BT-474 Tam1), whereas T-47D Tam1 & Tam2 displayed changes in metabolism associated genes, especially in those of the cholesterol pathway (Study III, Table 2, Figure 2A). The results of the ZR-75-1 Tam2 and BT-474 Tam1 needs to be viewed with caution as the adjusted p-value is low due to a low amount of input genes (Study III, Table 2).

Interestingly, we also observed an increase of cholesterol
pathway related genes in the metastatic patient samples (Study III, Figure 2A). McBryan et al. did, however, remove all the liver-related genes from their analysis as the breast cancer metastasis samples were taken from the liver (McBryan et al. 2015). Nevertheless, our results raise the hen and egg question: What came first, the changes in cholesterol pathway related genes in breast cancer cells as a resistance mechanism or the settlement of the metastatic breast cancer cells in the liver and subsequent adjustment of cholesterol pathway related genes due to the liver environment? The reprogramming of lipid metabolism, however, is currently emerging as a player in metastatic cancer (reviewed in Luo et al. 2017) and in drug resistance (C.-L. Chen et al. 2016).

2.5 Drug responses of tamoxifen-resistant cell lines

When drug-resistance develops and signaling pathways in tumor cells change, new therapy vulnerabilities or even co-resistance to other drugs may arise. Therefore, we investigated in study II the drug responses of the seven tamoxifen-resistant and their parental cell lines towards 279 investigational and approved oncology drugs. We identified common, cell type-, and cell clone-specific sensitivities and co-resistances by calculating the DSS for each compound and comparing parental with resistant cell line (Study II, Figure 2, 3, 4, 5, 6, 7, Additional file 2, 5, 11, 12). We drew drug response networks by integrating drug targets with copy number changes (Study II, Figure 4, 5, 6, 7, Additional file 11 and 12). However, as the majority of the copy number changes are heterozygous deletions, their role in the drug responses are potentially minor. The networks are highly cell line specific, confirming once more that dif-
ferent resistance mechanisms develop in these cell lines.

We observed only a few common effector drugs (drugs with DSS difference < |5|) across the cell lines (Study II, Figure 2, 5A and 5B). As the MCF-7 Tam 1 cell lines developed a rather overwhelming co-resistance profile, there were no drugs to which all the resistant cell lines became more sensitive (Study II, Figure 5A). However, one of the drugs to which the other resistant cell lines were more sensitive, was VX-11e (Study II, Figure 5A and 5B). It has been shown that VX-11e induces ERK1/2 phosphorylation (Chaikuad et al. 2014, 1). The resistant cells had reduced ERK1/2 phosphorylation compared with the parental cell line, which was then increased under VX-11e treatment (Study II, Figure 5C). Interestingly, a study by Zhou et al. observed that tamoxifen induces cell death when ERK1/2 is continuously activated (J.-H. Zhou et al. 2007). As VX-11e also induced cell death in the parental cell line when supplemented with 1 µM tamoxifen (Study II, Additional File 5), the proposed mechanism by Zhou et al. might explain the increased sensitivity to VX-11e.

On the other spectra, all of the tamoxifen-resistant cell lines developed a co-resistance towards paclitaxel (Study II, Figure 5A and 5B), but also other chemotherapeutics were less effective in a more cell-specific manner (Study II, Figure 3, 4, 6, Additional file 11 and 12). As stated in study II, ER has been associated with chemoresistance (Shi et al. 2014; Kurebayashi et al. 2010; Tabuchi et al. 2009), but in the case of the tamoxifen-resistant cell lines we assume the lower effectiveness of chemotherapeutics is rather due to the cells' slow growth. Indeed, in study III we performed another DSRT, including paclitaxel, with the T-47D cells (Study III, Figure 4A, Additional file 9). Paclitaxel was more effective in the T-47D Tam2 cells in this screen than in study II. However, we also observed an increased proliferation in the T-47D Tam2 cells (Study III, unpub-
lished) compared to our first screen (Study II). Importantly, T-47D Tam1, which grow at the same speed as in the first screen, confirmed the results in study II of reduced sensitivity towards paclitaxel (Study III, Figure 4A, Additional file 9).

In the HER2 overexpressing BT-474 cell lines, we saw that HER2/EGFR inhibitors (gefitinib, ibrutinib, neratinib) were even more effective in the tamoxifen-resistant cells compared to the already sensitive parental cells (Study II, Figure 5, 6, Additional file 2). Interestingly, neratinib has been recently approved by the FDA for treatment of early-stage HER2-positive breast cancer (Center for Devices and Radiological Health, 2017). Analyzing the RNA-sequencing data, we detected changes in genes associated with protein modification (BT-474 Tam1 & Tam2), especially glycosylation (Study III, Table 2). Glycosylation has been connected to oncogenesis (Ng et al. 1987; Vavasseur et al. 1994) and shown to modulate the response of HER2+ breast cancer cells to chemotherapy and herceptin treatments (Peiris et al. 2017). Therefore, one could speculate that changes in glycosylation of the EGFR receptor family members potentially make them more accessible to their ligands, or in this case, drugs.

In addition to our results from the exome- and RNA-sequencing, the drug profiling confirmed that clone-specific responses underlay the tamoxifen-resistance and that different drugs are needed to overcome each resistance mechanism.

3 Lipid changes in T-47D and their consequences

In study III, we investigated the T-47D cell lines more in depth. RNA-sequencing indicated changes in cholesterol and lipid metabolism,
RESULTS & DISCUSSION which were found in the patient samples as well (Study III, Table 2, Figure 2A). We were able to observe an enormous increase in cholesterol and neutral lipids by immunofluorescence as well as with thin layer chromatography (Study III, Figure 2 and 4B). It has recently been reported that the use of cholesterol-lowering drugs in endocrine-therapy prevents breast cancer recurrence. Even though this study did not detect any benefits in the tamoxifen treatment only arm, cholesterol levels were decreased (Borgquist et al. 2017). This indicates that the increase of cholesterol does not seem to be a normal drug response and might indeed be a resistance mechanism. We further observed that the cholesterol accumulates in highly enlarged lysosomes (Study III, Figure 3A and B). Lysosomes are involved in mediating different types of cell death, such as apoptosis, necroptosis, necrosis, and ferroptosis, through lysosomal membrane permeabilization (LMP; Galluzzi, Bravo-San Pedro, and Kroemer 2014). Therefore, one could say that preventing LMP can lead to drug resistance. Indeed, when we treated the tamoxifen-resistant T-47D cells with LLOMe, an LMP inducing agent, we observed a decrease in the number of galectin-3 positive lysosomes compared to the parental cells (Study III, Figure 3C and D). Furthermore, it has been shown that accumulation of cholesterol (Appelqvist et al. 2011), an increase in lysosomal-associated membrane protein 1 (Lamp1) and Lamp2 (Fehrenbacher et al. 2008), as well as downregulation of cathepsins prevent LMP (Liaudet-Coopman et al. 2006). All of these LMP inhibiting mechanisms were seen in T-47D Tam1 and Tam2 (Study III, Figure 3).

We also measured an increase in triglycerides and lipid droplets in these cells (Study III, Figure 2C, D and 4B). However, when targeting the lipid phenotype with drugs affecting the lipid metabolism, we did not observe a reversal of the lipid phenotype or a
major reduction in cell viability (Study III, Figure 4, Additional file 9 and 10). We, therefore, suggest that the lipid droplets are mainly functioning more as energy storage than a resistance mechanism.

Compounds targeting pathways involved in regulating the reactive oxygen species (ROS), such as SOD1 inhibitor LCS-1, were able to induce cell death in all the T-47D cells. ROS are able to induce LMP (Aits and Jäättelä 2013) and we did see an upregulation of SOD1 expression in both of the tamoxifen-resistant cell clones (Study III, Additional file 7). SOD1 encodes for a superoxide dismutase, which turns superoxide into either O$_2$ or H$_2$O$_2$, lowering the ROS that could induce LMP. Also RSL-3, which inhibits the glutathione peroxidase 4 that prevents lipid peroxidation, was able to induce cell death in all the cell lines. In addition, we can see an upregulation of ceruloplasmin, an enzyme that converts Fe$^{2+}$ to Fe$^{3+}$ and thus prevents the Fenton-reaction, which would produce superoxide that would then induce LMP. When putting all these results in perspective, it seems that the tamoxifen-resistant T-47D cells circumvent tamoxifen-induced cell death by preventing LMP due to the reduction in superoxide, downregulation of cathepsin D, and an increase in cholesterol and LAMPs (Figure 5).
Figure 5: Proposed resistance mechanism of tamoxifen-resistant T-47D cells. The lysosomal membrane is stabilized by upregulation of Lamp1, Lamp2 and cholesterol. Upregulation of cerulopasmin (CP) and superoxide dismutase (SOD1) prevents the A Fenton as well as B Haber-Weiss reaction, respectively. Thereby ROS gets reduced and lysosomal membrane permeabilization (LMP) is not induced. LMP would, together with cathepsins, trigger various types of cell death. Lamp1/2, lysosomal-associated membrane protein 1/2; CAT, catalase; GPx, glutathion peroxidase

These results also show that resistance mechanisms are very well ingrained and targeting a particular resistance mechanism might not always be successful or harbor only a small window of opportunity.
CONCLUSION

Tamoxifen is one of the most widely used targeted therapies for ER-positive breast cancer. However, patients that initially respond to the therapy can acquire drug resistance and the cancer ultimately relapses. A thorough understanding of the complex mechanisms of drug resistance is needed in order to use tamoxifen more effectively. This understanding can guide development of combinations with other therapies to prevent drug resistance. It can also enable the detection resistance when it occurs, and present an opportunity to switch therapies or target the resistance mechanism.

In my thesis work I, therefore, investigated the genomic and transcriptomic changes of seven tamoxifen-resistant cell lines, tried to identify compounds that can target those changes, and explored the possibility of fusion genes as possible prognostic or diagnostic markers. It became evident relatively quickly that each of the resistant cell lines had developed its own drug resistance profile, or as I would say I discovered the “Seven shades of tamoxifen resistance”. I observed the different molecular changes in the genetic-, transcriptomic- and drug-profile with limited overlaps. I identified that the MCF-7 resistant cell line mainly regulated their growth properties and displayed multi-drug resistance. The resistant ZR-75-1s still need a thorough investigation about their mechanisms, but there are hints of cell cycle and adhesion protein modification. The resistant BT-474s cells modified their protein glycosylation and adhesion according to RNA-Sequencing analysis. And last but by no means least, the T-47D cells prevented lysosomal membrane permeabilization by upregulating their cholesterol metabolism. The example of the T-47D cell lines additionally show how difficult it is to target the resistance mechanisms, and one might need to look beyond the field of cancer...
research in order to understand and combat them.

On one hand, it is confirming that the models represent already known resistance mechanisms, as well as sharing similarities with patient samples, making them appropriate models to study the molecular mechanisms of resistance development and discover possible drug vulnerabilities. On the other hand, seeing the diversity of these mechanisms in only seven resistant cell lines enormously highlights the need of personalized diagnosis and therapies in treatment of tamoxifen-resistant breast cancer.

Whether fusion genes can be used as diagnostic markers or even drug-targets as in hematological cancers such as CML, can be doubted because the diversity of breast cancer is so high. Nevertheless, maybe one of the recurrent fusion gene partners could be utilized as a therapeutic target and on a personalized therapy level they could function as cancer progression markers and indicate possible therapy success.

I was once asked by a scientist that actually works in this field if this breast cancer field is highly competitive. I pointed to my results and said whoever develops another resistance model will find other mechanisms and only when we have enough of the data freely available we might be able to stratify patients. However, more important than models would be the existence and access to clinical data sets, such as the set published by McBryan et al., which not only contains the primary tumor information, but also the information of diagnosed acquired tamoxifen resistance with response/survival information. This would enable researchers to compare the relevance of their models and prove that the resistance mechanism of interest exists in human cancer.
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