PROSPECTIVE COHORT STUDY USING THE BREAST CANCER SPHEROID MODEL AS A PREDICTOR FOR RESPONSE TO NEOADJUVANT THERAPY

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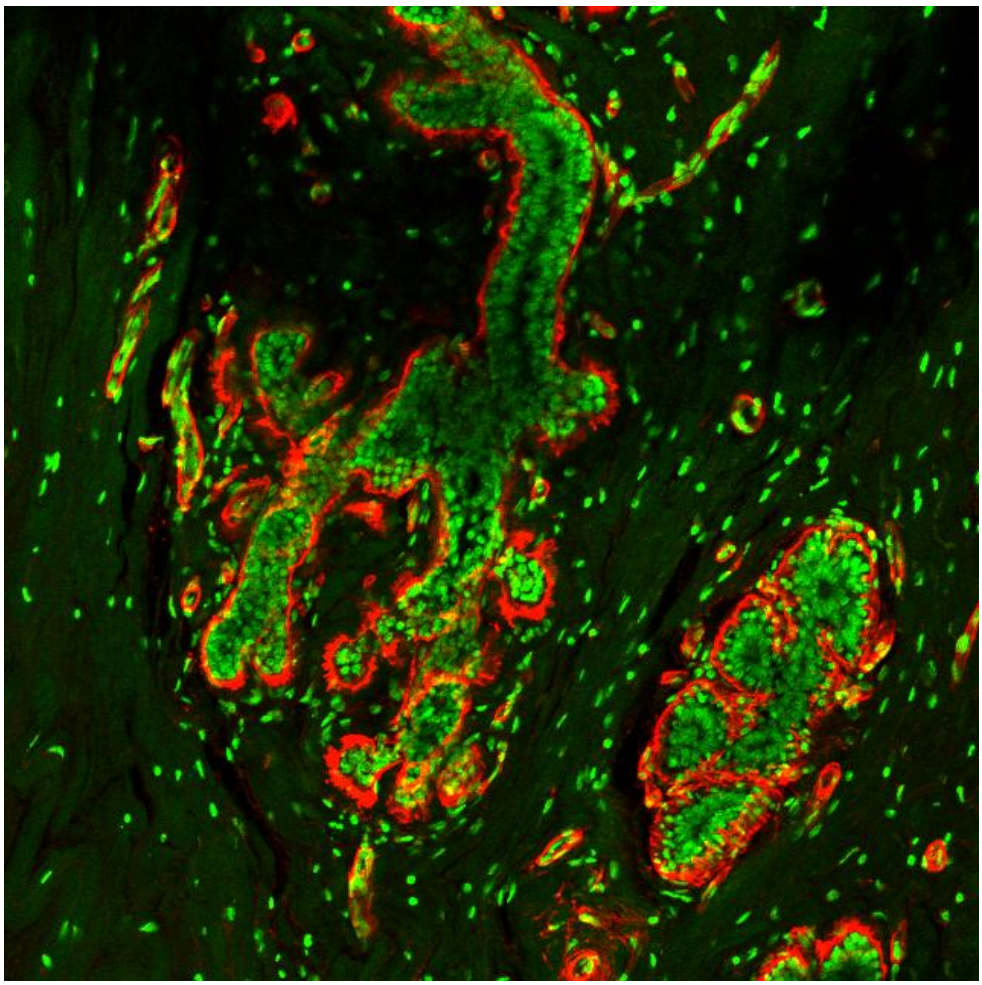
The SpheroNEO STUDY



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Prospective cohort study using the breast cancer spheroid model as a predictor for response to neoadjuvant therapy - The SpheroNEO study

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ABSTRACT

PURPOSE: Prior to genetic characterization, “breast cancer” was considered a uniform diagnosis and was treated in a standardized and uniform way. The discovery of the disease’s heterogeneity in recent years led to the subsequent development of a multitude of new drugs and drug combinations. Since every breast cancer patient has to be considered unique, with each tumor behaving in a distinctive manner, treatment selection should be just as singular. This is why a diagnostic tool to accurately predict the treatment efficacy of each tumor would be a key factor in this decision-making process. Ideally, this assay should replicate each component involved in tumor treatment and progression with a minimum of confounding factors.

A three-dimensional breast cancer cell culture model was proposed and introduced, generated from patient biopsy material that includes cancer cells as well as the surrounding tumor microenvironment on an individual patient level.

The aim of this prospective study was to predict treatment response in breast cancer patients receiving neoadjuvant chemotherapy by using the *in vitro* breast cancer spheroid model. We propose that the predictive potential of such an *in vitro* model system may simplify in the decision, which drugs or drug combination are most helpful in the treatment of patients on an individual basis.

PATIENTS AND METHODS: Three-dimensional spheroids were generated from fresh tumor biopsies of 78 primary breast cancer patients eligible for neoadjuvant drug treatment. Cell survival following 96 h *in vitro* exposure to the clinically equivalent therapeutic agents was measured using a standard metabolic assay. Treatment results were correlated with pathological complete response (pCR, i.e. ypT0 ypN0) determined following the completion of chemotherapy. The sensitivity and specificity of the collected variables was quantified using receiver operator characteristic (ROC) analysis and compared according to the accuracy of predicting pCR.

RESULTS: A mean cell survival of 21.8% was found in the breast cancer spheroid model for 22 patients with pCR. The mean cell survival *in vitro* was 63.8% in 56 patients without pCR (p =.001). The area under the ROC curve to predict pCR was 0.86 (95% CI: 0.77 to 0.96) for cell survival *in vitro* compared to 0.80 (95% CI: 0.70 to 0.90) for a combined model of conventional factors (hormone- and HER2 receptor,

and age). A cutoff at 35% cell survival in the spheroid model was proposed. Out of the 32 patients with values below this threshold, 20 patients (62.5%) and one patient (1.8%) with a cell survival of >35% achieved pCR respectively; sensitivity 95.5% (95% CI: 0.86 to 1.00); specificity 80.4% (95% CI: 0.70 to 0.91). In addition, extent of residual disease (ypT) was significantly associated with increased cell survival (p < .001).

CONCLUSION: The high rate of patients without pCR means that roughly two thirds of the patients may have undergone suboptimal chemotherapy and emphasizes the need for a pre-screen *in vitro* model to improve chemotherapy success. The breast cancer spheroid model proved to be a highly sensitive and specific predictor for pCR after neoadjuvant chemotherapy in breast cancer patients.

ZUSAMMENFASSUNG

EINLEITUNG: Vor der genetischen Charakterisierung wurde Brustkrebs als einheitliche Erkrankung betrachtet, die mithilfe von ungerichteten, standardisierten Methoden behandelt worden ist. Dies schien nach dem damaligen Verständnis der beste Ansatz zu sein, um die bestmöglichen Heilungschancen zu erzielen. Die Erforschung der zugrundeliegenden Heterogenität und die daraus folgende Entwicklung einer Vielzahl neuartiger Medikamente und Kombinationstherapien, legte zudem nahe, die therapeutische Herangehensweise zu überdenken. Jede Patientin ist einzigartig und jeder Tumor verhält und entwickelt sich nach einem individuellen Muster. Demzufolge sollten die diagnostischen Tests zur Vorhersage des Ansprechens einer Therapie ebenso auf einer individuellen Basis beruhen. Im Idealfall sollte ein Test die einzelnen Komponenten und Systeme der Tumorentwicklung, mit einem Mindestmaß an konfundierenden Faktoren, möglichst vollständig wiederspiegeln und beinhalten

Dreidimensionale Zell-Kultur-Modelle bestehenden aus Patienten-eigenem Gewebe repräsentieren die heterogenen Zellpopulationen und einzelne Komponenten des Tumormicroenvironment wieder. Im Gegenteil zu einem Model aus einem zweidimensionalen Monolayer Assay wird hierbei die ursprüngliche Tumorachitektur ohne den Einfluss des murinen Microenvironment beibehalten.

Ziel dieser prospektiven Diagnostik-Studie war die Untersuchung der Prädiktivität eines *in vitro* Brustkrebs-Sphäroid-Models bei neoadjuvant behandelten primären Brustkrebs-Patientinnen.

PATIENTEN UND METHODIK: Von 78 primären Brustkrebs-Patientinnen, die aufgrund von klinischen Faktoren potentiell für eine neoadjuvante Chemotherapie geeignet erschienen, wurden aus entnommenen Biopsien zur Diagnosestellung 3D Sphäroide generiert. Nach einer Inkubationszeit von 48 Stunden mit der geplanten Kombination zytostatischer Wirkstoffe und entsprechender Lösungsmittelkontrolle, wurde die metabolische Restaktivität mittels eines standardisiertem ATP oder MTS Assay gemessen. Die gewonnenen Werte wurden anschließend mit klinischen und histologischen Faktoren (pathologische Komplettremission, ypT0/ypN0) nach erfolgreichem Behandlungsende und anschließender Operation, miteinander verglichen.

ERGEBNISSE: Der Vergleich von 22 Patientinnen mit und 56 ohne pCR, zeigte eine mittlere metabolische Restaktivität von 21.8% im Gegensatz zu einem Mittelwert von 63.8% bei Patientinnen mit verbleibendem vitalen Tumorgewebe (p =.001). Der ermittelte area under the curve Wert in einer ROC Analyse ergab 0.86 (95% CI: 0.77 – 0.96) für die metabolische Restaktivität im Sphäroid-Modell. Im Vergleich dazu betrag die AUC eines multifaktoriellen Models aus konventionellen klinischen Faktoren bestehend aus Hormon- und HER2-Rezeptorstatus, sowie Alter bei Erstdiagnose 0.80 (95% CI: 0.70 – 0.90).

Aus den gesammelten Daten wurde ein Trennwert von 35 % metabolische Restaktivität ermittelt um individuelle Patienten mit vollständigem und unvollständigem Behandlungserfolg zu differenzieren. Insgesamt 32 Patientinnen erreichten einen Wert unter diesem Trennwert, 20 Patientinnen (62.5%) aus dieser Subgruppe zeigten ebenfalls eine Komplettremission. Eine Probe mit einem Wert oberhalb 35% zeigte ebenfalls pCR, was eine Sensitivität von 95.5% (0.86 – 1.00) und Spezifität von 80.4% (95% CI: 0.70 – 0.91) des Sphäroid-Modells ergab. Analyse der zusätzlichen Ausgangsfaktoren ergab eine ebenfalls signifikante Korrelation der Daten des Sphäroid-Modells und des Umfangs der residualem Tumoranteile (ypT, p < .001).

SCHLUSSFOLGERUNG: Das Sphäroid-Modell erwies sich als hoch spezifischer und sensibler Prädiktor für pCR bei neoadjuvant behandelten Brustkrebs-Patientinnen. Zum einen werden die individuellen Eigenschaften eines Tumors berücksichtigt und zum anderen stimmen die ermittelten *in vitro* Behandlungswirksamkeit mit den klinischen Ergebnissen überein.

INTRODUCTION

Due to an increasingly aging population, the number of women diagnosed with breast cancer has continually increased, especially in western countries where it remains the most common type of cancer to afflict women [1, 2]. Mortality rates in women are second only to lung cancer, with especially high rates found in developing countries [3]. Overall prognosis has, however, continually improved as a result of emerging treatment options, and a more in depth understanding of the underlying tumor biology. Today, a diagnosis of breast cancer is no longer considered the death sentence it once was, and is increasingly viewed as a chronic illness for the majority of patients. Epidemiological data have reported 5-year survival rates between 93 – 100%. However, there remain to be patients with rapid tumor progression and poor long-term prognosis. For example the 5-year survival rate of advanced or metastasized breast cancer currently lies around 22% [4, 5]. Identifying these susceptible patient subgroups and selecting the most effective drug treatment is a main goal of current research today. In order to do this several aspects of the disease and its treatment must be identified and considered as detailed in the following sections.

Characterizing tumors of the breast

The breast consists of several layers of tissues and structures, the main components are fatty and glandular tissue. Different phases of development (childhood, puberty, pre- and post-menopause) and pregnancy are associated with differences in tissue consistency and makeup due to changes in hormone production [6]. Figure 1A-C show confocal images of normal breast tissue taken from a postmenopausal woman [7]. Malignant tumors originating in the breast most commonly stem from the epithelium of the glandular tissues, consisting of lobules or ducts. Invasive ductal carcinoma is the predominant histological type of breast cancer and is collectively referred to as “invasive breast cancer of no special type” (NST) [8, 9]. The lobular histology, found in approximately 10% of cases, is distinctly different in comparison to tumors with NST histology and has an overall worse prognosis. Differences were observed in metastatic pattern (primary visceral metastasis), lower rate of pathologic complete response, and a higher incidence of multifocal and contralateral tumors [10-12]. Figure 1D-F show microscopic images of sections taken from an invasive lobular carcinoma. Both ductal and lobular neoplasms can also occur as precursor lesions (duc-

tal carcinoma *in situ* DCIS, lobular carcinoma *in situ* LCIS), which are considered a risk factor in developing invasive cancer [13, 14]. Less frequent histological types are classified according to WHO criteria [15]. In one and the same tumor, a heterogeneous pattern of histological subtypes may be found [16-18].

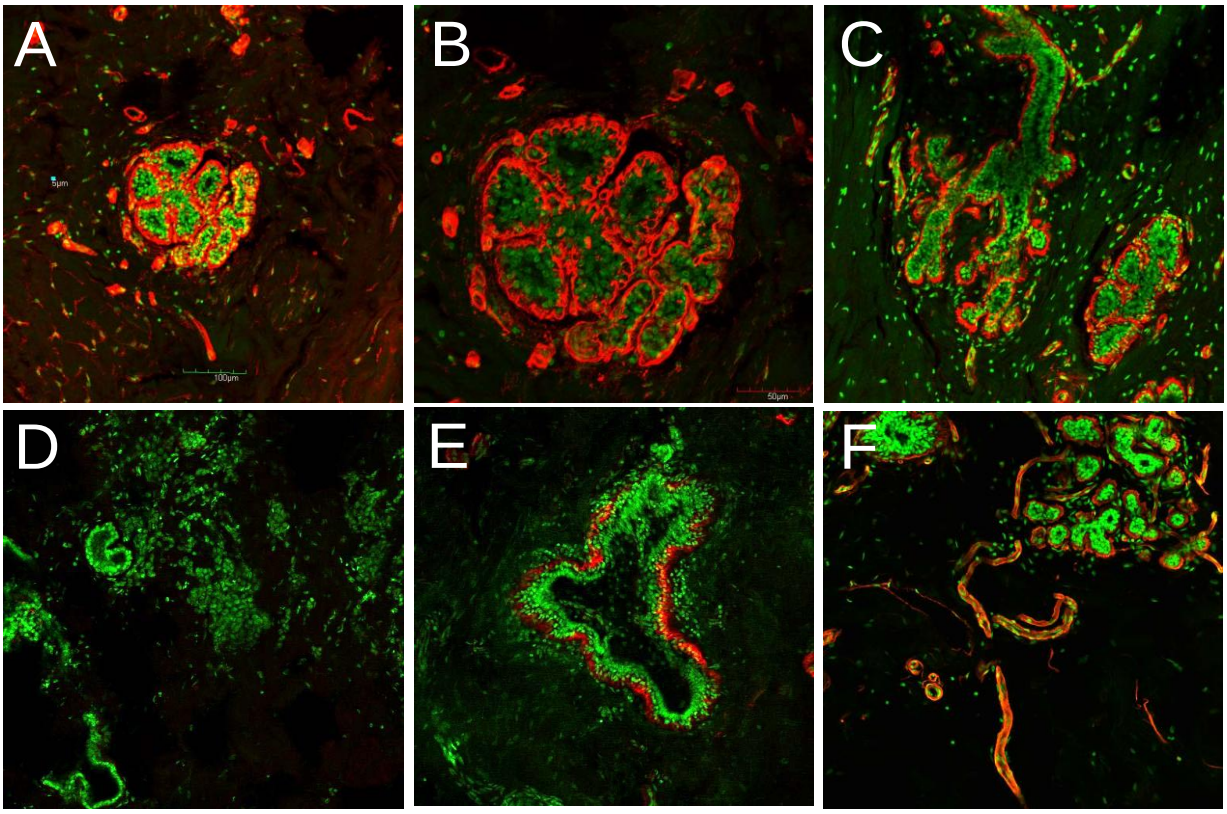


Figure 1: Confocal images of normal breast tissue A-C antibody staining using collagen IV (red) and DAPI (green). A) 20x magnification B) 40x magnification of a lobule with adjacent ducts. Connective tissue is seen in the surrounding area and fatty tissue is not stained at all. C) 20x magnification of a duct leading to lobules. Green stained cells in the surrounding area are fibroblasts. D-F show tissue from a lobular carcinoma stained using DAPI (green) and tenascin (red). D) Control section negative for tenascin showing tissue without invasive components. E) Positive red stain indicating cancerous stroma surrounding a lobule. F) Typical cell formation for lobular carcinoma aligned in single-row at 20x magnification.

The initial histopathological analysis is done using microscopic tissue sections from small core or fine-needle biopsies, or surgical tissues which are stained using hematoxylin and eosin. Additional immunohistochemistry markers may be further used to distinguish between benign and malignant lesions (e.g. myoepithelial markers), as well as determining the histologic type (e.g. E-cadherin).

During the microscopic analysis of the cancerous tissue the grade of differentiation is also determined. Long-term studies have shown that the grade of differentiation is highly associated with overall survival (OS). Well differentiated (G1) tumors show a

better prognosis compared to poorly differentiated ones (G3) [19]. In breast cancer the majority of tumors is moderately (G2) or poorly differentiated [20, 21].

The tumor biology is characterized via the steroid hormone receptors (HR) status, consisting of the estrogen (ER) and the progesterone receptor (PR). Both receptors are part of the subfamily 3 of the nuclear receptors, more specifically ERs are part of the ER-like, and PR of the 3-ketosteroid receptors groups. One or both are overexpressed in approximately 70% of tumors [22]. George Beatson first made the association between ER overexpression and cancer in the 19th century following observations of changes in tissue composition in lactating livestock. He later went on to successfully treat breast cancer patients by removing the tubules and ovaries [23]. Analyses of the prognostic implications showed that patients with ER positive (ER+) tumors have a lower rate of recurrence compared to patients with ER negative (ER-) tumors, independent of other clinical factors associated with prognosis [24, 25].

ER and PR are both expressed as two subtypes α and β, which may combine to form homo- and heterodimers. Both ER subtypes seem to have an opposing effect on carcinogenesis. The ERα receptor is associated with resistance to antihormonal treatment and estrogen-induced proliferation, while ERβ has been shown to limit proliferation and invasiveness of cancer cells through its influence on ERα regulated genes [26]. For each ERα and β subtype, several isoforms have been identified. In a recent publication a low ERβ1 and high ERβ2 expression was associated with higher risk of recurrence in patients with ERα positive tumors following endocrine therapy [27].

The expression of the PR is induced by estrogen and characterizes an intact ER signaling pathway. PR may also effect ERα action, although the exact modes of co-regulation, as well as the implications for endocrine therapy are not yet fully clear [28, 29]. PR positive (PR+) tumors, similar to ER+ tumors, show a better prognosis than negative tumors (PR-). Tumors that are both ER+/PR+ have a better response to endocrine therapy and patients an overall improved prognosis [30].

The hormone dependency found in the majority of tumors has resulted in the development of effective antihormonal or endocrine treatment. The cutoff to distinguish between hormone receptor positive (HR+) and negative (HR-) tumors is set at 1%

immunoreactive cells, and a decision for an endocrine drug strategy is made accordingly [31].

An additional subset of 15-30% of patients is characterized by HER2 receptor overexpression (HER2+), a condition that results in an especially aggressive type of breast cancer [32, 33]. The HER2 receptor, also referred to as receptor tyrosine-protein kinase Erb-B2, is composed of an extracellular and a transmembrane region with a cytoplasmic tyrosine kinase domain. The HER2 receptor, as well as others of the same protein family (HER1, HER3, and HER4) is overexpressed in several solid tumors such as ovarian and gastric cancer, as well as neurodegenerative diseases [34]. However, out of these four ErbB-receptors only HER2 remains in a constant state of activation [35]. Several pathways are activated by HER2 receptor signaling, including the mitogen-activated protein kinase (MAPK), phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K, through binding with HER3), and signal transducer and activator of transcription (STAT) pathways. Cellular growth and differentiation are also stimulated [36, 37].

Through mRNA characterization two additional HER2+ breast cancer subtypes where recently identified: the HER2 enriched and luminal subtypes, each with a prevalence of 50% [38]. The HER2 receptor has proven to be a valuable target for specifically designed drugs, and its discovery marks a major milestone in cancer treatment (i.e. trastuzumab, pertuzumab, TDM-1, etc., see section below) [39].

Test results for receptor overexpression via protein expression analysis are grouped according to number of receptors per cell, percentage of positive cells, and the degree of membrane staining. Tumors are scored 0, 1+, 2+, or 3+ with a score of 3+ indicating a medical need for anti-HER2 therapy, whereas a score of 0 or 1+ does not (HER2-). Additionally tests using *in situ* hybridization methods, mainly FISH, are required for a score of 2+ in order to quantify the number of HER2 genes per tumor cell [40]. Some researchers argue that patients with a score of 1+/2+ might profit from anti-HER2 therapy as well, especially in patients with a score of 2+ regardless of the results from FISH analysis [41].

Ki67 is a nuclear protein that has only recently been included in the initial assessment of the tumors, and is currently under discussion as a biomarker for tumor proliferation. This protein can be found in dividing cells during the active phase of the

cell cycle but not during the G_0 phase. The percentage of positive stained cells 0 – 100%) is sometimes referred to as the Ki67 labelling index. A threshold to differentiate between low, medium, and high proliferating tumors has been proposed, but current guidelines recommend that Ki67 should best be used as a continuous variable. Luminal A and B (both HR+ and HER2 negative) are breast cancer subtypes that are separated through a low (luminal A), or high (luminal B) Ki67 index. Nearly all conducted studies have shown some prognostic and predictive relevancy of Ki67 [25, 42]. However, Ki67 is only predictive of treatment outcome in the neoadjuvant chemotherapy setting, not for survival following adjuvant therapy. The cause may be an underlying heterogeneity in tumor biology in the previously conducted studies. It has been proposed that on one hand some highly proliferative tumors respond well to treatment, thus leading to a good prognosis. On the other hand, highly proliferative tumors with a poor response to neoadjuvant therapy lead to a bad prognosis. Tumors with a low Ki67 expression tend to be associated with a good prognosis. This observed interaction between treatment response and proliferation serves as an explanation for the observed differences in the prognosis for individual patient subgroups. While a statistical relevance for Ki67 continues to be found in many clinical trials, there is still an ongoing discussion regarding the ideal clinical implementation of this biomarker. Problems such as intraobserver variability and intratumeral heterogeneity have prevented Ki67 from being fully validated. In addition, the immunohistochemistry procedure (MIB-1 antibody) used to quantify Ki67 has not been standardized [42, 43].

The molecular characterization of tumors was first proposed with the discovery *BRCA1* and *BRCA2* genes in the 1990s and some two decades later *BRCA3* [44]. These genes were identified while studying families where breast cancer was passed onto female family members with a higher frequency compared to idiopathic cases. In their normal function, the BRCA genes are implicated in the repair mechanism of damaged DNA. The genes lose their original tumor suppressor function in the mutated form, leading to the development of invasive breast cancer in around 5-10% (*BRCA1* and *2*) and 0.3% (*BRCA3*) of cases. The likelihood for the development of ovarian cancer is also increased 10 to 30 fold in women who carry these mutations. Sporadic tumors with triple negative tumor biology (HR- and HER2-)

share characteristics with these hereditary tumors and are referred to as “BRCAness” [45].

Aside from the germline BRCA mutations, a genetic characterization by the Human Cancer Genome Atlas has revealed somatic mutations associated with breast cancer [46, 47]. Some of the up-regulated genes in invasive ductal cancer are *TP53*, *PIK3CA*, *GATA3*, and *MAP3KI* [46]. Based on these finding, a cluster of four molecular subtypes was proposed by Perou et al.: Luminal A (ER+ and/or PR+, HER2-, low Ki67); Luminal B (ER+ and/or PR+, HER2+ or HER2- with high Ki67); Basal-like (ER, PR-, HER2-); HER2 enriched (ER, PR-, HER2+) [48]. The luminal A subtype has shown the highest prevalence with 30-70% of all cases, luminal B and basal-like rank second with respectively 10-20% and 15-20% each. The HER2 enriched subtype is the least frequent with only 5-15% of all cases. The prognosis for these subtypes varies with luminal A and B having the highest 10-year OS rate of circa 90% and 88% respectively. Basal-like which share some similarities with serous ovarian cancer have the lowest survival rate of 75%, slightly below the HER2 enriched subtype (77%) [49-52].

The clinical and genetic characteristics of triple negative tumors (ER-, PR-, and HER2-) largely overlap with the above described basal-like molecular subtype. However, both groups are not identical and only 71% of triple negative tumors are also basal-like [53]. A recent cluster analysis of genetic data has suggested an additional subdivision of triple negative tumors into basal-like 1, basal-like 2, immunomodulatory, mesenchymal, mesenchymal stemm-like, and luminal androgen receptor [54].

Proteomic analysis, mainly done using immunohistochemistry (IHC) or mass-spectrometry may offer valuable insight into the differences in protein expression and/or additional modification (phosphorylation, acetylation, ubiquitination, etc.) between different breast cancer subtypes, as well as intrapatient differences between normal and malignant tissue [55]. The same applies for data obtained on the small-molecule composition (metabolomics) or the analysis of mRNA (transcriptomics) [56, 57]. The combination of several methods of characterization into one systems approach (proteomics, metabolomics, transcriptomics, and genomics) is currently a very promising area of interdisciplinary research. Through an

increasingly smaller subcategorization of breast cancer patients, these newer methods could lead to increasingly refined drug targets and treatment strategies. However, these methods are currently not fully validated for reproducible biomarker/patient subgroup identification. Reliability also remains a critical issue, especially when new antibodies are used. The massive amounts of data collected in these analyses present major statistical and bioinformatics challenges.

Chemotherapy

Early treatment mainly relied on radical and oftentimes crippling surgical techniques to treat breast cancer. Today, treatment may include surgery, radiation, and/or drug treatment [58, 59]. Due to years of extensive clinical and basic research, three approaches to drug treatment have evolved: cytotoxic chemotherapy, endocrine therapy, and targeted therapy.

The accidental discovery of the cytostatic effects of mustine, an analogue of mustard gas, was the first use of a chemical compound in treating cancer. In the following years other alkylating drugs (cyclophosphamide), as well as folate antagonists (methotrexate) were discovered to have an antineoplastic effect. Additional substance groups are antimetabolites, anti-microtubules, topoisomerase inhibitors, as well as drugs with antibiotic effect [60, 61]. The efficacy of the applied drug or combination of drugs often depends on the disease stage, tumor biology, or prior cytostatic treatment [62-64]. In addition, the mode (oral vs intravenous) and timing (number/length of the applied treatment cycles) may vary.

Treatment may be administered with curative or palliative intention. The central aim of palliative treatment is to improve the quality of life for the patient by minimalizing secondary tumor symptoms such as pain and discomfort. Breast cancer patients with potentially curable disease receive chemotherapy either in the adjuvant (following surgery) or in the neoadjuvant setting (prior to surgery) with proven equivalent outcome regarding disease-free survival (DFS) and OS [65, 66]. The EORTC trial showed similar results using FEC combination treatment (5-FU+Epirubicin+Cyclophosphamide) [67]. Neoadjuvant therapy was initially recommended for patients with large and locally advanced, as well as inflammatory breast cancer considered inoperable at diagnosis. Using a neoadjuvant approach the number of cases with breast conserving surgery instead of mastectomy has vastly

improved. Another advantage is a direct *in vivo* observation of the cytostatic effect of the applied substances through tissue or imaging analysis during the course of the treatment. When the disease and treatment setting allow, a tissue sample may be obtained before, shortly after beginning, and following treatment completion for a longitudinal analysis of molecular and protein data. For example, a study conducted in 2007 found a significant correlation between consistently high levels of Ki67 during neoadjuvant endocrine treatment and poorer prognosis [68]. Other factors such as *GATA3* mutation and reduced DUSP4 expression were likewise discovered to be associated with resistance to endocrine and standard chemotherapy [69, 70].

Primary endpoint for neoadjuvant therapy is pathologic complete response (pCR), a potential surrogate marker for survival. The current overall rate of pCR lies around 20-30% [71, 72]. In a clinical trial pCR and/or survival endpoints may be recorded. Survival can be measured as the time between the end of treatment until death from any cause (OS), or until the disease or symptoms of the disease return (DFS)/worsen (progression-free survival, PFS). Pathologic complete response is defined as no remaining tumor (ypT + ypN0) or non-invasive lesions (ypT0/is + ypN0) in breast and lymph nodes. The association with event-free survival (EFS) and OS is less clear when positive lymph nodes (ypT0/is + ypN0/+) or microinvasive tumor residuals (ypTis/mic + ypN0/+) are included. However, using pCR as a surrogate for survival remains controversial since a significant correlation on an individual patient-level has only been found for luminal B/HER2-, HER2-enriched, and triple negative disease. A trial-based level of correlation between the two parameters has not yet been verified [49, 71].

Most chemical based cytostatic drugs target the frequently observed high rate of proliferation characteristic for tumor cells by interfering with the cell cycle, thus leading to a stagnation of tumor growth. Depending on the drug or drugs being administered side effects such as cardiotoxicity, alopecia, inflammation of the intestines, anemia, and immune weakness may occur. Cardiotoxicity is most frequent in patients receiving antibiotic chemotherapy drugs called anthracyclines, especially doxorubicin [73, 74]. PEGylated liposomal-encapsulated (Caelyx®) and non-pegylated liposomal doxorubicin (Myocet®) were developed to reduce these side effects. Significantly improved rates of treatment-induced cardiotoxicity were found for both substances, although a high rate of hand-foot syndrome was observed in patients receiving

Caelyx®. The combination of Myocet®, paclitaxel, and trastuzumab was found to achieve a pCR rate of 40% in women with HER2+ tumors, with few observed side-effects [75].

Anthracyclines are produced by genetically altered *Streptomyces* through fermentation. Compounds from this substance group have four different modes of action against tumor cells: through a) DNA intercalation, which prevents the replication of DNA and RNA, b) stabilization of the topoisomerase II complex, which is responsible for separating the DNA double helix during replication and causing the breakdown of DNA, or c) the production of free oxygen radicals [74]. In breast cancer, anthracycline-based combination treatment, mainly including epirubicin or doxorubicin, are a central component of nearly every chemotherapy regimen [76]. Results obtained in the NSABP B-18 study showed that adjuvant or neoadjuvant application of anthracycline-based chemotherapy is equivalent regarding prognosis [65, 66]. In addition, the ADEBAR Study showed that a higher dose of anthracycline (i.e. epirubicin) does not increase the rate of observed toxicity [77, 78].

The compound doxorubicin also belongs to the topoisomerase inhibitors and is frequently combined with cyclophosphamide, an alkylating agent [79]. These compounds act by directly binding an alkyl group of the DNA and by crosslinking DNA strands, thereby interrupting DNA synthesis via P53 [80, 81]. Cyclophosphamide is considered a pro-drug and is enzymatically processed into phosphoramide mustard. Initially, cyclophosphamide was part of the combination treatment cyclophosphamide-methotrexate-fluorouracil (CMF) which remained the standard treatment for breast cancer until the publication of the NSABP B-15 study in which the combination doxorubicin + cyclophosphamide was found to be equally effective [82]. Considering the increased risk of leukemia following prolonged treatment with cyclophosphamide, the anthracycline-based regimen was subsequently preferred due to the shorter application period (63 days vs 6 months) [83].

The platinum-based compounds carboplatin or cisplatin are referred to as alkylating-like agents. These compounds form a covalent bond with the DNA, thereby causing DNA strands to crosslink and impairing DNA conformation and synthesis [84, 85]. In recent trials carboplatin was found to be effective in the treatment of metastatic and locally advanced triple negative breast cancer [86, 87]. Cisplatin is currently being

studied for its efficacy in the treatment of hereditary breast cancer, based upon promising data obtained from *in vitro* and *in vivo* models, as well as small clinical trials [88]. Especially *BRCA1* mutation carriers achieved good results [89, 90]. Comparison between the two platinum compounds, showed a slightly higher rate of pCR for carboplatin. In a meta-analysis, anthracycline-taxane based neoadjuvant chemotherapy including any platinum compound resulted in a pooled pCR rate of 48.4%. In the triple negative group, the overall rate of pCR was twice as high compared to other tumor subtypes. While promising, an optimal chemotherapy regime for platinum compounds remains under discussion [91].

Unlike the previously mentioned groups of chemotherapy drugs, antimetabolites are specific for the S-phase phase of the cell cycle. DNA synthesis takes place during this phase and the antimebolitic drugs act as competing substitutes for naturally available substances such as purine and pyrimidine. The drug 5-fluorouracil (5-FU) for example acts on RNA synthesis by inhibiting the thymidylate synthase thereby causing DNA damage [92]. It remains one of the most commonly used drugs in the treatment of several solid tumors, either as single-agent or combination therapy. However, current German therapy guidelines no longer recommended it for the primary treatment for breast cancer due to results from the GIM-2 study which showed that the addition of 5-FU does not improve the overall efficacy of anthracycline- or taxane-based therapy [63, 93]. Recently conducted studies comparing FEC to anthracycline-taxane treatment (i.e. EC – Doc) showed equivalent results for OS, and hematologic and serious adverse events were more frequent in the FEC study arm [77]. In contrast, another study found more toxicities for the anthracycline-taxane combination, although treatment efficacy was also higher [94]. The addition of a taxane was shown to increase efficacy to FEC combination treatment [95, 96]. 5-fluorouracil or its prodrug capecitabine may be an alternative for patients intolerant to taxane-based treatment. Capecitabine is taken orally and is converted to 5-FU through enzymatic processing. In metastatic breast cancer capecitabine is currently mainly prescribed as single-agent chemotherapy, and less frequently in combination with docetaxel, the microtubule inhibitors ixabepilone and eribulin, or the multi-kinase inhibitor sorafenib where it has shown a high response rate and good tolerability. It is also recommended for patients resistant to, or heavily pretreated with first-/second-line taxane and/or anthracycline chemotherapy [97, 98].

Gemcitabine is also an antimetabolite and induces apoptosis by replacing the nucleic acid base cytidine [99, 100]. It is mainly used in the treatment of metastatic disease where it has shown mixed results when combined with standard chemotherapy [101-103]. Studies with high risk primary breast cancer patients have also not any improvement in pCR through the addition of gemcitabine to anthracycline-taxane based chemotherapy [104, 105].

Another mode of action in anticancer treatment targets the microtubule function, more specifically the assembly and disassembly during the S-phase of the cell cycle. Two different drugs used in the treatment of breast cancer fall under this category, the taxanes and the vinca alkaloids. The precursor substance for the taxanes paclitaxel and docetaxel are extracted from the needles of the yew tree, and chemically altered to their final pharmacologic form [106]. Taxanes work by stabilizing microtubule formation, thus blocking disassembly and obstructing the cell cycle [107, 108]. Multiple clinical trials have demonstrated the high and equivalent efficacy of both paclitaxel and docetaxel. Combinations including one of the two include DocAC [109], FEC-Doc [94, 110], and EC- Doc [111, 112] both in the neoadjuvant and adjuvant setting. The addition of a taxane to anthracycline-based chemotherapy was shown to both improve the rate of breast conserving surgery (BCS) and pCR [113, 114]. Currently, the anthracycline-taxane based chemotherapy remains the standard of care, although there are still ongoing trials to determine the ideal treatment timing and sequence [115, 116]. A modified form of paclitaxel, nanoparticle albumin-bound (nab-) paclitaxel, has also been subject of several clinical trials for the treatment of metastatic disease. The goal behind the development of this compound was to reduce neurological side-effects by eliminating the solvent chromophore EL, as well as increasing drug availability through improved systemic distribution [117]. Several trials have found a clinical benefit and improvement in the rate of pCR for albumin-bound paclitaxel compared to regular taxanes [118-120].

Vinca alkaloids also act through competitive binding to tubulin, the building blocks of the microtubules, subsequently preventing the formation of the mitotic spindle. From this group of substances, vinorelbin is most commonly used for pretreated metastatic cancer [121, 122]. Older patients may tolerate treatment with vinorelbin better than taxane or anthracycline-based treatment. However, in monotherapy vinorelbin was found to be less effective compared to taxanes. Compared to 5-FU/capecitabine and

other combination treatments an equivalent response was observed in the neoadjuvant, as well as the adjuvant treatment setting [121].

Although efficacy to these untargeted cytostatic drugs (meaning, proliferation is a sufficient but not exclusive criteria of cancer cells) is high, some patients may nevertheless show tumor progression due to drug resistance. In metastatic disease, the success rate of second-line chemotherapy is as low as 20-30% following disease progression after first-line treatment [123]. Tumors may be intrinsically resistant or develop an acquired resistance to a specific compound. In some cases an acquired resistance may extend to include other compounds with different modes of action, referred to as cross-resistance. The main mechanism implicated in resistant tumors is via increased drug efflux. Multi-resistant tumors have an upregulation of ATP-binding cassette (ABC)-membrane transporter proteins (i.e. P-glycoprotein, multi-drug-resistant protein 1, breast cancer resistance protein) in the plasma membrane, which leads to reduced intracellular drug concentrations. Other mechanisms leading to drug resistance are mutations, alterations, or overexpression of tubulin isotypes or tubulin-associated proteins (resistance to taxanes), changes in topoisomerase II (anthracycline resistance), cytoplasmic drug-metabolizing enzymes (cyclophosphamide resistance), or aldehyde dehydrogenase isoform expression (taxane resistance). On a molecular level the changes in the tumor-suppressor gene p53 (resistance to doxorubicin), resistance to apoptosis through the *survivin* gene, and abnormal mismatch repair mechanisms were all associated with drug resistance [124, 125]. Currently different strategies of overcoming drug resistance are being studied such as resistance-reversing drugs, new drugs with altered targets, or resistance predicting biomarkers [126].

Overcoming resistance and improving treatment outcome may also be achieved using dose-dense or dose-intensified chemotherapy. In dose-dense regimens, standard compounds are given at a higher dose, while dose-intense regimens are given at a higher dose and shorter between cycle intervals. Promising results have been found for patients with a high risk profile (lymph node metastasis ≥ 4), both in the adjuvant and neoadjuvant treatment setting [127]. However, the optimal dosing scheme is still being discussed. The frequency of observed toxic side-effects was also increased. In order to limit the subsequent myelotoxicity and neutropenia in-

duced by the increased chemotherapy dose, granulocyte colony-stimulating factor is simultaneously administered [128].

Endocrine treatment

Some types of cancers such as breast, prostate, as well as endometrial cancers can be treated using a whole other strategy, by exploiting the tumor's hormone dependency [129]. There are three treatment strategies which target HR+ tumors: inhibition of hormone synthesis, competitive antagonists, and hormone substitutes [26, 130]. The choice of treatment depends on the disease stage and the menopausal status of the patients [131, 132]. In pre-menopausal women the estrogen and progesterone production by the ovaries can be suppressed through an analog of the gonadotropin-releasing hormones (GnRH) which acts on the pituitary gland [133]. The aromatase inhibitors (AIs) inhibit hormone production through the aid of an enzyme called aromatase in other tissues as well. This enzyme catalyzes the transformation of androgens into estrogen. Letrozole, anastrozole, and exemestane are among the substances that are used to inhibit the function of aromatase [64, 131].

The second class of anti-hormone drugs is the competitive antagonists, such as the selective ER modulators (SERMs) and the antiandrogens. However, only the SERMs are relevant for the treatment of breast cancer. Tamoxifen is the most commonly prescribed of these drugs and can be used in the treatment of both early, locally advanced, and the metastatic cancers. Tamoxifen binds to the ER and inhibits the subsequent signaling pathway [134-136]. In general, this type of therapy may be administered as neoadjuvant, or more commonly as adjuvant maintenance therapy. In the neoadjuvant setting, an increase in objective response rates and BCS, especially in older, postmenopausal women was found [137].

In a few cases when all other substances have failed to be effective or are contraindicated, estrogen or progesterone can be administered as a supplement. This may lead to a down-regulation of ERs through a negative feedback mechanism. This treatment is, however, outdated and not frequently used [138-140].

The positive response rate to endocrine therapy lies around 75% [141]. Current data on resistance mechanisms suggest a hyper activation of the PI3K/PTEN/AKT/mTORC1 (PTEN, phosphatase and tensin homolog, mTORC1,

mammalian target of rapamycin complex 1, AKT, protein kinase B) and MAPK pathways mediated through growth-factor stimulation (HER2, insulin-like growth factor 1 receptor, fibroblast growth factor receptor 1). Resistance to tamoxifen is also induced through an altered or decreased expression of ERα or changes in the enzymes that metabolize the drug (CYP2D6). Overexpression of the cofactor AIB1 and subsequent binding to the ERα transcription complex may also lead to tamoxifen-stimulated tumor growth. Similar drug efflux mechanisms are active in the resistance to untargeted chemical compounds and are also implicated in endocrine resistance (Pgp, MDR1) [142, 143]. In recurrent tumors loss of HR overexpression was also observed [144, 145]. A combination of mTOR inhibitors (everolimus, see also below) and endocrine therapy drugs (tamoxifen, aromatase inhibitors) may be used to treat patients with HR+/HER2- primary and metastatic tumors resistant to endocrine therapy [146-148].

Targeted drugs

A third major group of drugs, monoclonal antibodies and small molecule drugs target specific properties that only cancer cells exhibit, thereby increasing efficacy and decreasing unwanted side effects.

Compounds with low molecular weight, otherwise known as small molecule drugs, target tyrosine or serine/threonine kinases. Unlike the larger antibody-based drugs, small molecule compounds may function by intervening in both intracellular and extracellular processes. However, the half-life of these drugs is also shorter compared to antibody drugs [149]. The two main drugs which are part of current breast cancer treatment are lapatinib and everolimus. Lapatinib is a tyrosine kinase inhibitor targeted against HER2 overexpression and acts through the inhibition of the EGFR pathway [150, 151]. In clinical trials, lapatinib combined with standard chemotherapy was found to be effective both for primary and metastatic disease, both as a single, and a dual HER2 blockade with trastuzumab. The treatment effect on survival remains less clear with results from follow-up from neoadjuvant and adjuvant trials showing only small or no improvement [152, 153].

Everolimus, on the other hand, works by blocking the mammalian target of rapamycin (mTOR) and is used to treat tumors that are HER2- or exhibit an acquired resistance to anti-HER2 drugs. It is also used for several other solid tumor manifes-

tations such as renal and pancreatic carcinoma. The drug binds to the FKBP12 receptor, and inhibits downstream signaling pathways involved in cell proliferation through subsequent interaction with mTORC1 protein complex. Research suggests that everolimus may reverse resistance to endocrine and anti-HER2 therapy as mentioned above [154, 155]. The recommended treatment setting for everolimus is limited due to an increase in adverse events and mortality observed among clinical trial participants, and currently the drug is only approved for patients with metastatic or advanced disease [156, 157].

Poly (ADP-ribose) polymerase (PARP) inhibitors interfere in the DNA repair pathways and work by preventing homologous recombination following binding through the replication fork and double-strand break. This inhibition is especially effective in BRCA-mutated tumors where this mechanism is impaired, thereby leading to cell death of mutation carrying cells. For this reason PARP inhibitors are a promising alternative for BRCA mutation carriers and BRCA-associated tumors. Currently, olaparib and similar PARP inhibitors (veliparib, niraparib, talazoparib, rucaparib, and CEP-9722) are being studied in phase III clinical trials to assess their efficacy and toxicity in breast and other solid tumors [158].

Compared to small molecule drugs monoclonal antibodies such as trastuzumab, bevacizumab, and the more recently developed pertuzumab, have been more successful. Trastuzumab and pertuzumab are both humanized monoclonal antibodies that bind to the HER2 antigen presented on the surface of cancer cells. Cell growth is prevented through the interference with the signaling pathway, the internalization and subsequent degradation of the receptor heterodimer, prevention of dimerization, downregulation of HER2 expression, induction of cell cycle arrest, and inhibition of irregular HER2 cleavage [159]. A second mode of action of trastuzumab is mediated through the innate and adaptive immune system [160]. The drug is given either simultaneous to regular chemotherapy, and/or as maintenance therapy for one year after surgery in the neoadjuvant or adjuvant setting for HER2 positive (HER2+) early, locally advanced, and metastatic disease [161].

Targeting of the HER2 receptor can be done with a single compound or dual targeting using trastuzumab, pertuzumab, the small molecule compound lapatinib, as well as antibody-drug conjugates [162-164]. Due to the affected signaling pathways a

dual inhibition of the HER2 receptor leads to an accelerated intracellular degradation [37]. The reasoning behind a dual blockade is to prevent tumor resistance through parallel targeting of two different cellular mechanisms. Lapatinib for example, targets both HER1 and HER2 as an ATP competitor, thereby preventing PI3K/AKT and MAPK signal transduction [165]. Pertuzumab on the other hand prevents the HER2 receptor from binding to other receptors from the HER family. Both drugs have been found to be equally effective given concomitant with standard chemotherapy. This dual approach is especially effective for tumors which are also HR-.

This treatment option has caused the rate of pCR among women with HER2+ tumors to rise up to 53.2% [166] through single-agent inhibition, and up to 66.2% using dual inhibition [167-170]. Supplementing trastuzumab to neoadjuvant chemotherapy resulted in an increase in pCR regardless of the applied chemotherapy regimen (FEC [167], CMF [171], EC – Doc [172]). The observed OS rate was also improved from 75.2 to 84%, DFS likewise by 40% [173, 174]. A main concern regarding this combination treatment is the simultaneous application of anthracyclines and trastuzumab, especially regarding cardiotoxicity. For this reason trastuzumab is mainly given simultaneous to taxane therapy. The study of the underlying cellular pathways in cardiac dysfunction induced by trastuzumab has however shown that these effects are not persisting, unlike those brought on by anthracycline application [175].

A full and long-lasting response to HER2 inhibition is achieved in circa 30% of patients [176-178]. Potential biomarkers to predict resistance to anti-HER2 treatment via lower rates of pCR are alterations in the oncogene PI3KCA (mutation, loss, amplification, copy number gain, increased expression, and PTEN loss) [179]. Mechanisms of resistance are via p95HER2 (shortened HER2 receptor, extracellular binding site for trastuzumab missing), IGFR (through interaction with HER2), abnormal c-MET (signaling pathway leading to resistance still unclear), Src (implicated in intrinsic and acquired resistance, phosphorylated form), signaling with the ER, and low immune response (low levels tumor-infiltrating lymphocytes, TILs, low PD-L1). Identified resistance mechanisms may represent potential druggable targets to overcome resistance. For example, inhibitors to the heat shock protein Hsp90 are currently being investigated in clinical trials (ganetespib, tanespimycin) [180-182].

A conjugate of trastuzumab and emtansine (T-DMI), an anti-microtubule agent, has recently been approved for patients with metastatic tumors resistant to regular trastuzumab formulation. Via intracellular delivery of emtansine, T-DMI was found to increase OS in patients previously treated with trastuzumab and taxane [183]. Through the intracellular mode of action, the safety-profile was also improved [184].

Compared to a reversible block from the previously described drugs, new agents that irreversibly block the HER2 receptor such as afatinib and neratinib may increase the anti-HER2 treatment efficacy even further [185, 186]. However, a recently conducted study was ended prematurely due to a high rate of observed adverse events in the afatinib study arm [187].

Bevacizumab is humanized monoclonal antibody that is used to treat the remaining cases that are HER2-. The target is the vascular endothelial growth factor A (VEGF-A), a protein that is associated with angiogenesis and vasculogenesis. Both factors have been implicated to play a major role in tumor growth [188-191]. The ability of a tumor to attract existing blood vessels or form new blood vessels has been identified as a major factor in the differentiation of normal to malignant cells [191]. Bevacizumab is currently being used in the treatment of primary and advanced cancers with a HR+ or triple negative tumor biology. An improvement in progression-free survival was especially evident in metastatic disease [192]. Results regarding OS and pCR were less clear, with the GeparQuinto trial reporting especially favorable results for HR+ tumors while Bear et al reported greater benefit for HR- disease [193, 194]. However, the approval of the drug had been under great scrutiny due to an unfavorable risk/benefit ratio in clinical trials. The approval for the drug was revoked 2010 in the United States by the Food and Drug Administration for the treatment of breast cancer, the European Agency has upheld their initial approval [195].

Predictive and prognostic biomarkers

In breast cancer treatment decision-guiding algorithms are based on the results of a long history, and a multitude of clinical trials summarized in national and international guidelines. These are successfully applied in the treatment selection process by an interdisciplinary team of treating physicians for the systemic management of the disease [196]. Routine clinical and pathological criteria such as TNM criteria, HR and HER2 status, grading, histology, and age are important factors in identifying basic

breast cancer subtypes. For the majority of patients, treatment stratification based on these criteria will be sufficient, but in certain cases and circumstances a more individualized approach is essential. This includes patients not responding to treatment, recurrent or metastatic disease, as well as those with a high risk of recurrence. Also, in some cases a certain treatment cannot be administered because of a persisting medical condition, drug intolerance, or other conflicting comorbidities. Here a similarly effective treatment option needs to be identified. For these reasons, preclinical and clinical diagnostic methods, biomarkers, or factors predicting individual treatment efficacy and overall prognosis remain an important aim of translational medicine [197]. This is especially important considering the overwhelming multitude of available and emerging treatment options as described in the previous sections.

Per definition, diagnostic factors, assays, or biomarkers are variables that consist of a single measurement or a combined score obtained from the joint analysis of several measurements. Regardless of the type of variable, the results may be predictive for survival (prognostic) and/or treatment outcome (predictive). The difference between the predictive and prognostic biomarkers is the association with the applied treatment: A marker is predictive if the difference between its presence/absence marks a higher effect for one of the treatment arms, it is prognostic if its presence or absence is the same for both arms equally [198]. Biomarkers may also detect and diagnose a disease, as well as provide surveillance data. A proposed biomarker is categorized using levels of evidence (LOE) ranging from strong I to weak V. In order to obtain a LOE I the biomarker must be validated in a randomized controlled trial using prospective data [199, 200]. The validity of the biomarker describes the agreement of the measured data with the actual clinical outcome (i.e. clinical validation), also important factors are the reliability or analytical validation of the data consistent across repeated measurements [201].

Established clinical predictive factors that are associated with a higher rate of pCR following neoadjuvant chemotherapy are high tumor grade (G3), young age at diagnosis [202], HER2+ [72, 203], and HR- status [71]. Young age at diagnosis is also prognostically favorable, along with other factors such as negative lymph node status, low tumor grade, HR+ and HER2+ status, pCR, and early response to neoadjuvant treatment [204, 205]. These have been extensively studied and are easily measured, due to their implementation into routine clinical practice.

Emerging factors that may function as potential biomarkers and drug targets include dysregulation of cyclin D1 [206], topoisomerase IIα [207], circulating tumor cells or tumor cell-derived DNA [208, 209], as well as the ERβ [210, 211]. All of these markers were found to have prognostic relevancy, however research data is not conclusive and the mode of implementation into clinical routine remains unclear at this time.

Ki67 and the serine protease urokinase plasminogen activator and its associated serpin inhibitor plasminogen activator inhibitor-1 (uPA/PAI-1) on the other hand, are already part of most routine histopathological analysis. As described above, Ki67 is a potential surrogate marker for cellular proliferation that has been highly associated both as a predictive and prognostic factor in all breast cancer subtypes. The combined assessment of uPA/PAI-1 has been validated as LOE I in lymph node negative breast cancer as a prognostic and predictive marker [212]. The uPA system is found to be upregulated in several types of cancer and is associated with an increased cellular migration and lessened adhesion via changes in the extracellular matrix (ECM). The disrupted ECM thus initiates local and distant invasion through cancerous cells and therefore a high uPA score is a marker for higher risk of recurrence [213]. For early intermediate risk ER+/G2 breast cancer patients, the assessment of uPA/PAI-1 was proven to aid in risk stratification regarding the necessity for adjuvant chemotherapy [214]. While being very cost-efficient drawbacks include the requirement of fresh/frozen tissue samples. Validation and standardization of the assay using fixed tissue samples remains a key issue, a reason why the clinical implementation of this assay has only slowly caught on.

Molecular profile assays, imaging methods, as well as immune-related factors are other central research areas [215-220]. As described in the section on the characterization, molecular profiles obtained through cDNA microarray analysis provide data on patient risk stratification by identifying the tumor subtype. Patients are characterized by their risk of relapse with or without systemic chemotherapy, thereby avoiding potential overtreatment. The commercially available molecular profiles are PAM50, MammaPrint, and OncotypeDX. The difference between these tests are the underlying genes that are used as classifiers, whereas OncotypeDX uses a set of 21 genes for its profile, PAM50 uses 50, and MammaPrint 70 genes. MammaPrint was the first

test of its kind available to patients, and is also the only test currently approved by the FDA.

The OncotypeDX has been successfully validated for ER+/LN- primary breast cancer patients, and classifies patients into low/intermediate/high risk for relapse. Systemic chemotherapy is considered unnecessary for patients with a low risk profile. The identified risk profiles are also associated with differences in response to neoadjuvant chemotherapy [221]. However, the clinical relevancy of the large amount of generated data is not yet fully understood and the implications for intermediate- and high-risk profiles remain less clear. Also, the currently available tests are costly and only analyze exons are analyzed, lacking information on the underlying driver mutations [222].

A closely related subgroup classification may be done using the routine histopathological tissue assessment. The combined score is referred to as IHC4 and combines HR and HER2 status, as well as a tumor grade. In contrast to cDNA microarray, IHC-based analysis is scored manually using semi-quantitative methods, results are therefore more difficult to standardize and less reliable. On the other hand this approach is already part of the routine histological analysis, making it a feasible and cost-efficient option even in developing countries. A comparison of the IHC4 assay with the equivalent microarray assay PAM50 resulted in a similar categorization with the exception of the HER2-/LN- patient subgroup [223].

The available tests have in common that they distinguish between patients who should receive chemotherapy and patients who should not [47, 224, 225]. Similarly, circulating tumor cells have proven to be of prognostic value [226], however, a predictive value has not been shown [227, 228].

Preclinical research models

Only a small percentage of drug candidates screened successfully in preclinical research studies are subsequently approved for patient treatment, oftentimes due to issues regarding safety and clinical efficacy [229, 230]. This high attrition rate suggests that current preclinical strategies are not fully representative of the characteristics exhibited by patient tumors. A refined preclinical strategy through improved models may reduce costs and expedite the time until market approval.

Preclinical research models and tools consisting of *in vivo* animal and *in vitro* cell culture models are an essential component in identifying new and promising drug targets, underlying pathways, and predicting short-/long-term treatment efficacy. Animal models were first developed during the 1930s in parallel with the development of the first chemotherapy drugs, the most widely used animal being tumor-bearing mice. In comparison to other available animal models the genetic makeup of the mouse is more closely related to humans than non-vertebrae, amphibian or fish models. The advantage of the mouse is its small size and efficient breeding methods compared to larger mammals. All pharmacological and physiological effects of a candidate drug can be studied, including its toxicology. The entire organism is represented, which is why *in vivo* models make up a central component in the current drug approval process [231, 232]. In breast cancer research mouse models for both primary and metastatic disease stages have been successfully implemented, and were fundamental in developing important drugs such as tamoxifen [233]. A widely-used model for breast cancer progression and metastasis is the MMTV-PyMT model [234]. In general, treatment efficacy in mouse models can be analyzed using defined biomarkers, imaging methods such as PET-CT, NMR, and ultrasound. Alternatively surrogates for survival can also be assessed [235]. Individual processes such as tumor dissemination, for example, may be simulated by injecting tumor cells directly into the circulatory system of the animal [236].

Mouse models may be classified according to spontaneous or induced tumor growth [60, 237]. For example skin tumors may be induced through the application of carcinogenic substances such as 7,12-dimethylbenzo[a]anthracen (DMBA) and croton oil [238]. Inoculation with microorganisms, such as infection with *H. pylori* allow the study of gastric tumorigenesis [239].

Genetically engineered mouse models (GEM) are bred carrying specific loss or gain of function mutations in a single or multiple tumor suppressor gene(s) or oncogene(s) (i.e. *TP53*, *BRCA*), thus leading to the development of tumors. Through the controlled manipulation deregulated pathways contributing to tumorigenesis and its effects on treatment efficacy and survival may be studied without the confounding influence of outside carcinogenic factors [240, 241]. Unlike xenograft models, GEM models maintain a competent immune system.

Alternatively, cancer cells may be injected, transplanted subcutaneously or orthotopically into the animal. These xenographic and patient-derived xenograft (PDX) models utilize nude or NOD-SCID mice with an immature immune system so the transplanted tissue will not be rejected by the host animal. Components of the patient's hematopoietic immune cells may also be transplanted resulting in so called "Avatars" [242]. In another approach the mammary fat pads of the mouse may be cleared and injected with human fibroblasts, epithelial and stromal cells to mimic the human tumor microenvironment [236, 243]. Both organ site-specific orthotopic and heterotrophic tumors where tumor cells are subcutaneously implanted into the flank of the animal, develop within several weeks or months following implantation. The successful implantation rate lies around 75% [244, 245]. Through transfer from one animal to the next a tumor may be cultivated and expanded for an extended period of time. Studies using patient tissue from breast, colon, pancreatic and lung cancer have shown that results may be predictive for treatment efficacy in the clinic. Several aspects of the individual patient tumors such as tumor histology and phenotype, gene expression profiles, SNPs, and copy number variants are retained in PDX models [243-245]. PDX models more closely represent the multiple and heterogeneous mutations of the majority of human tumors whereas GEM models are more related to tumorigenesis associated with genetic predispositions, thus making the PDX approach more relevant for preclinical drug selection [246].

The main problem of PDX models is the sensitivity of the immunodeficient mice, making the breeding and caretaking costly and time-consuming [242, 247-249]. In addition, some criticize that these in general mouse models do not accurately mimic the tumorigenesis and heterogeneity of human tumors due to inherent inter-species distinctions [250]. For example, telomerase activity and metastatic behavior are two points that show marked differences [246]. The murine, or rather loss of the human tumor microenvironment represents a significantly confounding factor even when this component is supplemented using patient material [251].

These may be the underlying reasons why preclinical drug efficacy studies using the PDX model have not been as successful as initially predicted [252]. Problematic remains the high-throughput practicability and clinical implementation. It should also be noted that not all patient tumors are successfully implanted, with a higher success rate observed for more aggressive tumors. Following successful implantation the

time span for tumor development in the mouse (several weeks) exceeds the time between a patient's diagnosis and the start of chemotherapy [253]. Although models for breast cancer have been well established and validated there are currently no models available for other solid neoplasms such as ovarian, brain, kidney, and malignant melanoma [254].

A vast amount of research has been done using established breast cancer cell lines. These are isolated from excised primary or metastatic tissue, pleural cavity fluid or ascites of cancer patients. The primary tumor cells become immortalized using *in vitro* techniques or due to intrinsic mutations [255]. All cells of a specific cell line are clones and therefore behave similarly when exposed to comparable conditions and compounds, making it a simple and inexpensive method for preclinical drug selection, as well as to study various aspects of cancer biology [256, 257]. The most widely used cell lines in breast cancer research are the MCF-7 [258], T-47D [259], and MDA-MB-231 [260] cell lines. All three cell lines were obtained from the pleural effusion of patients with metastatic disease [261]. Cell lines may be used in adherent single-layer culture or suspension culture conditions. Data comparing the cell lines with the primary tumors or tumors of similar characteristics shows that phenotype, hormone receptor status, genomic aberrations, and changes in protein expression may remain stable even after prolonged culture [262-264].

However, others have shown that this is not true for all characteristics. In some instances experimental data obtained from the same cell line may even differ between laboratories [265]. Various cell lines cultured in one laboratory may have more in common with each other than with the same cell lines stored in another facility due to location-specific cell culture conditions. Treatment efficacy results obtained from cell lines do not consistently translate to clinical treatment outcome. Likewise, a comparison of the pathways involved in multi-drug resistance showed that results were inconsistent with clinical data [266].

The reason behind this may be long-term cell culture which favors cells that are more adapted to these conditions. Upon transfer from the patient to the laboratory, cellular heterogeneity, genetic complexity, and extracellular and stromal signaling are reduced through the removal of the supporting tumor microenvironment [267]. In

addition, due to repeated passaging of the cells genetic aberrations accumulate thereby causing the cells to lose some of their original tumor characteristics [268].

Compared to freshly isolated primary tumor cells cell lines also show a higher level of genome amplification. The reason behind this might be the tissue source, which is mainly metastatic pleural effusions or tumors with triple negative tumor biology [269, 270]. In comparison to other cancer cells, breast cancer cells are particularly difficult to maintain due to the slow rate of proliferation and contamination with normal cells and fibroblasts. Only around 100 cell lines are permanently available and the majority of research data is based on the three most commonly used lines. Another complicating factor is tumor necrosis, reducing the net yield of isolated cells obtained from surgical tissue. There are currently only a small number of cell lines obtained from early stage, primary breast tumors, HCC1937 remains the only cell line with a *BRCA1* mutation. For the luminal A and B breast cancer subtypes there is a lack of representative cell lines [271-273]. This leads to the suggestion that human breast cancer cell lines may only represent a small spectrum of the heterogeneity found in patient tumors both in phenotype and genotype [268].

Regardless of whether cell lines or primary cancer cells are used, a main criticism of standard 2-dimensional (2D) cell culture has been the absence of the tumor microenvironment, which may have an important influence on tumor development, progression, and drug efficacy [274, 275]. The tumor vasculature, stromal and immune cells, signaling molecules such as growth factors and cytokines, as well as the extracellular matrix (ECM) are all key factors in the perpetual interaction between the growing tumor and the surrounding tumor microenvironment [276]. Several of the body's defense mechanisms must be overcome or modulated in order for a tumor to grow in size and spread to distant sites, such as formation of blood vessels, degradation of the surrounding ECM and its components, and transitioning from an endothelial to mesenchymal phenotype. The underlying signals originate from the tumor microenvironment [277].

Through the increasing tumor mass the amount of available oxygen is also decreased, thereby creating a hypoxic environment which is associated with greater genetic instability and increase in angiogenesis. Due to the lack of available oxygen the Warburg effect causes tumor cells to produce lactate leading to an acidic envi-

ronment, although the cancer cells themselves retain a neutral pH. These changes may lead to the dysfunctional behavior of epithelial cells or vice versa [278, 279]. The hypoxic environment has also been implicated in the commissioning of tumor-associated macrophages (TAMs) and mesenchymal stem cells; thereby acting immunosuppressive [280, 281].

Compared to benign breast tissue that contains normal stromal fibroblasts, specific cancer associated fibroblasts (CAFs) are found in malignant breast tumors. These CAFs are involved in tumor progression by secreting growth factors and chemokines, through protease secretion cancer cells may infiltrate the surrounding benign tissue [282, 283]. The origin of these CAFs has not yet been definitely determined; however the current hypothesis states that they may evolve from local fibroblasts, mesenchymal stem cells, and/or epithelial cells. CAFs and other fibroblasts (myofibroblasts and adipocytes) are involved in tumor growth, angiogenesis, promoting endothelial-mesenchymal transition (EMT), remodeling or destroying the ECM, and via the reverse Warburg effect feeding lactate to cancer cells [284].

In addition, through the production of myelogenous cells such as TAMs and tumor-infiltrating lymphocytes (TILs) act as opposing forces in tumor progression. TAMs lack cytotoxic efficacy and are linked to a mild chronic inflammation often found in solid cancer. Regarding treatment efficacy, TAMs may be either beneficial or harmful. A chemoresistance to several compounds has been observed through the induction of TAM-induced survival pathways. Signaling involves cytokines (e.g. TNF, IL-1, IL-10, and IL-17), cathepsins, growth factors, and the recruitment of additional macrophages. Especially the M2 TAM subcategory is associated with an anti-inflammatory and immune suppressive phenotype, leading to a reduced response to chemotherapy (e.g. anthracyclines, platinum compounds, and taxanes). Conversely, a shift toward a M1 macrophage subtype is considered beneficial for treatment efficacy, suggesting a potential target for new targeted drugs [285-287].

TAMs also hide cancer cells through the secretion of IL-10, promote angiogenesis through VEGF + NOS, support tumor growth (EGF), and remodel ECM. An increase in TAMs is associated with a worse prognosis. TAMs have also been found to suppress T-cell activity, thereby actively suppressing an immune response against the tumor.

An increase in TILs found in the surrounding stroma and/or intratumoral compartments is considered beneficial regarding overall prognosis in triple negative and HER2-positive breast cancer [288, 289]. Furthermore, TILs are considered a predictive marker for neoadjuvant therapy in breast cancer, with high levels significantly associated with an increased likelihood for pCR [216, 290].

Cancer cells and the TME restrict the accumulation of TILs through the secretion of exosomes (FasL and TRAIL), potentially CAFs, TAMs and myeloid-derived suppressor cells (MDSCs) are also involved. Apart from suppressing accumulation, survival, reproduction and stromal migration are also restricted. This is mediated through the tumor vasculature, chemokines, induction of apoptosis through Fas-L, IDO, and PD-L1.

These factors all represent potential targets for therapy which is the reason they are essential components of a preclinical assay system which ought to represent the entire tissue not just individual cancer cells. Single-cell suspension and cell monolayer assays using cell lines or patient-tissue derived cells [291-294] cannot fully recapitulate these factors. Assays based on this type of cell culture are currently not sufficiently validated for clinical implementation [295].

In comparison, data suggests that 3-dimensional (3D) spheroid models have the advantage of incorporating components of the tumor microenvironment, as well as other critical factors such as cell-cell and cell-matrix interaction, while maintaining the original tumor architecture with similar physiochemical gradients [296]. In comparison to standard 2D monolayer cell culture, 3D models offer the advantage of a more tissue-like complexity and heterogeneity, tissue-like cellular polarity and interactions. The 3D architecture also results in the formation of a penetration barrier. Cancer cells grown in 3D have demonstrated similar behavior, structure, and organization compared to *in vivo* tissue, making it an ideal model to study treatment efficacy [297]. Several approaches have been studied in regard to predicting drug response *in vitro* [298, 299]. Spontaneous or induced spheroid formation has been observed in cancer cells derived from colon [300-302], gastric [303], lung [304, 305], and breast patient tissue [306-308]. In addition there are several cell lines which form spheroids or aggregates in suspension culture such as MCF-7, T-47D, and BT20 among others

from different tumor types. The aggregation is initiated by the secretion of extracellular matrix components by the cells themselves [303, 309].

This can be achieved with the aid of several methods, such as with the aid of a scaffold, matrigel, hydrogel, microfluidics, cell-derived matrix, extracellular matrix-free spheroid culture, or self-aggregating cells, which exhibit a natural tendency to form close contact with neighboring cells. The type of method used to generate the 3D cell culture as well as the subsequent mode for culturing the engineered construct depend on the aim of the research being done [310, 311].

The main 3D cell culture models that are currently being applied in cancer research are organotypic explant cultures, tumor on a chip, and multicellular spheroids in mono- or co-culture with other cells such as fibroblasts.

In organotypic explant cultures the human or animal tissue in its 3D form remains intact and is the most basic and least technically challenging type of 3D cancer cell culture. Small biopsy or minced surgical patient tissue samples are cultured on top of or embedded in a collagen matrix for a specific length of time to test drug efficacy. While preserving the original tumor architecture of the patient tissue is a key advantage of this culture method, while on the other hand interfering with standardization due to interpatient heterogeneity [255, 312].

The tumor on a chip approach combines a microfluidic system where µm-sized spheroids or tissue slices are cultured for extended periods of time in a replicated model of the human tumor microenvironment. Using interconnected channels, compounds or circulating tumor cells can be applied to the tumor samples and the biochemical composition of the resulting fluid may be assessed in regard to drug transport, target receptor binding, and overall cellular response [313]. Taking this approach one step further allows the incorporation of one or more additional organ-specific compartments. Thereby simultaneously modelling drug efficacy and toxicity [314, 315]. The substance in- and efflux can be minutely controlled to study the effect of different flow properties. Changes in tissue composition may be visualized using among others real-time confocal imaging, viability assays, on-chip cytometry [316, 317]. However, while technologically interesting this type of system currently lacks *in vivo* and clinical validation regarding treatment efficacy outcome.

The nomenclature for multicellular 3D spheroid models varies greatly as summarized by Weiswald et al [318]. As suggested in this review the available methods may further be categorized into four groups: multicellular tumor spheroids (MCTS), tumorspheres, tissue-derived tumor spheres, and organotypic multicellular spheroids. Cells derived from non-malignant tissue or cell lines are also capable of forming a 3D structure in culture and these are termed organoids. Whereas MCTS are mainly generated from cancer cell lines, the other three methods may be applied to cells obtained from patient or xenograft tissue. Tumorspheres may be obtained using small numbers of cells which are allowed to proliferate in stem cell medium until a sphere-like structure is formed *in vitro*. The methodology for both organotypic multicellular spheroids and tissue-derived tumor spheres begins with the mechanical dissociation of the obtained tissue sample. This is followed by enzymatic digestion which leads to a single-cell suspension. The length of the subsequent culture and cell type composition effects the size and compaction of the generated spheroids. Generally a more prolonged culture leads to spheroids larger in diameter with a more compact morphology. In a scaffold-based natural or synthetic hydrogel, and hydrogel-coated plates approach in spheroid culture, the external factors may be manipulated to study cell-cell and cell-matrix interaction as well as cellular migration. While post-experimental harvesting of the spheroids is facilitated, the handling of the hydrogel requires some practice in order to obtain uniform experimental settings and the confined space may limit outward growth and increase necrosis.

Using a spinner or rotating flask, a large number of spheroids can be generated simultaneously. Due to the continuous stirring or rotating movement, the cells cannot attach to the surface of the culture vessel and all elements of the culture medium are evenly distributed. The cells in culture, however, show a tendency to drift toward the sides of the vessel where they form spheroids.

Methods without a scaffold consist of the hanging drop [319], or liquid overlay technique [320], as well as using ultra-low attachment plates. All three methods have in common that cells are self-aggregating and the generated spheroids are less compactly organized compared to scaffold-based methods, which may be attributed to a differential production of fibronectin [321, 322]. Regardless of the method used to generate spheroids, there are several methods available for characterization, such

as microscopic and imaging analysis, direct viability assessment through flow cytometry or via a surrogate measurement in biochemical assays [297, 323, 324].

Aim and objective

Preclinical models that correctly predict treatment outcome on an individual patient basis remain an unmet need in the current drug development process. Contrary to the 2D cell culture models 3D patient-derived spheroids reflect the tumor biology much more accurately and incorporate several aspects of the tumor microenvironment absent in other models and assays [300, 301, 325-328]. The spheroid-based assay proposed herein, was assessed as a diagnostic tool to aid in therapeutic decision-making for primary breast cancer patients receiving neoadjuvant chemotherapy. The objective of the SpheroNEO study detailed in this manuscript was to test whether *in vitro* treatment efficacy results obtained in the breast cancer spheroid model are associated with pCR in primary breast cancer patients undergoing neoadjuvant therapy.

PATIENTS AND METHODS

Machines and laboratory equipment

Refrigerators:	
a) KTe 1740 b) Liebherr Profi Line	Liebherr, Bulle, CH
Incubators:	
a) CO_2 Incubator Series CB210 (53L)	Binder GmbH, Tuttlingen, DE
Laminar Flow:	
a) Laminar Flow Gelaire® Flow Laboratories – Model BSB-6A b) Laminar Flow BDK-SKV 1200	BDK Luft- und Reinraumtechnik GmbH, Sonnen-buehl-Genkingen, DE
Microscope:	
a) Zeiss Axiovert 10 b) Zeiss Axiovert 25 CFL	Carl Zeiss AG, Oberkochen, DE
c) Olympus IX50	Olympus Deutschland GmbH, Hamburg, DE
Pipettes:	
a) Eppendorf Research ® 0.5 – 10 µl b) Eppendorf Research ® 2 – 20 µl c) Eppendorf Research ® 20 – 200 µl d) Eppendorf Research ® 200 – 1000 µl	Eppendorf AG; Hamburg, DE
e) Multichannel pipette Acura ® 851 5 - 50 µl f) Multichannel pipette Acura ® 855 20 - 200 µl	Socorex, Ecubien, CH

Pipetboy:	
a) Accu-jet ®	Brand GmbH & Co KG, Wertheim, DE
b) Pipetus ®	Hirschmann Laborgeräte GmbH & Co KG, Eberstadt, DE

Centrifuges:	
a) Hettich centrifuge Rotixa / RP – Model 4200	Andreas Hettich GmbH & Co. KG, Tuttlingen, DE
b) Eppendorf centrifuge type 5415 C c) Eppendorf centrifuge type 5810R	Eppendorf AG; Hamburg, DE

Photometer:	
a) FilterMax™ F3 – Multi Mode Microplate Reader b) Microplate reader VersaMax	Molecular Devices, LLC, Sunnyvale, CA, USA
c) Tecan Elisa-Reader Ultra384	Tecan Deutschland GmbH, Crailsheim, DE

Scales:	
a) Precision scale Model 2100g	Sartorius, Göttingen, DE
b) Scale model 440 21N	Kern & Sohn GmbH, Balingen-Frommern, DE

Water bath:	
a) Water bath type 1002	Gesellschaft für Labortechnik mbH, Burgwedel, DE
b) Water bath type OLS200	Grant Instruments, Cambridge, UK
c) Water bath type 3047	Köttermann GmbH & Co KG, Uetze/Hänigsen, DE

Materials:	
a) Neubauer Improved cell counting chamber (chamber depth = 0.100 mm)	LO Labor Optik GmbH, Friedrichsdorf, DE
b) Safe – Lock tubes 1.5 ml, 2 ml, 5 ml c) Ep Dualfilter T.I.P.S. d) ep T.I.P.S. Reloads	Eppendorf AG; Hamburg, DE
e) Falcon™ conical centrifuge tubes 50 ml	Corning Inc., Corning, NY, USA
f) Centrifuge tubes 15ml	TPP Techno Plastic Products AG, Trasadingen, CH
g) Disposable reagent reservoir 50ml h) Polystyrene serologic pipettes 2, 5, 10, 25 ml i) Sterile, nylon cell strainer 40, 70, 100 µm	Corning Inc., Corning, NY, USA
j) Surgical disposable scalpel nr. 22	Braun, Aesculap AG, Tuttlingen, DE
k) Costar® assay plate, 96 well, with lid, flat bottom, sterile, white polystyrene	Corning Inc., Corning, NY, USA
l) Glass petri dish	Manufacturer unknown
m) Pasteur pipettes Volac®	Sigma-Aldrich Chemi GmbH, Heidenheim, DE
n) Plastic petri dish easy grip Ø 60, 100 mm sterile o) Syringe BD Discardit™ II	Becton Dickinson GmbH, Heidelberg, DE
p) Sterile forceps	VWR International GmbH, Darmstadt, DE

Chemicals and reagents:	
a) Trypan blue solution 0.4%	Sigma-Aldrich Chemie GmbH, Heidenheim, DE
b) DMEM F12 (1:1) L-glutamine, 1.2g/l $NaHCO_3$, with glucose c) Fetal bovine serum (FBS) d) Non-essential amino acids (NEAA) without L-glutamine e) Vitamin 100x concentrate	Pan-Biotech, Aidenbach, DE
f) Denatured Ethanol 80%	Pharmacy University Hospital LMU, DE
g) Metronidazol 5 g/l	B. Braun Melsungen AG, Melsungen, DE
h) Amphotericin B 0.25 g/l	Biochrom GmbH, Berlin, DE
i) Ampicillin 50 g/l j) Gentamycin 40 g/l	Ratiopharm®, Ulm, DE
k) Ciprofloxacin 2 g/l	Fresenius Kabi Deutschland GmbH, Bad Homburg, DE
l) Dulbecco's phosphate buffered saline solution, without calcium or magnesium	Pan-Biotech, Aidenbach, DE
m) Liberase™ TM (Thermolysin Medium) Research Grade	Roche, Basel, CH
n) ATP Reagent CellTiter-Glo® Luminescent – Cell Viability Assay o) CellTiter 96® AQ_{ueous} MTS Reagent Powder	Promega, Mannheim, DE
p) Phenazine methosulfate	Sigma-Aldrich, Steinheim, DE
q) Methocoel (proprietary composition)	SpheroTec GmbH, Martinsried, DE

Cell culture media

a) Supplemented culture medium/shipping medium (proprietary composition)

- 500 ml DMEM F12
- 10% FBS
- 2.5x/ml NEAA
- 2.5x/ml Vitamin concentrate 100x
- Ampicillin
- Amphotericin B
- Ciprofloxacin
- Gentamycin
- Metronidazole

b) Medium for enzymatic digestion

- 500 ml DMEM F12
- 2.5x/ml NEAA
- 2.5x/ml Vitamin concentrate 100x
- Liberase TM Research Grade

c) Culture medium

- 500 ml DMEM F12
- 10% FBS
- 2.5x/ml NEAA
- 2.5x/ml Vitamin concentrate 100x

Study design

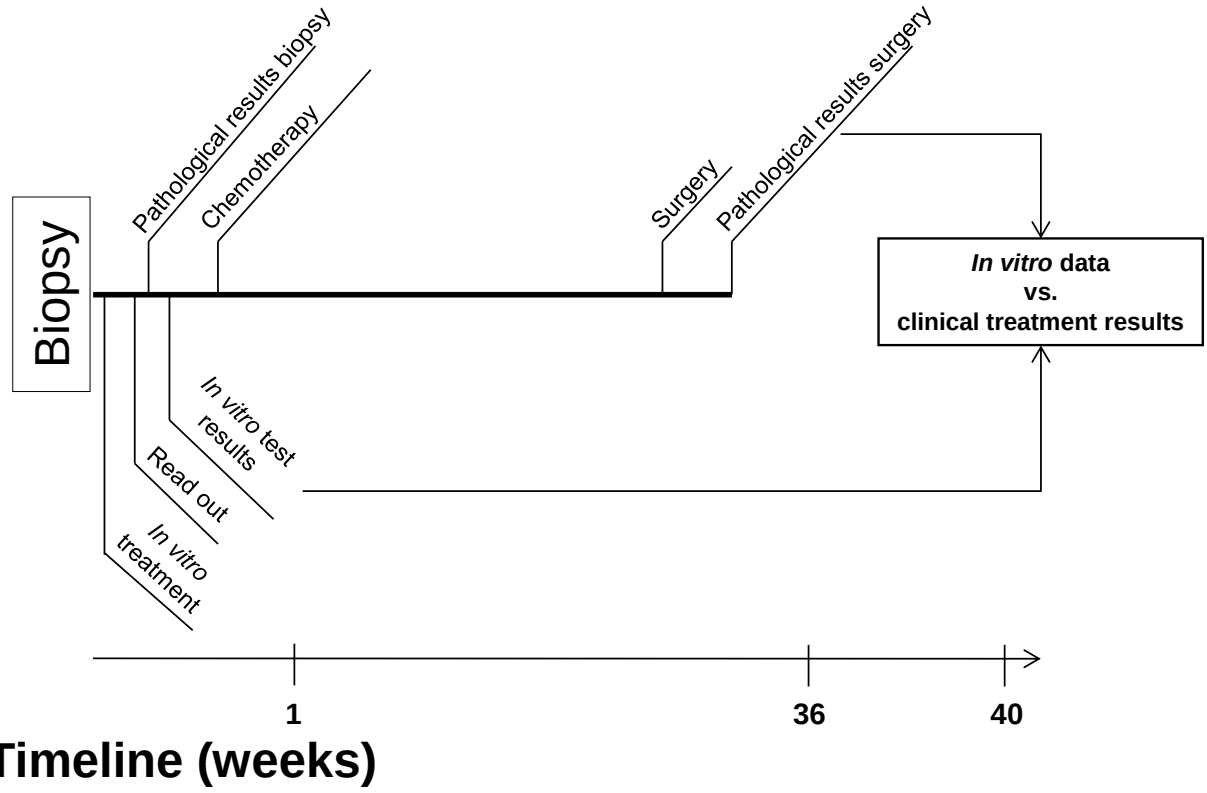


Figure 2: Study design overview, timeline. Study screening procedure begins during the initial biopsy. Patient informed consent is obtained prior to the start of the biopsy procedure. Duration of the individual steps in the course of the clinical treatment is dependent on the selected chemotherapy regimen and other clinical and patient factors.

The SpheroNEO study was designed as a prospective, non-interventional cohort study. Primary breast cancer patients (N=202) eligible for neoadjuvant chemotherapy were consecutively recruited for the study starting from October 2009 until September 2012 (Figure 2).

In a feasibility assessment prior to the beginning of the study, breast cancer centers in Germany were asked to fill out a questionnaire estimating the number of potential patients eligible for neoadjuvant treatment. Sixty of the sites addressed for study feasibility, 27 responded and 15 were interested in study participation. A total of seven met criteria for sufficient patient numbers and were initiated. An additional 7 sites in close association with the coordinating LMU University Hospital were also asked to participate without any prior feasibility assessment. All 14 study sites were asked to specify their preferred neoadjuvant chemotherapy regimen and to supply a ranking of the most frequently applied treatment schemes. However, during the course of

this study this information proved unreliable, and a patient-by-patient recommendation made by the treating physician the day of the biopsy procedure was more likely to correspond to the actual clinical treatment the patient received (data not shown). The complete list of all participating breast cancer centers can be found in the attachment (Table 12). The study was performed in accordance to all applicable GCP regulations and approved by all applicable ethics committees (ethics committee reference Nr. 278/04, Ethikkommission bei der LMU München). Patients were asked to participate and recruited to the study following clinical diagnosis either through palpation and/or radiological examination prior to the start of chemotherapy. Biopsy samples were obtained simultaneously for the SpheroNEO study and pathological confirmation of an invasive tumor. A written consent was obtained from all eligible patients prior to the biopsy procedure.

The in- and exclusion criteria were as follows:

Inclusion criteria:

- Standard clinical, radiological, and pathological treatment
- Women >18 years old
- Histological confirmed case of breast cancer
- Written and oral consent to participate in the study
- Ability to cooperate and understand the aims of the study
- **Laboratory:** Cellular vitality of the study sample > 50 %

Exclusion criteria:

- Medical history of breast cancer
- Previous or simultaneous malignant tumor (exceptions: squamous-cell or basal-cell carcinoma of the skin or cervical cancer *in situ*)

- **Laboratory:** Contamination of the spheroids by end of therapy ≥ 50 %
- **Laboratory:** Study sample shipment > 72 h
- **Laboratory:** Less than 4 wells with 10 000 cells each are available in cell culture for therapy prior to treatment

Patients with a metastasized disease were also excluded for the final analysis. The clinical treatment for each patient was performed according to the standard neoadjuvant protocol [329-331] of each study site in accordance to national guideline recommendations. The final treatment decision was made by a tumor board consisting of all treating physicians from the respective site following the completion of all relevant clinical and pathological examinations. The resulting recommendation for a particular treatment combination was communicated to the laboratory and arrived together with the tumor biopsy sample. An equivalent *in vitro* treatment was applied accordingly.

Results obtained in the breast cancer spheroid model were not communicated to the treating physician(s) and had no impact on the clinical treatment of the patients. A comparison of the treatment efficacy *in vitro* and clinical outcome, documented in the pathological report of the surgical specimen, was performed after all patients had completed neoadjuvant drug treatment and underwent the subsequent surgery.

Breast cancer spheroid model

The method to generate patient-derived spheroids used here can be referred to as a modified liquid overlay technique, which is described in previous publications [320, 332]. The method is patent-protected, for this reason some details are proprietary.

A freshly prepared sterile shipping medium was sent to the participating study sites each week with an expiration date set to two weeks following production. The tumor tissue was immediately transferred into medium after excision and shipped at 4-8°C to the laboratory (SpheroTec GmbH, Martinsried, DE) for cell isolation.

The entire laboratory procedure was performed according to standardized, quality-controlled (ISO9001:2008) operating procedures. All cell culture steps were per-

formed in the central laboratory under a laminar flow-hood for semi-sterile conditions. Upon arrival, the study samples were transferred into fresh medium and stored at 4°C for 1.25 – 162.75 min until the start of the cell isolation procedure. Subsequently, the tumor samples underwent mechanical and enzymatic digestion using an enzyme cocktail (Roche, Penzberg, DE) to generate a single-cell suspension. Cell number and viability were quantified using the trypan-blue exclusion test (Sigma Aldrich, Heidenheim, DE). The isolated cells were then evenly distributed on a 96-well plate together with a proprietary non-scaffold medium substrate. Spheroid formation took place in the subsequent 48 h under standard culture conditions (37° C, 5% CO_2). Following this period of incubation, the morphology was assessed using bright-field microscopy.

The spheroids were treated *in vitro* with the treatment combination recommended by the responsible tumor board of each study site. The peak plasma concentration (ppc) was used. The applied ppc was based on a literature search documenting the results of phase one and/or pharmacokinetic studies of the same or other tumor types. For each experiment/study sample control wells were included using the solvent control of the applied substance(s). Both solvent controls and ppc for each substance are listed in Table 1 below. In order to control for material artefacts of the utilized plates and medium, negative control wells without cells were also included. Values obtained from this blank control were subtracted from measured absolute data.

Table 1: Overview of the utilized compounds and the corresponding ppc concentration.

Compound	1 ppc [µg/ml]	Molecular weight [g/mol]	1 ppc [µmol/l]	Solvent	Literature source
Carboplatin	40.843	371.254	110.014	H_2O	[333]
Cyclophosphamid	41.000	261.086	157.036	NaCl	[334]
Docetaxel	2.180	807.8792	2.698	EtOH	[335]
Doxorubicin	1.640	543.5193	3.017	NaCl / H_2O	[336]
Epirubicin	1.005	543.5193	1.849	NaCl	[337]
5-FU	100.000	130.0772	768.774	H_2O	[333]
Paclitaxel	1.530	853.9061	1.792	EtOH	[333]
Trastuzumab	88.000	145531.5	6.047	H_2O	[338]

ppc, peak plasma concentration, NaCl, Sodium chloride, EtOH, Ethanol, 5-FU, 5-Fluorouracil.

Following the 96 h drug incubation period, treatment efficacy was determined using a standard assay measuring metabolic activity (Promega, Mannheim Germany) to quantify cell survival *in vitro*, MTS (fluorescence) or ATP (luminescence). Both viabil-

ity assays were performed according to manufacturer's instructions. Irrespective of the applied readout technology, mean cell survival was expressed as percent of metabolic activity relative to control wells. Laboratory test results were available after eight days.

Data management and statistics

Clinical and laboratory data was recorded in a case report form (CRF) and collected from all clinical sites at the end of the study. Study documentation was done in part by on-site staff and external personnel. A clinical research organization was commissioned with monitoring activities and the collection of the completed CRF. The clinical and laboratory data was subsequently entered into a relational database specifically designed for the trial data (Access 2010). Correct clinical data entry was verified by two physicians, and laboratory data was verified by several members of the laboratory team. Statistical analysis was done in cooperation with the Institute for Medical Informatics, Biometry and Epidemiology (IBE) in Munich. The data was extracted from the database to a SPSS file and provided to an independent statistician for analysis. The patient cohort was analyzed as intention-to-treat, meaning the treatment, which each patient initially began was used for the subsequent correlation irrespective of change in regimen, incomplete number of cycles, or dose reduction. Clinical and laboratory data was described separately for patients with and without pCR using appropriate measures of location and dispersion. The discriminatory power of the spheroid model and traditional risk factors were analyzed using receiver operating characteristic (ROC) curves and the corresponding c statistics (areas under the ROC curves, AUC). The ROC curves were calculated with the logistic regression procedure using the R package penalized. A potential cutoff was calculated based on the 95th percentile of the residual activity in women with pCR and the resulting sensitivity and specificity. The respective 95% confidence intervals were considered for each factor. A calculation of the Youden Index resulted in the same cutoff value. Statistical tests for categorical factors were done using Pearson's chi-square or Fisher's exact tests, ANOVA and t-tests for numerical variables. Strength and type of correlation between two numerical factors was done using the Pearson's product-moment correlation and Pearson's r is reported. Associations for ordinal factors were tested using the Spearman's rank correlation coefficient. Odds ratios for cell survival and frequently used clinical predictors of pCR were calculated using univariate and

multiple regression models. Odds ratios in the multiple-regression models were calculated using a forward selection based on Wald tests. Single factor regression analysis was done using a linear regression model and the change in R^2 was determined. All hypotheses tested were two-sided on an alpha level of 5%. Analyses were performed using the Statistical Analysis System SAS, version 9.2 for Linux (SAS Institute, Cary, NC, USA), R version 2.12.2 (The R Foundation for Statistical Computing, Vienna, AT), as well as SPSS Statistics version 22.0 (IBM, Armonk, NY, USA).

RESULTS

Halfter K, Ditsch N, Kolberg HC, Fischer H, Hauzenberger T, von Koch FE, Bauerfeind I, von Minckwitz G, Funke I, Crispin A**,** Mayer B *et al*: Prospective cohort study using the breast cancer spheroid model as a predictor for response to neoadjuvant therapy - the SpheroNEO study. *BMC Cancer* 2015, 15:519.

Study participant in- and exclusion

A total of 202 patients were enrolled in the SpheroNEO Study from 14 clinical sites associated with the University Hospital of the LMU, as well as other breast cancer centers located in the central and southern part of Germany. The mean drop-out rate was 65.15% with a range of 25-100% per study site. A total of 15.25 patients were enrolled and screened in the study per month, the recruitment per site ranging from 0.25 to 3.33 patients per month, data is shown in detail in Table 12 of the supplementary material. The study was generally well-received due to its simple management for the on-site hospital staff.

Exclusion criteria for the screened patients as shown in the study flowchart (Figure 3) are separated into clinical and laboratory criteria. The two main clinical reasons for exclusion related to the selection of the treatment regimen. A total of 30 patients did not receive neoadjuvant treatment as initially planned, instead underwent surgery and/or adjuvant chemotherapy, anti-hormone treatment. Patients with a diagnosis of metastatic disease (N=5) were also excluded from the final analysis.

The second main reason for exclusion (N=21) was the discrepancy between clinical and *in vitro* treatment selection. The initial recommendation for the *in vitro* treatment regimen was communicated upon shipment of the tissue specimen. At this time point, the final pathological report as well as the staging examination had not been completed and no final treatment decision had been made. In some cases, data obtained through these staging examinations caused the tumor board of the respective clinical study site to recommend a different treatment regimen from the one initially recommended.

A prior malignant disease lead to the exclusion of 7 patients, this criteria applied to malignant neoplasms which appeared in the patients' medical history at least 5 five years prior to the current diagnosis. A total of 6 patients were considered loss to follow-up; reasons were incomplete clinical documentation, continuation of treatment in

the patient's country of origin, or a patient's decision to switch medical facilities for subsequent treatment. Two samples were obtained from suspicious lesions which were diagnosed as benign upon further inspection.

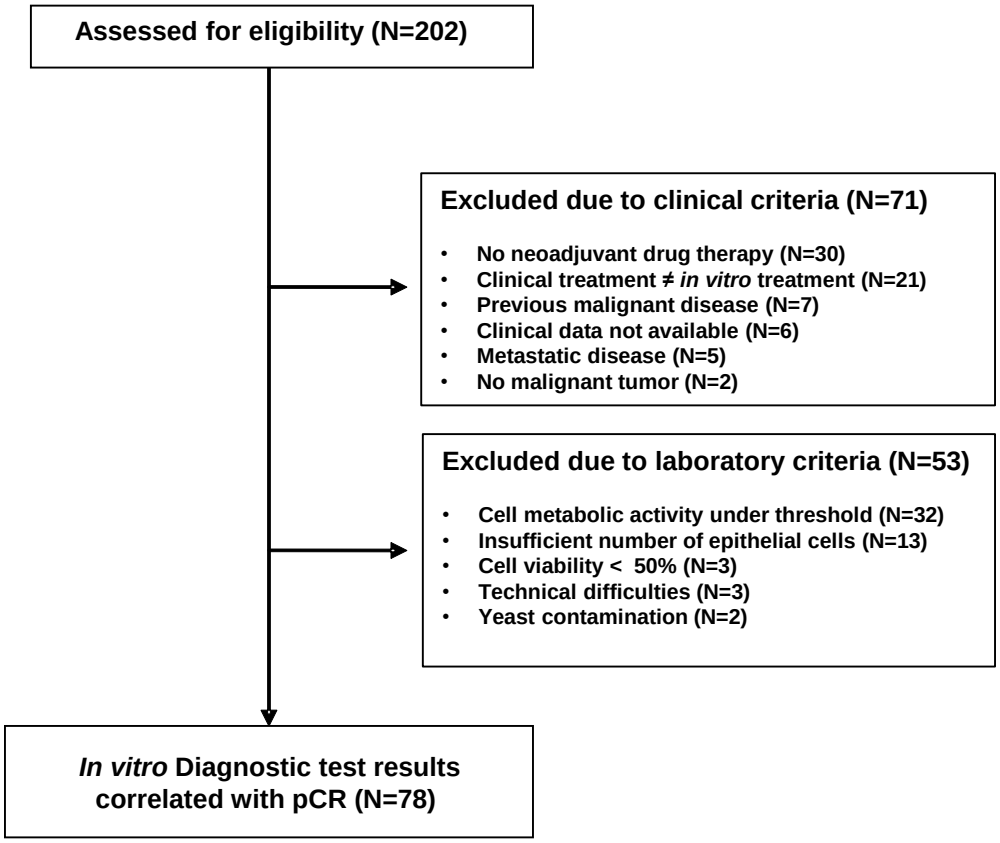


Figure 3: Study flowchart depicting the screening process of the patients in the study cohort. ≠, unequal to, pCR, pathologic complete response.

The main reason for laboratory exclusion was low baseline metabolic activity. A threshold twice the mean luminescence as measured in the untreated controls was considered sufficient for differential results (107.5 cps). Metabolic activity below this threshold was found for 32 study samples, which was significantly associated with a low sample weight (r(175) =.177, *p* =.019). The number of necessary biopsy cylinders was not specified in the initial study protocol and varied between patients and study sites. Site staff reported problems such as bleeding, patient discomfort, and difficulty maintaining a good biopsy trajectory as reasons for low number of additional biopsy cylinders available for the study. A mean of 3.55 cylinders were received per study sample with a range of 1-12. A total of 13 patients were excluded due to the insufficient number of isolated cells (12 000 total isolated cells per study sample).

Cell viability over all received samples was good with a mean of 85.92% (range 6.10 – 100%), three patients were excluded with a viability < 50%. In the final analysis the threshold of 70% cellular viability as previously specified in the study synopsis was determined to be an insufficient determinant for inclusion given the diverse tissue composition of the study samples, this led to the subsequent inclusion of two patients with a cellular viability <70%. One patient was included in the final analysis despite low viability at 26.2% since the number of vital cells was sufficient to obtain successful assay readout (3.41 $*10^6$ total isolated cells). Reason for low viability were insufficient shipping conditions and handling of the study samples (transportation in NaCl solution (N=2), or a long period before transfer to culture medium). Yeast contamination of the cultured cells was observed for two samples. Technical difficulties led to the exclusion of three study samples. During the initial phase of the study the laboratory criteria for a successful assay were defined broadly to compensate for problems in the initial setup of the trial logistics. In the later phase of the study, laboratory criteria were amended according to the experience gained from the starting phase. The inclusion and exclusion criteria were amended to compensate for the information gained during the beginning of the study (Amendment III approved 10.11.2011) by expanding the age of eligibility to patients older than 18 at diagnosis, excluding patients with a tumor diagnosis simultaneous to or ≤ 5 years prior to study enrollment, as well as the addition of laboratory inclusion criteria (cellular vitality of <70%, study sample shipment duration of <48h).

The final analysis included 78 patients, who qualified according to both clinic and laboratory criteria. The anonymized patient list is found in the supplementary materials with applicable exclusion criteria for all of the screened patients (Table 13).

Factors influencing successful assay outcome

A successful assay outcome, defined as valid readout results distinctly discerning cell survival from the applied cytostatic treatment to the solvent control, was significantly associated with several laboratory factors as shown in Table 2. Out of all samples, a total of 129 (63.9%) met all laboratory eligibility criteria. The following analysis compares these 129 samples to the remaining 73 that did not meet all criteria. The most prominent factors for assay success were directly or indirectly correlated with the absolute number of cells isolated from the study samples. The absolute

number of isolated cells per mg and metabolic activity of the solvent controls per cell was also highly correlated with assay success (successful assay outcome with mean 0.515 *10^6 vs 0.097 *10^6 cells; p =.000; 0.306 vs 0.052 cps/cell, p =.023). Total samples weight (mean 102.02 vs 44.28 mg, p =.000), number of biopsy cylinders received (mean 3.87 vs 2.94, p =.000), total enzyme used during tissue dissociation (mean 1.32 vs 0.98 units, p =.001), cell viability following digestion (87.48% vs 82.54%, p =.008), number of cells per spheroid (mean 23 977 vs 11 762.55 cells per spheroid, p =.000), number of generated spheroids (mean 7.18 vs 4.55, p =.000), number of treatment options applied per study sample (mean 1.72 vs 1.20, p =.000), and readout method used following treatment *in vitro* (mean 90.9% MTS vs 67.8% ATP, p =.009) were all significantly correlated to a valid assay outcome.

Regression analysis showed that the absolute number of cells isolated per study sample (all biopsy cylinders received per patient) allowed a fairly accurate estimate for the number of treatment options which could be tested in the assay (Figure 4A, R^2=0.264, p =.000). This factor is critical for the comparison of several clinically relevant treatment options in the assay, using the resulting linear equation a minimum of 2.984 *10^6 cells are necessary to test four treatment options in the assay. This cell number corresponds to a total samples weight of 248.08 mg. As previously mentioned, the number of isolated cells also factors highly in the successful readout at the end of the assay. Sample characteristics figuring most importantly in the total number of isolated cells were total sample weight and weight per received biopsy cylinder. A regression analysis of both factors resulted in a highly significant estimation of net cell yield (Figure 4B and 4D, total sample weight, R^2=0.764; weight per biopsy cylinder, R^2=0.166, both p =.000). The number of biopsy cylinders received per study sample was also associated with net cell yield but the association was not significant (Figure 4C, R^2=0.032, p =.053).

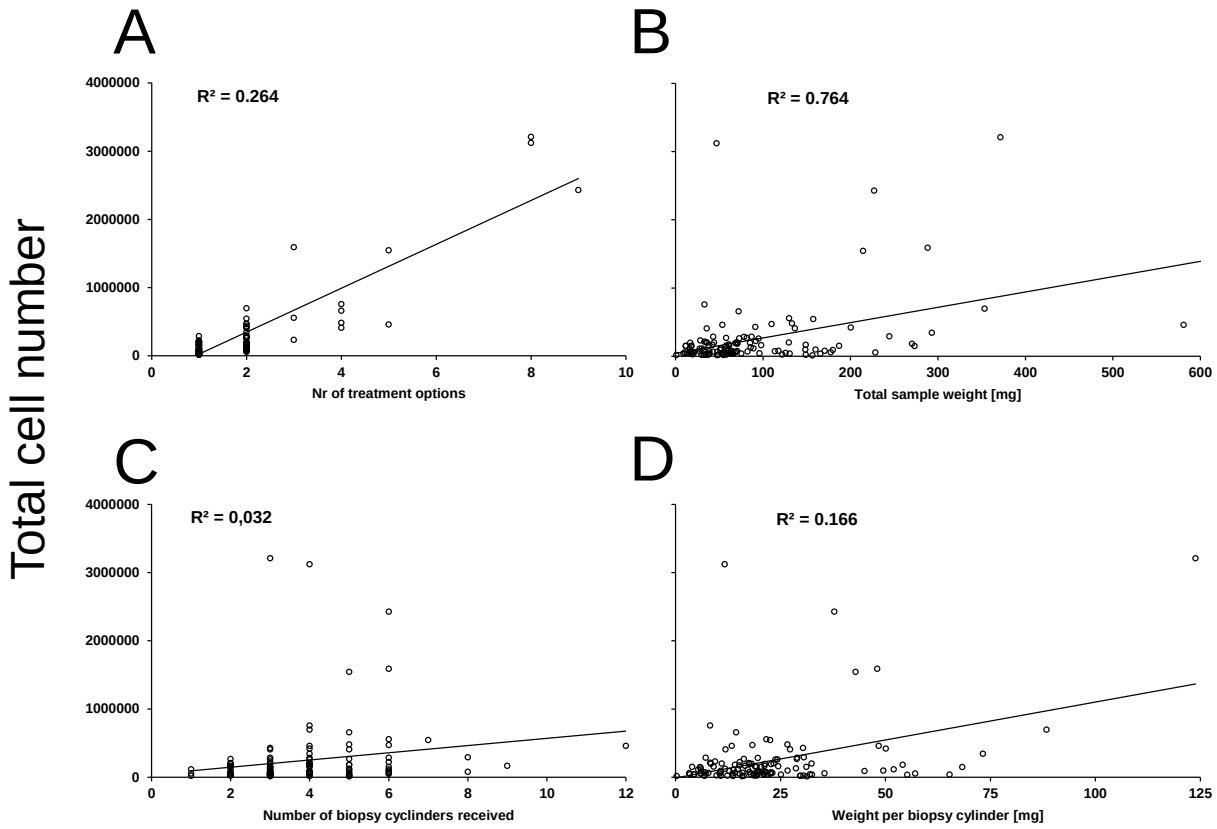


Figure 4: Factors influencing assay outcome, relation between the absolute number of isolated cells per study sample (N=129 eligible according to laboratory criteria) and A) number of treatment options, B) total sample weight, C) the number of biopsy cylinders received per patient, and D) weight per individual biopsy cylinder. The coefficient of determination as obtained through regression analysis is displayed at the top of each graph.

Table 2: Overview of factors influencing assay outcome**,** overall received study samples and compared according to successful assay outcome.

				Lab criteria fulfilled				
				Yes		No		
		N	%	N	%	N	%	*p*-value
All Study samples		202	-	129	63.9	73	36.1	
Tissue and logistic criteria								
No. of biopsy cylinders received								**.000**
	Mean	3.55		3.87		2.94		
	Range	1-12		1-12		1-6		
Weight study sample [mg]								**.000**
	Mean	82.573		102.02		44.28		
	Range	0.90–1568.00		8.90-1568.00		0.90-176.50		
Biopsy method								.427
	Core needle biopsy	198	98.0	126	63.6	72	36.4	
	Vacuum-assisted biopsy	2	0.99	2	100	0		
	Excision biopsy	2	0.99	1	50	1	50	
No. of technical replicates								.939
	Mean	2.19		2.20		2.14		
	Range	1-6		1-6		2-5		
Transport in medium								.153
	Yes	181	89.6	121	66.9	60	33.1	
	No	9	4.5	5	55.6	4	44.4	
	Not documented	12						

Transport medium not expired								.460
	Yes	153	75.7	101	66.0	52	34.0	
	No	21	10.4	16	76.2	5	23.8	
	Not documented	28	13.9					
No. of days TM expired [d]								.768
	Mean	7.33		8.19		4.60		
	Range	1-61		1-61		1-9		

Characteristics cell isolation procedure

Time diff. biopsy – begin lab procedure [h]								.447
	Mean	27.23		25.80		30.05		
	Range			1.25-87		7.25-162.75		
Mechanical isolation [min]								.868
	Mean	11.57		11.81		11.10		
	Range	2-45		2-45		3-23		
Enzymatic isolation [min]								.711
	Mean	158.42		156.99		161.30		
	Range	103-285		103-275		107-285		
Total time isolation procedure								.836
	Mean	169.99		168.80		172.40		
	Range	112-295		112-287		119-295		
Total enzyme added [units]								**.001**
	Mean	1.21		1.32		0.98		
	Range	0.65-5.20		0.65-5.20		0.65-2.6		
Total No. of vital cells [*10^6]								**.000**
	Mean	0.383		0.515		0.097		
	Range	0-34.092		0.012-34.092		0-1.420		
Cell viability [%]								**.008**
	Mean	85.92		87.48		82.54		
	Range	6.10-100		26.2-100		6.1-100		
No. of cells per spheroid								**.000**
	Mean	20206.29		23977.68		11762.55		
	Range	852-187500		2841-187500		852-80000		
No. of cells per spheroid								**.000**
	< 10 000	68	33.7	32	47.1	36	52.9	
	10 000 – 20 000	45	22.3	36	80.0	9	20.0	
	> 20 000	69	34.2	59	85.5	10	14.5	
	Not documented	20	9.9	2	10.0	18	90.0	
No. of spheroids generated								**.000**
	Mean	6.34		7.18		4.55		
	Range	0-60		2-60		0-35		

Cellular characteristics *in vitro*

Incubation of generated spheroids [h]								.853
	Mean	50.22		49.97		50.86		
	Range	36-94		36-79		46-94		
No. of treatment options								**.000**
	Mean	1.57		1.72		1.20		
	Range	1-9		1-9		1-6		
Metabolic activity solvent controls [cps/cell]								**.023**
	Mean	0.235		0.306		0.0516		
	Range	0.000 – 6.648		0.000 – 6.648		0.000 – 0.620		
Readout method								**.009**
	ATP	143	70.8	97	67.8	46	32.2	
	MTS	33	16.3	30	90.9	3	9.1	
	No readout	26	12.9					

No., number of, TM, transport medium, d, days, h, hours, min, minutes, cps, counts per second, ATP, adenosine triphosphate, MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

Factors related to the logistics of the sample shipment (biopsy method, transportation medium, expiration thereof) as well as characteristics of the cell isolation procedure (time difference between tissue excision, duration of mechanical, enzymatic, total duration of tissue isolation) did not show any significant impact on assay success, see Table 2. The time difference between tissue excision and the beginning of

the laboratory procedure (cell isolation, p =.526), as well as the number of technical replicates also did not have an impact on assay outcome (p =.939).

As described in the methods section above, spheroid formation was confirmed following a mean of 50.22 h (range 36-94 h) incubation. Differences in incubation time were not significantly associated with assay success (49.97 vs 50.86 h, p =.853).

Clinical factors were also tested in regard to assay success: only age was significantly associated with assay success (p =.007). A higher percentage of patients over 50 years were excluded due to metabolic activity below threshold (20 out of 32 excluded patients). However, an association with laboratory exclusion criteria was not found (p =.123). All other tested factors were not associated with assay success, as shown in Table 3.

Table 3: Overview of association of clinical factors with successful assay outcome.

		Laboratory criteria fulfilled				
		Yes		No		
		N	%	N	%	*p*-value
All patients		129	63.9	73	36.1	
Clinical characteristics						
Age at diagnosis [y]						.183
	≤50	62	69.7	27	30.3	
	>50	67	59.8	45	40.2	
Mean		53.2		56.1		.123
Range		21-85		32-82		
BMI						.604
	Under/Normal weight	56	67.5	27	32.5	
	Overweight/Adipose	55	72.4	21	27.6	
	Not documented	18	41.9	25	58.1	
Effected side						.083
	Left	71	68.3	33	31.7	
	Right	47	57.3	35	42.7	
	Not documented	11	68.8	5	31.2	
Centricity/focality						.651
	Multifocal	30	58.8	21	41.2	
	Multicentric	14	70.0	6	30.0	
	Unicentric	75	64.1	42	35.9	
	Not documented	10	71.4	4	28.6	
Quadrant						.409
	Central	7	70.0	3	30.0	
	Upper-inner	14	77.8	4	22.2	
	Lower-inner	2	33.3	4	66.7	
	Upper-outer	28	65.1	15	34.9	
	Lower-outer	7	77.8	2	22.2	
	Several overlapping quadrants effected	10	76.9	3	23.1	
	Caudal	1	50.0	1	50.0	
	Quadrant not specified	1	50.0	1	50.0	
	Not documented	-		1	100	
Tumor stage						1.000
	cT1/T2	90	64.3	50	35.7	
	cT3/4	31	64.4	17	35.4	
	Not documented	8	57.1	6	42.9	

						.222
Nodal status						
	cN+	65	69.9	28	30.1	
	cN-	58	61.1	37	38.9	
	Not documented	6	42.9	8	57.1	
UICC						.251
	IA	10	66.7	5	33.3	
	IIA	47	63.5	27	36.5	
	IIB	31	57.4	23	42.6	
	IIIA	19	86.4	3	13.6	
	IIIB	7	63.6	4	36.4	
	IIIC	0	-	1	100	
	IV	5	71.4	2	28.6	
	Not documented	10	55.6	8	44.4	
Clinical tumor size at diagnosis, largest diameter [cm]						
MRI (N=69)						
	Mean	3.4		3.8		.361
	Range	0.7-9.5		1.0-8.0		
Mammography (N=137)						
	Mea	3.2		2.9		.238
	Range	1.0-10.0		0.1-7.0		
Sonography (N=169)						
	Mean	3.0		2.8		.273
	Range	1.15-8.0		0.11-6.0		

Histopathological characteristics

Grading						.123
	G1/2	65	19.5	40	80.5	
	G3	56	37.1	29	62.9	
	Not documented	8	66.7	4	33.3	
Histologic type						.361
	Ductal invasive	81	62.8	48	37.2	
	Ductal invasive + DCIS component	14	56.0	11	44.0	
	Lobular invasive	16	69.6	7	30.4	
	Invasive ductal/lobular	1	100	0	-	
	Medullary	2	100	0	-	
	Inflammatory	2	50.0	2	50.0	
	Other	10	90.9	1	9.1	
	Not documented	3	42.9	4	57.1	
ER status						.732
	Negative	38	69.1	17	30.9	
	Positive	81	65.3	43	34.7	
	Not documented	10	43.5	13	56.5	
PR status						.428
	Negative	52	70.3	22	29.7	
	Positive	69	64.5	38	35.5	
	Not documented	8	38.1	13	61.9	
HER2 status						.593
	Negative	94	18.6	47	81.4	
	Positive	28	62.5	17	37.5	
	Not documented	7	43.8	9	56.2	
Triple negative						.432
	Yes	27	73.0	10	27.0	
	No	85	64.4	47	35.6	
	Not documented	17	51.5	16	48.5	
Ki67 (biopsy)						.230
	<=30% (median)	39	22.2	29	77.8	
	> 30%	31	42.3	13	57.7	
	Not documented	59	65.6	31	34.4	
Mean		37%		28%		.084
Range		3-99%		1-85%		
HR/HER2						.853
	HR+/HER2+	20	66.7	10	33.3	
	HR+/HER2-	62	65.3	33	34.7	
	HR-/HER2+	7	63.6	4	36.4	
	HR-/HER2-	27	73.0	10	27.0	
	Not documented	13	44.8	16	55.2	

y, years, BMI, body mass index, UICC, Union for International Cancer Control, MRI, magnetic resonance imaging, DCIS, ductal carcinoma *in situ*, ER, estrogen receptor, PR, progesterone receptor, HER2, human epidermal growth factor receptor 2, HR, hormone receptor.

Laboratory baseline factors of the study cohort

The laboratory baseline data is shown in

Table 4. A mean of 4 biopsy cylinders were received per study sample with a range of 1-12. The weight per cylinder averaged at 22.71 mg, ranging between 3.28 to 123.87 mg for the samples in the final analysis. The total weight of tumor tissue per received sample averaged to 114.1 mg (range 8.9 – 1568.0 mg). The majority of study samples were obtained through a core needle biopsy procedure (96.2%); however two samples were obtained through a vacuum-assisted biopsy and one through an excisional biopsy procedure.

Table 4: Summary of laboratory baseline characteristics and correlation with the mean cell survival of the clinically relevant cytostatic treatment *in vitro* as measured in the breast cancer spheroid model.

		N	%	% cell survival	*p*-value
All Study samples		78	100		
Cell survival					
	Mean		51.97		
	Range		3 – 103		
Tissue and logistic criteria					
No. of biopsy cylinders received					.486
	Mean		4		
	Range		1-12		
Weight study sample [mg]					.150
	Mean		114.1		
	Range		8.9-1568.0		
Biopsy method					*
	Core needle	75	96.2	53.8	
	Vacuum-assisted	2	2.6	7.3	
	Excision	1	1.3	4.1	
Transport in medium					*
	Yes	74	94.9	53.0	
	No	3	3.8	35.3	
	Not documented	1	1.3	-	
Transport medium not expired					.208
	Yes	61	78.2	54.6	
	No	12	15.4	42.7	
	Not documented	5	6.4	-	
No. of days TM expired [d]					.742
	Mean		10		
	Range		1-61		
Characteristics cell isolation procedure					
Time diff. biopsy – begin lab procedure [h]					.526
	Mean		26.3		
	Range		4.75-85.5		
Mechanical isolation [min]					.579
	Mean		11.76		
	Range		3-45		
Enzymatic isolation [min]					.937
	Mean		160.6		
	Range		103-275		
Total time isolation procedure [min]					.971
	Mean		172.3		
	Range		112-287		

Total enzyme added [units]					.520
	Mean		1.4		
	Range		0.65-5.2		
Total no. of vital cells after filtration					.102
	Mean		0.686 x10[b]		
	Range		13200-34.092x10[b]		
Cell viability [%]					.121
	Mean		87.2		
	Range		26.2-97.6		
No. of cells per spheroid					.827
	Mean		24646		
	Range		2841-187500		
No. of cells per spheroid grouped					.841
	< 10 000	22	28.2	55.5	
	10 000 – 20 000	20	25.6	50.8	
	> 20 000	36	46.2	50.5	
No. of spheroids generated					**.008**
	Mean		7		
	Range		2-60		
No. of technical replicates					**.023**
	Mean		2		
	Range		1-6		
Incubation of generated spheroids [h]					**.010**
	Mean		50		
	Range		36-75		
Metabolic activity [cps/cell]					**.023**
	Mean		0.322		
	Range		0.000 – 6.648		
No. of treatment options					**.002**
	Mean		2		
	Range		1-9		
Readout method					**.000**
	ATP	62	79.5	42.2	
	MTS	16	20.5	89.9	

* No statistics available due to sample size < 5 in the subgroup comparison. No., number of, TM, transport medium, d, days, h, hours, min, minutes, cps, counts per second, ATP, adenosine triphosphate, MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

The time between biopsy harvesting and the start of the cell isolation was between 4.75 and 85.5 h, with a mean of 26.3 h. Twelve samples (15.4%) were shipped in expired media with a range between 1 to 61 days past expiration date, however this was not associated with any differences in assay quality or outcome. Mechanical isolation varied with tissue consistency and percentage of included connective tissue, a mean of 11.76 min was documented (range 3 – 45 min). The following enzymatic isolation continued for another 160.6 min (range 103 – 275 min) with a total of 1.4 units of enzyme (range 0.65 – 5.2). The complete isolation procedure totaled to a mean of 172.3 min (range 112 – 287 min) until a single cell suspension was obtained.

A mean yield of 0.686 x 10^6 cells were obtained from the study samples (range 13200 – 34.092 x 10^6 cells per sample) with a mean of 3330 cells per mg sample weight (range 131 – 22927 cells/mg).

Mean overall cell viability was 87.2% (range 26.2 – 97.6%). A total of 7 spheroids were generated per sample (range 2 – 60) with a mean of 24646 cells per spheroid

(range 2841 – 187500 cells/spheroid). Seeded cells were subsequently kept in culture for a total of 50 h (range 36 – 75 h).

Two separate assays measuring cell viability were used to assess cell survival following cytostatic treatment *in vitro*; the majority of the samples (79.5%) samples were analyzed using the ATP assay.

Baseline clinical characteristics of the patient cohort

Baseline clinical characteristics of the patient cohort are shown in Table 5. A comparison of this cohort to other large trials in the neoadjuvant treatment setting showed that main characteristics such as age, clinical tumor stage, tumor histology and receptor status were representative [71, 339]. Comparison of the patients that were included in the final analysis to those excluded showed no significant differences in factor distribution with the exception of age. Older patients were more likely to be excluded (supplementary

Table 14). Patients older than 50 were less likely to receive neoadjuvant chemotherapy (19 out of 30 excluded patients) and treatment often varied from the initial recommendation due to existing medical conditions (14 out of 20 excluded patients).

Table 5: Clinical and pathological characteristics of the analyzed cohort. Presented data was collected at the time of the initial diagnosis.

				pCR				
				Yes		No		
		N	%	N	%	N	%	*p*-value
All Patients		78	100	22	28.2	56	71.8	
Clinical Characteristics								
Age at diagnosis [y]								.207
	≤50	43	55.1	15	34.9	28	65.1	
	>50	35	44.9	7	20.0	28	80.0	
Mean		51		46		53		**.045**
Range		21 - 78		21-65		25-78		
BMI								.620
	Under/Normal weight	39	50.0	10	25.6	29	74.4	
	Overweight/Adipose	38	48.7	12	31.6	26	68.4	
	Not documented	1	1.3	-		1	-	
Effected side								.801
	Left	46	58.9	13	28.3	33	71.7	
	Right	29	37.2	9	31.0	20	69.0	
	Not documented	3	3.9	-		3	-	

Centricity/focality								.067
	Multifocal	19	24.4	6	31.6	13	68.4	
	Multicentric	10	12.8	6	60.0	4	40.0	
	Unicentric	44	56.4	10	22.7	34	77.3	
	Not documented	5	6.4	-		5		
Quadrant								.088
	Central	5	6.4	0	-	5	100	
	Upper-inner	12	15.4	5	41.7	7	58.3	
	Upper-outer	20	25.6	8	40.0	12	60.0	
	Low er-outer	3	3.8	2	66.7	1	33.3	
	Several overlapping quadrants effected	6	7.7	0	-	6	100	
	Quadrant not specified	32	41.0	7	21.9	25	78.1	
Tumor stage								.779
	cT1/T2	56	71.8	16	28.6	40	71.4	
	cT3/4	21	26.9	5	23.8	16	76.2	
	Not documented	1	1.3	1	4.5	-		
Nodal status								1.000
	cN+	42	53.8	12	28.6	30	71.4	
	cN-	35	44.9	10	28.6	25	71.4	
	Not documented	1	1.3	-		1	-	
UICC								.740
	IA	6	7.7	1	16.7	5	83.3	
	IIA	30	38.5	8	26.7	22	73.3	
	IIB	21	26.9	8	38.1	13	61.9	
	IIIA	15	19.2	3	20.0	12	80.0	
	IIIB	4	5.1	1	25.0	3	75.0	
	IIIC	-		-		-		
	IV	-		-		-		
	Unknown	2	2.6	1	-	1	-	
Clinical Tumor Size at diagnosis, largest diameter [cm]								
MRI (N=36)								
	Mean	3.6		3.4		3.3		.517
	Range	0.7-9.5		1.8-6.60		0.70-9.50		
Mammography (N=57)								
	Mea	3.2		2.9		3.3		.285
	Range	1.0-8.0		1.0-4.6		1.3-8.0		
Sonography (N=69)								
	Mean	3.0		3.0		3.0		.948
	Range	1.15-8.0		1.3-6.0		1.15-8.0		

Histopathological characteristics

Grading								.123
	G1/2	41	52.6	8	19.5	33	80.5	
	G3	35	44.9	13	37.1	22	62.9	
	Not documented	2	2.6	1	4.5	1	-	
Histologic type								.498
	Ductal invasive	53	67.9	17	32.1	36	67.9	
	Ductal invasive + DCIS component	7	9.0	1	14.3	6	85.7	
	Lobular invasive	8	10.3	1	12.5	7	87.5	
	Invasive ductal/lobular	1	1.3	-	-	1	100.0	
	Medullary	1	1.3	1	100.0	-		
	Inflammatory	1	1.3	-		1	100.0	
	Other	7	9.0	2	28.6	5	71.4	
ER status								.180
	Negative	26	33.3	10	38.5	16	61.5	
	Positive	49	62.8	11	22.4	38	77.6	
	Not documented	3	3.8	1	4.5	2	3.6	
PR status								**.002**
	Negative	36	46.2	16	44.4	20	55.6	
	Positive	41	52.6	5	12.2	36	87.8	
	Not documented	1	1.3	1	-	-	-	
HER2 status								**.001**
	Negative	59	75.6	11	18.6	48	81.4	
	Positive	16	20.5	10	62.5	6	37.5	
	Not documented	3	3.8	1	-	2	-	
Triple negative								1.000
	Yes	18	23.1	5	27.8	13	72.2	

	No	52	66.7	14	26.9	38	73.1	
	Not documented	8	10.2	3	-	5	-	
Ki67 (biopsy)								
	<=30% (median)	27		6	22.2	21	77.8	.148
	> 30%	26		11	42.3	15	57.7	
	Not documented	25		5	-	20	-	
Mean		30%		40%		36%		.644
Range		5-95%		5-80%		5-95%		
HR/HER2								**.001**
	HR+/HER2+	10		6	60.0	4	40.0	
	HR+/HER2-	39		5	12.8	34	87.2	
	HR-/HER2+	5		4	80.0	1	20.0	
	HR-/HER2-	18		5	27.8	13	72.2	
	Not documented	8	10.3	3	-	5	-	

Drug treatment

Drug treatment								**.001**
	AC -> T	57	73.1	11	19.3	46	80.7	
	AC -> TH	9	11.5	8	88.9	1	11.1	
	TCbH	7	9.0	2	28.6	5	71.4	
	AC -> TCb	3	3.8	1	33.3	2	66.7	
	AC	2	2.6	0	-	2	100.0	
Treatment adherence								**.016**
	Yes	60	76.9	21	35.0	39	65.0	
	No	18	23.1	1	5.6	17	94.4	

Endpoints

Residual tumor size								**.000**
	ypT0	19	24.4	19	86.4	-		
	ypTis	4	5.1	-		4	7.3	
	ypT1	26	33.3	-		26	47.3	
	ypT2	16	20.5	-		16	29.1	
	ypT3	8	10.3	-		8	14.5	
	ypT4	1	1.3	-		1	1.8	
	Not documented/ ypTx	4	5.1	3	13.6	1	1.8	
Surgical therapy								**.004**
	BCS Yes	54	69.2	20	90.9	34	60.7	
	No	22	28.2	1	4.5	21	37.5	
	Not documented	2	2.6	1	4.5	1	1.8	
Grade of regression								**.000**
	0	3	3.8	-		3	5.9	
	1	38	48.7	-		38	74.5	
	2	7	9.0	-		7	13.7	
	3	3	3.8	-		3	5.9	
	4	22	28.2	22	100.0	-		
	Not documented	5		-		5	8.9	

y, years, BMI, body mass index, MRI, magnetic resonance imaging, DCIS, ductal carcinoma *in situ*, PR, progesterone receptor; ER, estrogen receptor, HER2, human epidermal growth factor receptor 2, pCR, pathologic complete response, A, anthracycline, T, paclitaxel or docetaxel, C, cyclophosphamide; Cb, carboplatin, H, trastuzumab, BCS, breast conserving surgery.

The mean age of the enrolled patients was 51 with a range of 21-78 years at initial diagnosis. A slightly higher percentage of patients were 50 or younger (55.1%). Age was significantly associated with pCR (p =.045), although a grouped comparison using the cutoff of 50 years was not (p =.207). Assessment of body mass index (BMI) showed an even distribution of under/normal weight patients versus overweight/adipose patients (50.0% vs. 48.7%). The BMI did not have an impact on the outcome of the neoadjuvant therapy and pCR rates did not show a significant difference between the two groups under/normal weight and overweight/adipose patients. Regarding disease stage, the majority of the patients were stage UICC IIA (38.5%) with a slightly higher percentage of patients with a positive lymph node status

(53.8%) and a tumor size between 2-5 cm in diameter (cT2; 45 out of 77; 58.4%). These three factors however, had no impact on pCR in this group of patients. The tumor size, as measured through radiologic imaging either through magnetic resonance imaging, mammography, or ultrasound, had a mean diameter of 3.6 (N=36), 3.2 (N=57), and 3.0 cm (N=69) respectively. The diameter at initial diagnosis was not significantly associated with pCR, regardless of the applied imaging method. Study samples were mostly obtained from tumors of the left breast (58.9 %) located in the upper outer quadrant of the breast (25.6%). 19 cases of multifocal and 10 multicentric tumor disease were included in the study but none of these factors had any impact on treatment outcome.

Patient tumors were most frequently invasive ductal in their histology (N=53, 67.9%), 7 tumors were identified as invasive ductal with included components of ductal carcinoma *in situ*. Invasive lobular tumor histology was documented in a total of 8 patients. One patient exhibited a mixed invasive ductal/lobular histology. Medullary, inflammatory, metaplastic, neuroendocrine, squamous cell carcinoma histology was observed in one patient. Comparison of the pCR rates for the different types of histology showed no observable differences. A two-group comparison of invasive ductal and/or other histologic types (N=70, 89.7%) and lobular histology did not reveal any difference in pCR rates (p =.429).

An analysis of tumor biology revealed that 65.4% of tumors were HR+ with 62.8% (N=49) and 53.2% (N=42) positive for ER and PR respectively. A negative PR status (p =.002) was significantly associated with pCR, whereas the ER status was not (p =.180). HER2 overexpression was noted in 20.8% of the tumor samples. A positive HER2 status was also highly correlated with achieving pCR (p =.001). Classifying patients according to both HR and HER2 status showed that patients with a negative HR status as well as a positive HER2 status (N=4) were most likely to achieve pCR (80%) in this patient cohort, although a positive HR status only caused the percentage to drop to 60% (N=6). The combined HR/HER status was significantly related to pCR (p =.001), however group size was lower than 5 in three groups (HR-/HER2+, HR+/HER2+ and pCR no). A triple negative tumor biology was evident in 23.1% of the patients, the pCR rate for this group of patients was 27.8% and therefore slightly higher than the overall rate of pCR although not significantly so (p =1.000).

The observed mean rate of the Ki67 index was 30% positive cells with a range between 5-95%, correlation of the data did not reveal any difference in Ki67 for patients with pCR and those without (p =.644). Analysis of patient subgroups with values above and below the observed mean did not reveal any differences between the patients with pCR and those without, although more patients with pCR also had a Ki67 value above 30%. However, data was only available for 53 (68%) of the study patients.

Regarding chemotherapy regimen, all except two patients received taxane-based chemotherapy combined with either anthracycline or carboplatin. The mean duration of the complete treatment period starting from biopsy to histopathological analysis of the surgical specimen was 6.6 months (range 3.2 – 9.4 months) with a mean of 8.9 received cycles (range 2 – 16) depending on choice of treatment regimen. All HER2+ patients received trastuzumab (H), combined with either carboplatin (6 cycles TCbH, N=7) or an anthracycline/taxane combination regimen: 4x Epirubicin + Cyclophosphamide (EC) followed by 4xDocetaxel (Doc, N=1)/12xPaclitaxel (Pac, N=8). HER2-negative patients mainly received an anthracycline/taxane combination regimen of 4 cycles EC followed by 4 cycles of Doc (N=39) or 12 weekly cycles of Pac (N=5). Carboplatin was added to the taxane treatment (Pac, N=2, Doc, N=1) in three cases following 4 cycles of anthracycline chemotherapy. Eight patients received the combination treatment consisting of Doc, doxorubicin, and cyclophosphamide (DocAC). Three patients were treated with three cycles of an anthracycline regimen containing 5-FU followed by three cycles of Doc. Two patients received 2 or rather 4 cycles of EC without the addition of a taxane. One patient was simultaneously enrolled in the GeparQuinto Study (German Breast Group) studying the effect of Everolimus (Eve) on non-responders following 4 cycles of neoadjuvant anthracycline treatment and received this compound in combination with Pac [340]. Another patient switched to nanoparticle albumin-bound paclitaxel (nab-paclitaxel) following three cycles of paclitaxel therapy with prior anthracycline treatment due to an allergic reaction.

Due to the small number of patients in each of the treatment regimen, patients receiving anthracycline/taxane combination therapy were combined in one group, excluding those patients also receiving carboplatin (N=59). In this subgroup of patients 19.3% achieved pCR, compared to the other treatment combinations that showed a lower rate of successful treatment outcome (p =.009). Patients whose treatment in-

cluded trastuzumab showed significantly higher rates of pCR: 9 out of 12, respectively 8 out of 9 patients with anthracycline/taxane regimen combined with trastuzumab and 2 out of 7 receiving TCbH (p =.001). Treatment adherence defined as receiving all scheduled cycles and doses of the prescribed chemotherapy drugs was documented for 60, whereas a total of 18 patients received a reduced dose (N=9), ended chemotherapy prematurely (N=9), and/or switched drugs (N=5). Reasons for a change in treatment were either due to side effects or allergic reactions (N=8), progressive disease or no response (N=5), or patient preferences (N=2). In three cases the reason for a change in the initially planned treatment was not documented. Treatment adherence was significantly associated with pCR (p =.016).

Predictive power of the breast cancer spheroid model for treatment outcome

Following the completion of the clinical chemotherapy for all patients, a comparison was made between cell survival as measured in the breast cancer spheroid model *in vitro* and the documented clinical endpoints (pCR, grade of regression, ypT). Figure

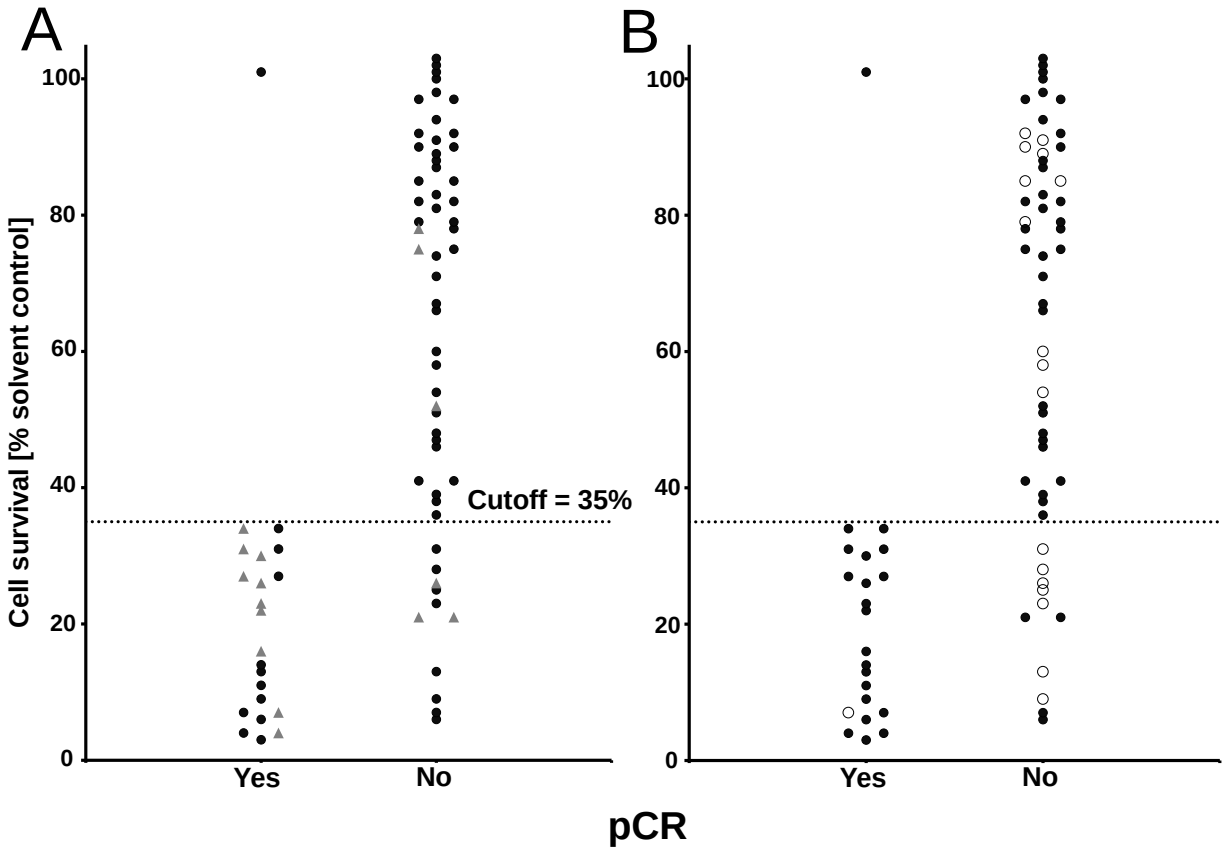


Figure 5: Dot plot showing the individual results of the analyzed sample plotted according to pCR Yes or No following clinical treatment. A) Patients receiving trastuzumab-based treatment are highlighted with grey triangles and in B) patients not adhering to the originally planned treatment regime are displayed as open circles. The dotted line represents the resulting cutoff at 35% cell survival *in vitro*. pCR, pathologic complete response

5A-B shows the individual values for the samples tested in the study grouped according to pCR yes or no. A significant association between the two factors was found (p =.001). A mean cell survival of 21.8% was found in the breast cancer spheroid model for patients with pCR versus 63.8% in non-pCR patients over all received clinical chemotherapy regimen. As can be seen in this figure a cutoff to correctly predict pCR using the assay was determined according to the 95th percentile of cell survival in patients achieving pCR at a value of 35% cell survival.

In Figure 5A the individual samples are highlighted to indicate patients receiving trastuzumab in addition to an anthracycline- or taxane-based regimen. For this subgroup a mean of 21.9% versus 45.4% cell survival was seen in patients achieving or not achieving pCR respectively (see Table 6, p =.085). The HER2 status was also closely associated with the postulated cutoff (p =.000). A total of 28.8% (N=17) HER2 negative patients showed values below 35% cell survival in the breast cancer spheroid model, with the remaining 71.2% showed values above this cutoff. Patients with a HER2+ tumor predominantly showed results below 35% cell survival namely 81.3% (N=13) out of the 16 HER2+ patients.

Figure 5B highlights patients not adhering to the originally planned clinical treatment since this factor was also associated with the rate of observed pCR in this cohort as mentioned above. However, this factor was not related to the detected cell survival *in vitro* (p =.929) or cutoff in the model (p =.789).

Hormone-receptor negative tumor samples showed a higher cytostatic treatment response compared to hormone-receptor-positive (p =.038). Refined subgroups according to ER and PR status showed the lowest response to treatment *in vitro* for ER/PR-positive tumors (62.49%), followed by PR+/ER- or unknown samples (47.82%). The best response was observed for ER+/PR- or unknown samples (36.10%). A closer analysis of ER and PR status separately showed that PR+ was significantly correlated with higher observed cell survival in the assay (p =.007), whereas ER status was not (p =.084). PR negative samples showed a higher cytostatic response (41.99%) compared to PR positive samples (61.77%). The distribution of the samples according to the cutoff value showed that the majority of samples with a negative PR status fell below the cutoff (N=20, p =.019). ER negative samples likewise responded better to treatment *in vitro* (43.40%), however the difference to ER positive samples was not significant (p =.097). Likewise, no significant association was found for ER positive and negative samples and cell survival above or below the cutoff (p =.224).

No significant correlation was seen between other documented clinical factors and cell survival such as age at diagnosis (p =.877), tumor stage at diagnosis (p =.181), lymph node status (p =.813), grading (p =.339), or histologic type (p =.569). Similarly,

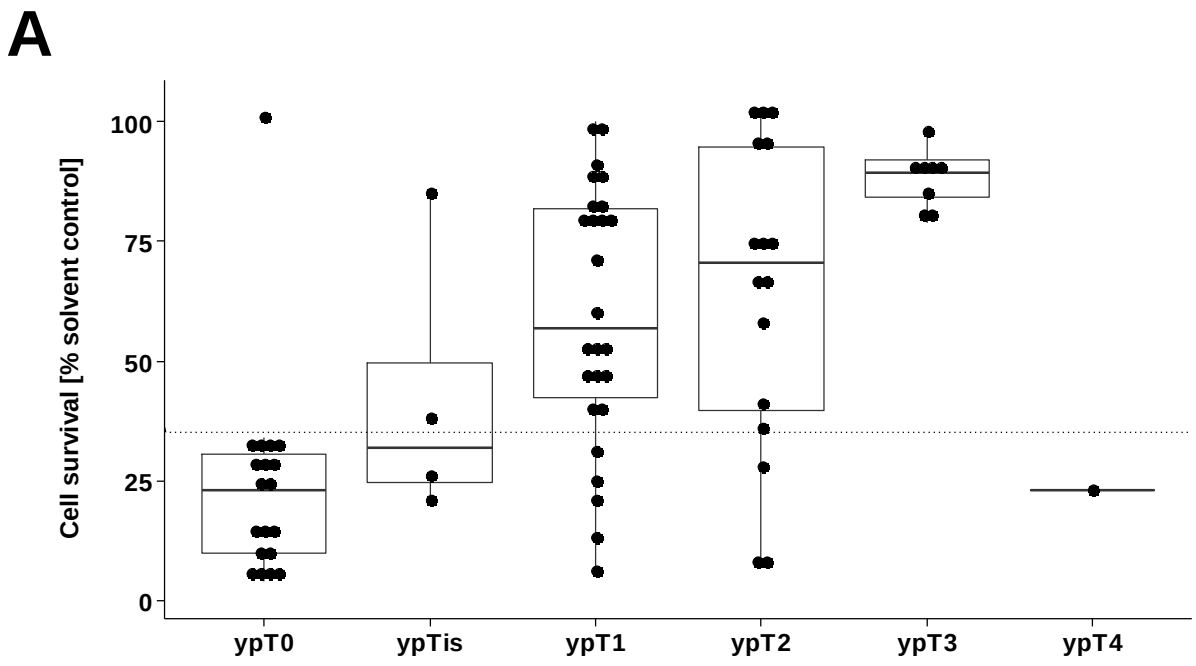
the number of patients with values below or above the cutoff also showed a similar distribution of values and no significant associations were found.

Table 6: Correlation of cell survival cutoff with clinical and pathological factors

			Cutoff				
			≤35%		>35%		
	Mean cell survival [%]	*p-value*	N	%	N	%	*p-value*
All Patients			32	41.0	46	59.0	
Age at diagnosis [y]		.877					1.000
≤50	51.44		18	41.9	25	58.1	
>50	52.61		14	40.0	21	60.0	
Mean			51		51		.817
Range			21-76		25-78		
Tumor stage		.181					.603
cT1/T2	49.40		24	42.9	32	57.1	
cT3/4	60.66		7	33.3	14	66.7	
Not documented	-		1	100			
Nodal status		.813					1.000
cN+	50.41		17	40.5	25	59.5	
cN-	52.20		15	42.9	20	57.1	
Not documented			-		1	100	
Grading		.339					.486
G1/2	55.34		15	36.6	26	63.4	
G3	48.09		16	45.7	19	54.3	
Not documented			1	50.0	1	50.0	
Histologic type		.569					.439
Ductal invasive/other	51.25		30	42.9	40	57.1	
Lobular invasive	58.29		2	25.0	6	75.0	
ER status		.084					.224
Negative	43.40		13	50.0	13	50.0	
Positive	57.10		17	34.7	32	65.3	
Not documented			2	66.7	1	33.3	
PR status		**.007**					**.019**
Negative	41.99		20	62.5	16	34.8	
Positive	61.77		11	34.4	30	65.2	
Not documented			1	100	-		
HR status		**.038**					**.042**
ER+/PR+	62.49		10	25.6	29	74.4	
ER+/PR-/Unknown	36.10		7	70.0	3	30.0	
ER-/unknown/PR+	47.82		1	50.0	1	50.0	
ER-/PR-	43.03		12	50.0	12	50.0	
HER2 status		**.000**					**.000**
Negative	58.34		17	28.8	42	71.2	
Positive	30.70		13	81.3	3	18.7	
Not documented			2	66.7	1	33.3	
Treatment adherence		.929					.789
Yes	51.79		24	40.0	36	60.0	
No	52.58		8	44.4	10	55.6	
ypT-stadium		**.000**					**.000**
ypT0	23.49		18	94.7	1	5.3	
ypTis	42.61		2	50.0	2	50.0	
ypT1	59.65		5	19.2	21	80.8	
ypT2	64.64		3	18.8	13	81.3	
ypT3	88.52		0	-	8	100	
ypT4	22.79		1	100	0	-	
Not documented	-						
Grade of regression		**.000**					**.000**
0	64.80		1	33.3	2	66.7	
1	69.95		5	13.2	33	86.8	
2	50.91		1	14.3	6	85.7	
3	28.35		2	66.7	1	33.3	
4	21.78		21	95.5	1	4.5	
Not documented	5						

y, years, PR, progesterone receptor; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; pCR, pathologic complete response.

Correlation of cell survival in the spheroid model with the ypT stage revealed that the breast cancer spheroid model was significantly related to the gradual response as seen in the surgical specimen after chemotherapy (Figure 6A; Spearman's rho= .548 *p* =.000). No residual tumor remaining in the surgical specimen (ypT0) or a remaining noninvasive component (ypTis) was associated with a lower cell survival in the assay (23.49% and 42.61% respectively) compared to mean cell survival for patients with remaining viable tumor cells following the completion of chemotherapy (ypT1, 59.65%; ypT2, 64.64%; ypT3, 88.52%; ypT4, 22.79%). One patient was categorized as ypT4 due to a previously undiagnosed inflammatory component in the surgical sample, thus explaining the outlier found for the ypT4 category in Figure 6A. A similar results was seen for the comparison between grade of regression and cell survival in the breast cancer spheroid assay (Figure 6B; Spearman's rho= -.611, *p* =.000), the higher the grade of regression the lower the recorded cell survival. Undetectable tumor cells in the surgical specimen (grade of regression = 4), as seen in 22 samples, was associated with a mean cell survival of 21.78%. An increasing amount of viable tumor tissue was highly associated with an increasing cell survival (grade of regression 3, 28.35%; grade of regression 2, 50.91%; grade of regression 1, 69.95%; grade of regression 0, 64.80%).



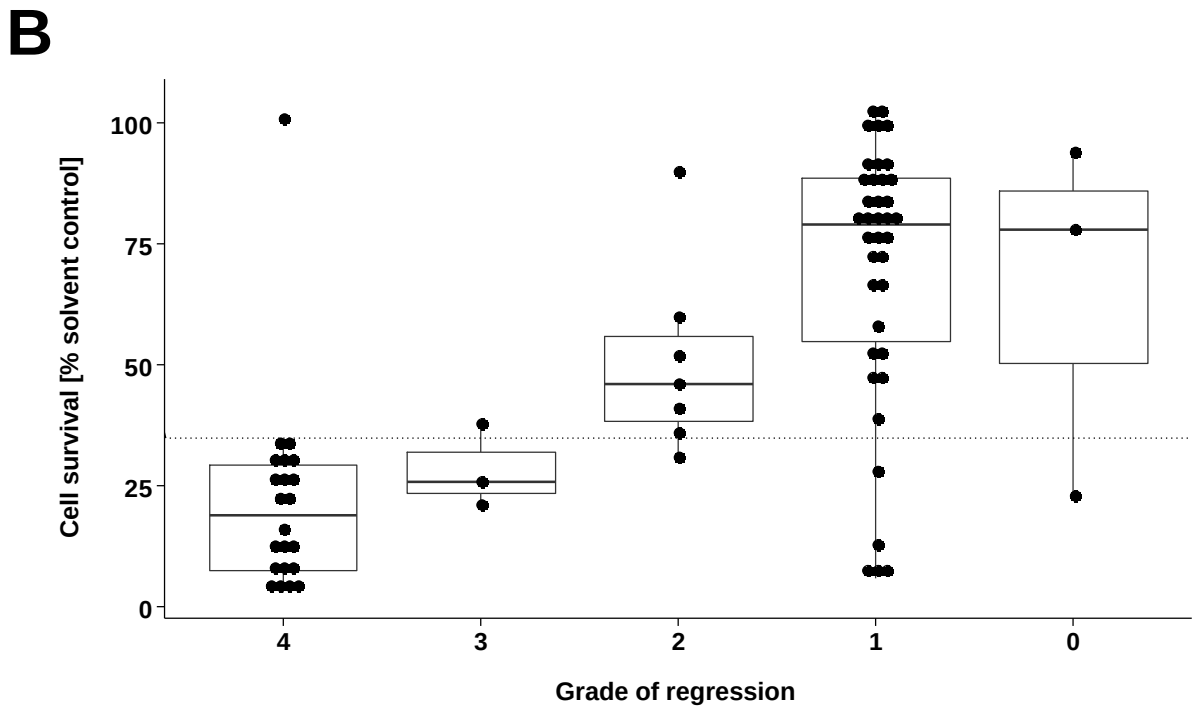


Figure 6: Box plot diagram with overlaying dot plot of each individual study sample showing mean cell survival in the breast cancer spheroid model after cytostatic treatment in comparison to the A) remaining tumor (ypT-stadium) and B) grade of regression as determined by the pathological assessment of the surgical specimen after chemotherapy. Cutoff of 35% is represented by the dotted line.

The sensitivity and specificity of the determined cutoff value was calculated, and relevant values are given in Table 7. Sensitivity or the rate of true positive treatment outcome prediction was calculated at 95.5% and specificity or the rate of the correct identification of non-pCR was determined at 80.4%. The positive predictive value of the test, also referred to as the precision value of a test, was determined to be 21 out of 32 cases which equals to 65.6% while the negative predictive value was calculated to be 45 out of 46 (97.8%). A calculation of the cutoff values according to the collected data using the Youden index resulted in the same value of 35% cell survival. The ratio between a true positive test result and false positive test results was determined at 4.86 for the positive likelihood ratio. The corresponding negative likelihood ratio was calculated to be 0.06. All six values can be used in this case, since the prevalence of pCR in this population is comparable to the rate of pCR in current literature and are therefore representative for the diagnostic accuracy of the assay.

Table 7: Sensitivity and specificity of the resulting cutoff, as well as other commonly used parameters used to assess the quality of diagnostic methods.

	pCR [N]		
Cell survival [% solvent control]	**Yes**	**No**	**Total**
≤ 35	21	11	32
> 35	1	45	46
Total	**22**	**56**	**78**

Diagnostic accuracy	
Sensitivity	95.5
Specificity	80.4
Positive Predictive Value	65.6
Negative Predictive Value	97.8
Positive Likelihood	4.86
Negativ Likelihood	0.06

pCR, pathologic complete response.

In order to further assess the predictive power of the breast cancer spheroid model the accumulated data was additionally analyzed in the context of established predictors using a receiver operator characteristic curve (ROC). This graph summarizes the cost/benefit analysis of the assay, the benefit being the correct identification of patients likely to achieve pCR against the potential cost of incorrectly identifying patients who will not achieve pCR but are classified by the assay to do so. In this case the breast cancer spheroid model shows a high accuracy in predicting treatment outcome (Figure 7A). The c statistic (area under the ROC curve) was calculated for the treatment results as well as for standard clinical parameters and the resulting value was compared. The c statistic is the probability of randomly selecting a set of patients from the pCR and non-pCR group and correctly classifying both. The values for the spheroid model are shown above the ROC curve in Figure 7A and Table 8. A comparable multivariate model using the clinical parameters age, ER, PR, as well as HER2 status resulted in a value of 0.7976. Combining the result obtained in the spheroid model with the factor of treatment adherence resulted in the highest AUC totaling to 0.9082, see Figure 7C. Calculating the sensitivity/specificity using only patients adhering to the initially planned protocol resulted in an improvement in the specificity of the assay to 89.7%, while the sensitivity remained the same. Several other factors were also analyzed in univariate and multivariate models, the corresponding c statistics are given in Table 8.

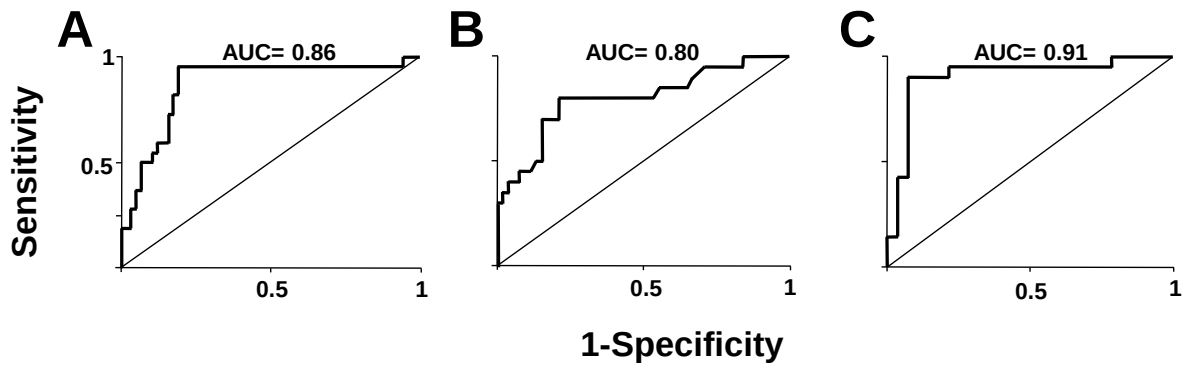


Figure 7: Receiver operator curves (ROC) displaying the sensitivity and specificity of A) the breast cancer spheroid model, B) a multifactor model with baseline predictive factors (ER, PR, HER2, and age) impacting pCR , and C), a model combining the breast cancer spheroid model with the factor of treatment adherence defined as treatment discontinuation, dose-reduction or change of treatment. The resulting area under the curve (AUC) is displayed at the top of each graph

Table 8: Corresponding c statistics from the ROC curve analysis

Criteria	AUC
ER, PR, HER2, triple neg	0.7817
ER, PR, HER2, Age	0.7976
Breast cancer spheroid model plus clinic criteria (ER, PR, HER2, Age)	0.8587
Lobular histology vs Others	0.8589
HER2 status	0.8589
PR status	0.8597
cT1/2 vs. cT3/4	0.8605
cN- vs. cN+	0.8620
HR status	0.8633
ER status	0.8633
Chemotherapy regimen containing anthracyclines	0.8636
Medication groups	0.8636
Age ≤50 vs. >50 years	0.8636
Breast cancer spheroid model	0.8636
Chemotherapy regimen containing trastuzumab	0.8774
Cutoff breast cancer spheroid model ≤35 vs. >35%	0.8791
Ki67 ≤30% vs. > 30%	0.8807
Breast cancer spheroid model + clinic criteria (ER, PR, HER2, Triple neg)	0.9058
Change in tumor diameter T_{Start} - T_{Change}	0.9071
Treatment adherence	0.9082
Change in tumor diameter T_{Change} - T_{End}	0.9219

ER, estrogen receptor, PR, progesterone receptor, HER2, human epidermal growth factor receptor 2, HR, hormone receptor, triple neg, triple negative.

The odds ratios (OR) and confidence interval (CI) were determined as a byproduct of the backward regression analysis to calculate the ROC curves and showed similar results, namely that PR status, HER2 status, treatment adherence, and cutoff in the breast cancer spheroid model had the most significant impact on pCR (see Figure 8). Other factors such as ER (OR 0.463, CI 0.164-1.306), HR status (OR 0.513, CI 0.180-1.465), and anthracycline-taxane-based chemotherapy (OR 0.244, CI 0.084-0.709) were not found to increase the probability of achieving pCR in this cohort of patients.

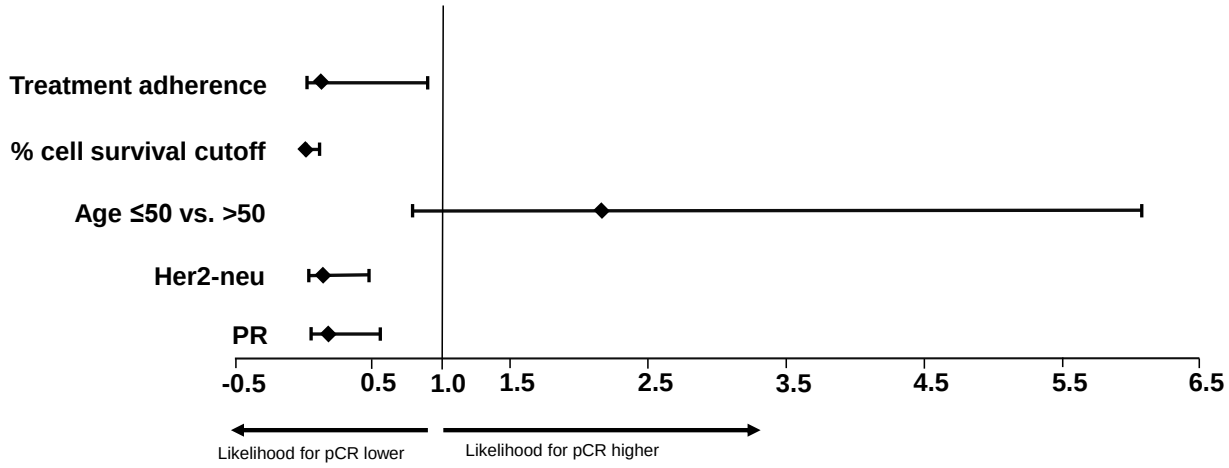


Figure 8: Odds ratios for significant factors impacting pCR. Diamond shape indicates the determined odds ratio and bars left and right the corresponding 95% confidence interval. pCR, pathologic complete response, HER2, human epidermal growth factor receptor 2, PR, progesterone receptor.

Differences in treatment efficacy *in vitro*

The spheroids in the assay were treated *in vitro* according to the national treatment recommendations relevant to the study recruitment period [341-343]. Figure 9 shows all individual study samples according to each *in vitro* treatment, patients with pCR are highlighted in black. The corresponding cell survival values are additionally specified in Table 9.

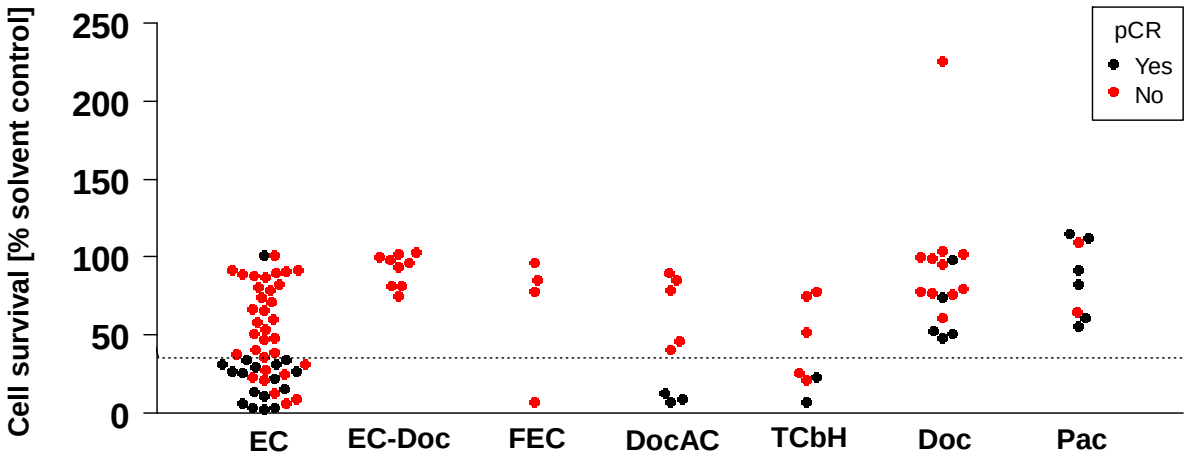


Figure 9: Dot plot showing the individual cell survival values per utilized treatment compound or combinations. Red dots represent patients without pCR following chemotherapy; black dots show patients with complete response. The dotted line shows the cutoff value at 35% cell survival. pCR, pathologic complete response, EC, epirubicin + cyclophosphamide, Doc, docetaxel, FEC, 5-fluourouracil + epirubicin + cyclophosphamide, DocAC, docetaxel + doxorubicin + cyclophosphamide, TCbH, paclitaxel, carboplatin, trastuzumab, Pac, paclitaxel.

Table 9: Overview of the cell survival for each tested compound or combination of compounds

			pCR				
			Yes		No		
	N	Mean cell survival ± Stdv [%]	N	Mean cell survival ± Stdv [%]	N	Mean cell survival ± Stdv [%]	*p-value*
Tested compounds							
EC	50	46.0 ± 30.4	17	24.6 ± 22.5	33	57.0 ± 28.2	.000
EC-Doc	9	92.7 ± 10.2	0	-	9	92.7 ± 10.2	-
FEC	4	66.8 ± 40.4	0	-	4	66.8 ± 40.4	-
DocAC	8	46.3 ± 34.7	3	10.0 ± 3.0	5	68.1 ± 23.0	.004
TCbH	7	40.3 ± 28.1	2	15.2 ± 11.1	5	50.3 ± 26.6	.145
Doc	16	88.9 ± 41.2	5	64.9 ± 21.2	11	99.9 ± 44.1	.118
Pac	8	86.9 ± 23.9	6	86.6 ± 24.7	2	87.6 ± 31.3	.966

pCR, pathologic complete response, Stdv, standard deviation, EC, epirubicin + cyclophosphamide, Doc, docetaxel, FEC, 5-fluourouracil + epirubicin + cyclophosphamide, DocAC, docetaxel + doxorubicin + cyclophosphamide, TCbH, paclitaxel, carboplatin, trastuzumab, Pac, paclitaxel.

The majority of samples were treated *in vitro* with EC (N=50), which resulted in a mean cell survival of 46.0%. In these samples, cell survival was significantly lower in patients with pCR (N=17, mean 24.6% cell survival) compared to those with residual tumor at surgery (N=33, 57.0% cell survival, p =.000). The addition of a taxane, namely Doc, was not as effective and resulted in a mean cell survival of 92.7%, none

of whom achieved pCR. The addition of 5-FU resulted in a slightly better treatment efficacy with a mean cell survival of 66.8%. Again, none of these patients showed a full response, therefore, a comparison with cell survival *in vitro* could not be done. The combination treatment DocAC showed a mean cell survival of 46.3%. Comparison with pCR status showed that treatment efficacy was significantly higher in patients with no residual tumor (N=3, 10.0%) versus 50.3% for all 5 patients with remaining residual tumor (p =.004). A similarly high difference in cell survival was seen following *in vitro* treatment with TCbH with a mean of 40.3% over all tested samples. A mean of 15.2% (N=2) and 50.3% (N=5) cell survival was observed for those with and without pCR respectively (p =.145).

Table 10 shows the association between the tested compounds and the number of samples showing values above or below the cutoff of 35% cell survival.

Table 10: Cross table showing the number of samples achieving values above and below the resulting threshold grouped according to the tested compounds

	Cutoff [% cell survival]				
Tested compounds	**≤ 35%**		**> 35%**		***p-value***
	N	**%**	**N**	**%**	
EC	24	48.0	26	52.0	.075
EC-Doc	0	-	9	100.0	
FEC	1	25.0	3	75.0	
DocAC	3	37.5	5	62.5	
TCbH	4	57.1	3	42.9	
Doc	0	-	16	100	
Pac	0	-	8	100	

EC, epirubicin + cyclophosphamide, Doc, docetaxel, FEC, 5-fluourouracil + epirubicin + cyclophosphamide, DocAC, docetaxel + doxorubicin + cyclophosphamide, TCbH, paclitaxel, carboplatin, trastuzumab, Pac, paclitaxel.

Analysis of laboratory factors influencing assay outcome were already discussed above (see Table 2). As shown in Table 11, additional testing using the determined cutoff showed that the number of spheroids (p =.009), but not the total number of isolated cells (p =.299) had a significant influence on whether or not cell survival was above or below 35%. Also impacting cell survival in this analysis was the number of tested treatment option *in vitro* (p =.011), the fewer spheroids generated or options tested in the assay, the higher the changes of values above 35% cell survival. This latter factor is, however, directly associated with the number of generated spheroids. The type of readout also had an impact on the outcome of the assay: all tested samples using the MTS assay resulted in values above the determined cutoff, while 48.4% only of the samples tested in the ATP assay did (p =.000).

Table 11: Laboratory factors influencing calculated cutoff of 35% cell survival.

		Cutoff				
		≤35%		>35%		
		N	%	N	%	*p-value*
All Study samples		32	41.0	46	59.0	-
No. of biopsy cylinders received						.547
	Mean	4		4		
	Range	1-12		1-9		
Weight study sample [mg]						.631
	Mean	148.9		89.4		
	Range	10.5-1568.0		8.9-288.3		
Biopsy method						.106
	Core needle biopsy	29	38.7	46	61.3	
	Vacuum-assisted biopsy	2	100.0	-	-	
	Excision biopsy	1	100.0	-	-	
No. of technical replicates						.118
	Mean					
	Range					
Transport in medium						.561
	Yes	29	39.2	45	60.8	
	No	2	66.7	1	33.3	
	Not documented	1	100.0	-	-	
Transport medium expired						.200
	Yes	22	36.1	39	63.9	
	No	7	5.8	5	41.7	
	Not documented	3	60.0	2	40.0	
No. of days TM expired [d]						.561
	Mean	13		7		
	Range	1-61		1-21		
Time diff. biopsy – begin lab procedure [h]						.646
	Mean	25.6		26.8		
	Range	17.5-46.8		4.75-85.5		
Mechanical isolation [min]						.721
	Mean	12.3		11.39		
	Range	4-45		3-40		
Enzymatic isolation [min]						.968
	Mean	160.3		160.8		
	Range	103-225		107-275		
Total time isolation procedure						.959
	Mean	172.6		172.2		
	Range	115-224		112-287		
Total enzyme added [units]						.856
	Mean	1.4		1.3		
	Range	0.65-5.2		0.65-2.6		
Total No. of vital cells						.299
	Mean	1.408 x10^{b}		0.173x10^{b}		
	Range	22950-34.092x10^{b}		13200-1.590 x10^{b}		
Cell viability [%]						.386
	Mean	85.9		88.2		
	Range	26.2-96.1		60.6-97.6		
No. of cells per spheroid						.972
	Mean	21732		26673		
	Range	4443-51000		2841-187500		
No. of cells per spheroid						.934
	< 10 000	8	36.4	14	63.4	
	10 000 – 20 000	9	45.0	11	55.0	
	20 000 – 50 000	14	42.4	19	57.6	
	> 50 000	1	33.3	2	66.7	
No. of spheroids generated						**.009**
	Mean	10		5		
	Range	4-60		2-25		
Incubation of generated spheroids [h]						.373
	Mean	48.5		50.5		
	Range	36-59		36-75		
No. of treatment options						**.011**
	Mean	2		1		
	Range	1-9		1-4		
Readout method						**.000**
	ATP	32	51.6	30	48.4	
	MTS	-	-	16	100.0	

* N per group <5, No., number of, TM, transport medium, d, days, h, hours, min, minutes, cps, counts per second, ATP, adenosine triphosphate, MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

Correlation of clinical factors with treatment efficacy *in vitro*

Since the group of samples tested with EC made up the largest number of cases, treatment outcome in the spheroid model was correlated with different clinical subgroups to determine if any differences in treatment efficacy *in vitro* could be identified. Of all tested parameters a significant difference was only observed in a comparison between tumors originating from the left or right breast (Figure 10A), as well as in regard to the HER2 status of the tumor (Figure 10B). Regarding sidedness, tumor originating in the right breast responded significantly better to EC treatment compared to tumors originating in the left breast (right breast, N=19, 33.77% vs left breast, N=29, 54.95% mean cell survival, p =.011). Similarly tumors with a positive HER2 status showed a higher treatment efficacy compared to tumors with a negative HER2 status in regard to response to EC (HER2+, N=9, 23.24% vs HER2-, N=39, 52.54% mean cell survival, p =.000). A similar result was seen in the comparison of the patient grouped according to HER2 status combined with HR status (Figure 10C, p =.010). In all cases HER2+ tumors responded significantly better to EC treatment compared to negative tumors, combined with a positive HR status treatment efficacy was improved further (HER2+/HR+, N=6, 20.35%, HER2+/HR-, N=3, 29.01% cell survival). Triple negative tumors showed the highest heterogeneity in treatment response to EC (N=13, mean 38.08%, range 3.00 – 101.00% cell survival). HR+, HER2 negative samples showed the lowest treatment efficacy to EC, with a mean well above the cutoff (N=25, 58.47% mean cell survival).

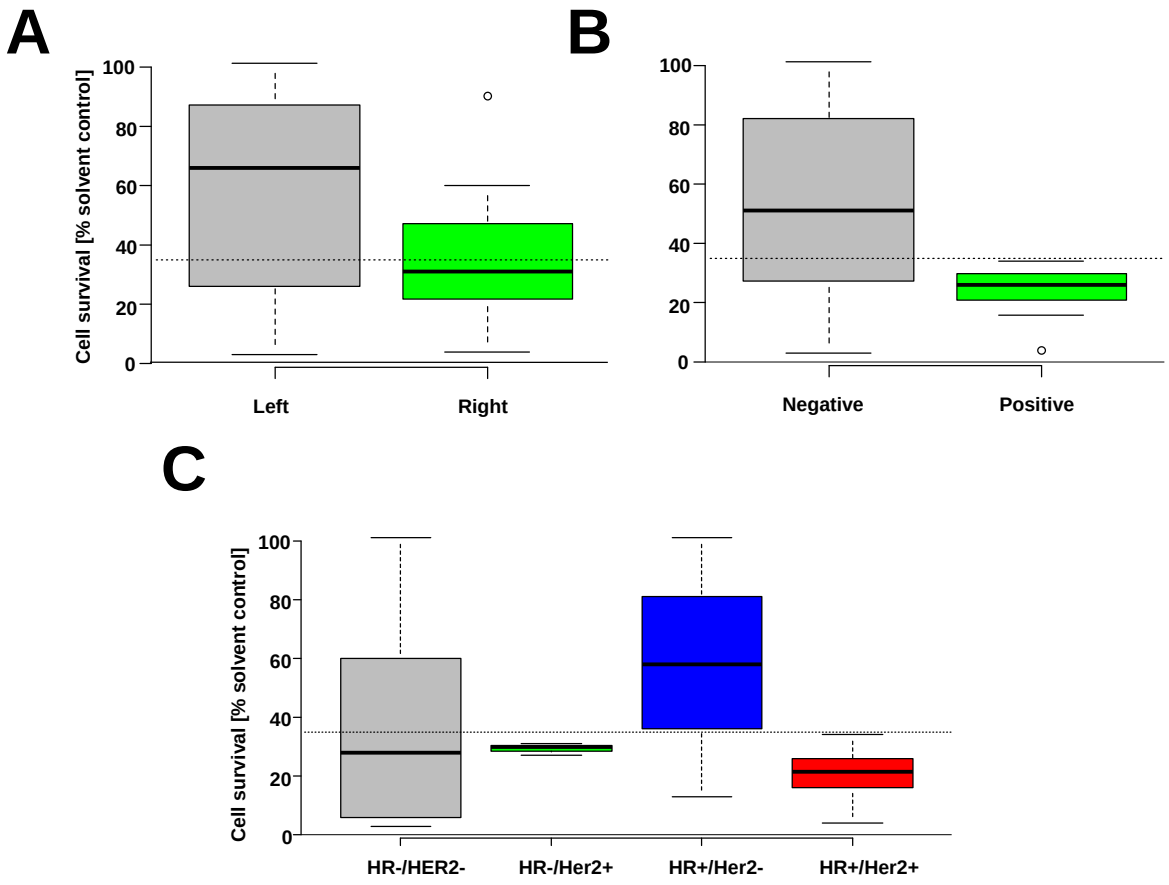


Figure 10: Subgroup of patients tested with EC combination treatment grouped according to resulting cell survival according to factors of tumor biology, such as A) tumor origin in left versus right breast, B) HER2 status, as well as C) combination of HR status with HER2 status. Dotted line represents the resulting cutoff at 35% cell survival. HR, hormone receptor, HER2, human epidermal growth factor receptor 2.

DISCUSSION

The main results of the SpheroNEO Study indicate that the breast cancer spheroid model correctly identified treatment outcome for clinically applied chemotherapy regimens on an individual patient basis with a high degree of sensitivity (95.5%) and specificity (80.4%). The primary endpoint pCR, and other clinical outcome variables such as residual tumor extent (ypT) and grade of regression, were all significantly associated with *in vitro* cell survival. A cutoff of 35% cell survival was calculated separating assay results according to higher or lower likelihood for pCR with a high degree of diagnostic accuracy. Corresponding ROC analysis and odds ratios of the assay results and the proposed cutoff further strengthened these findings. Comparison of the collected data with similar patient cohorts showed that both clinical and histological patient characteristics were representative. Patient exclusion was mostly due to changes in the initially intended treatment scheme. Quality criteria regarding patient tissue samples and assay methodology were consistently high. The main limiting factor for successful assay readout was the number of cells isolated per patient sample. Results obtained in this exploratory study will be used in the design of an interventional study.

Assay outcome and diagnostic accuracy

The overall rate of pCR in this study was 28.2%, which corresponds to data found in other studies where similar neoadjuvant chemotherapy combinations were used in the treatment of breast cancer [344, 345]. This rate of positive response increased to 65.6% (21 out of 32 patients) using the breast cancer spheroid assay. The addition of treatment adherence as co-factor caused this rate to increase to 83.3% (20 out of 24 patients). This indicates that the results from the *in vitro* assay are highly associated with treatment outcome as measured via pCR, and are further improved when patients complete the full drug treatment as recommended by national guidelines.

By comparison, many of the standard clinical parameters were much less effective in predicting treatment outcome. Age groups, cT stadium, LN status, tumor histology, as well as grading were all not associated with pCR. However, this was also found in a recent analysis of pooled neoadjuvant trials. Unlike in other neoadjuvant studies a higher odds ratio for pCR was not found for a HR- status, the main biomarker in breast cancer treatment [346, 347]. Also surprising was the significant association between pCR and the PR status, which was not evident for the ER status. However, it is highly likely that these finding are artifacts and mainly a result of the small sample size. As expected, HER2+ status significantly predicted treatment outcome. This can also be seen in the corresponding c statistics of the ROC analysis and odds ratios. A direct comparison of diagnostic accuracy of the breast cancer spheroid model to similar chemoresponse assays is difficult, since currently there are not many studies that are comparable in design and were conducted using a prospective approach. One such study by Singer et al showed parallels to the SpheroNEO study, such as a prospective approach and the use of the peak plasma concentration for the applied treatment *in vitro*. However, no association with pCR was reported, rather assay results are compared to the measured change in proliferative activity (Ki67) and clinical change in tumor size through imaging methods [348]. Using isolated primary cells obtained from patient tumors embedded in collagen and studying treatment efficacy using imaging analysis, Takamura et al were able to obtain a comparable level of accuracy (positive predictive value = 83.3, negative predictive value = 100.0 to treatment with EC) using breast cancer biopsies, however, as with the previous publication, a 3D cell culture model was not used and patient numbers were not powered to the specified outcome of clinical response [349].

Multivariate analysis combining the *in vitro* assay data with ER, PR, and HER2 status improved the diagnostic accuracy of the data to 0.908. This result was only surpassed by treatment adherence, which interestingly showed the highest level of sensitivity/specificity. The overall specificity of the breast cancer spheroid model was lower than the observed sensitivity by approximately 10%, but no common characteristics could be identified to explain this discrepancy. Treatment adherence was able to account for some but not all of the patients that were false positive in the assay. However, the overall accuracy of 84.6% was similar to other diagnostic methods such as mammography and ultrasound [350]. These factors, such as changes in tumor diameter assessed through standard radiological examinations (i.e. sonography, mammography, MRI) did show a high correspondence as well, however this data was not collected under standardized circumstances, and factors such as inter-examiner differences were not accounted for. Current reviews have shown that ultrasound examinations tend to underestimate tumor size while MRI examinations overestimate the actual tumor size [351, 352]. PET measurement of tumor metabolism would be the most appropriate clinical counterpart to an *in vitro* assay system. However, current data is inconclusive in regard to the sensitivity/specificity of this approach [353] and PET is expensive and not part of the routine clinical care for most breast cancer patients.

In vitro treatment efficacy comparing standard treatment combinations in this study recapitulate clinical treatment options as outlined in current guidelines [330, 341, 354, 355]. Mean cell survival was significantly lower for patients exhibiting pCR compared to those that did not. This association was independent of the applied treatment combination (compare Table 9), thereby strengthening the hypothesized relation between *in vitro* and clinical efficacy. The clinical treatment for the majority of patients consisted of an anthracycline/taxane-based combination chemotherapy analogous to current guideline recommendations, consisting of EC followed by either Doc or Pac with or without trastuzumab for the majority of patients (84.6%, 66 out of 78 patients). In this study (pCR rate 28.8%, 16 out of 66 patients), and other large-scale trials this treatment combination was found to be highly effective [356-359]. The same treatment *in vitro* was similarly effective, DocAC (mean 45.3% cell survival) and EC (mean 46.0% cell survival) achieving similar results. However, the TCbH combination (mean 40.3% cell survival) proved slightly more effective. These combi-

nations all proved far superior to EC-Doc (mean 92.7% cell survival) and taxane single compound treatment (Doc, mean 88.9% cell survival) or Pac (Pac, mean 86.9% cell survival). The addition of 5-FU to the anthracycline-based combination was somewhat more effective *in vitro* with a mean cell survival of 66.8%, although only a total of four cases were tested with this combination. This compound is currently under discussion in the treatment against breast cancer due to the results of several clinical trials that showed the addition of the 5-FU in tablet form did not improve treatment outcome or long-term prognosis [93, 110, 360]. However, in each of the studies a small percentage did respond to this treatment, and out of the four tested cases one showed a high treatment efficacy to FEC *in vitro*. Given the proven efficacy of 5-FU and its continued use in other tumor diseases, this combination should still be considered for some patients. Potentially a comparison of individuals with a high treatment response may be useful in identifying smaller patient subgroups that profit from this treatment regimen. Outliers were also found for the other tested combinations, larger sample sizes could determine common clinical or tumor biological characteristics of these potentially individual patients. A similar approach is currently being used in clinical research across different tumor diseases, identifying super responders profiting from drugs initially thought to be ineffective [361].

Overall cell survival correlated with HER2 status with all nine study positive samples showing a treatment efficacy below 35%, all of which were treated with trastuzumab. This finding corresponds to data found in a large meta-analysis where the more aggressive tumor biology found in HER2+ and triple negative tumors resulted in higher rates of pCR following anthracycline-based chemotherapy compared to hormone positive/HER2 negative tumors even without the addition of trastuzumab. This high chemosensitivity might be due to a higher proliferative activity, however correlation between Ki67, pCR, and survival have been inconclusive. Using smaller, molecular subgroups as a basis could potentially lead to a more definitive explanation [49, 362]. The potential of the assay to predict the HER2 status through the response pattern found *in vitro* could aid in this process. In a review of current literature, there were only a small number of publications using a similar approach to investigate a potential association between treatment efficacy *in vitro* and HER2 status. In a study by Woo et al a similar difference in treatment efficacy was found, however the difference between the two groups was only significant for the antimetabolite gemcitabine

[363]. In a second study from Korea a trend was observed towards a higher response for HER2+ patient samples in an ATP assay and a significant difference in the response to treatment with epirubicin was found [364]. Both studies were conducted using small patient numbers, and the findings were not consistent even though similar cytostatic compounds were tested. In the data presented above, the mean cell survival of triple negative samples was also below 35%, although the range of treatment response was much greater. In regard to the breast cancer spheroid model, high sample numbers will be necessary to determine if the assay could be applied as a functional biomarker to discriminate triple negative and HER2+ tumor biology based on treatment efficacy results.

High standard deviations were found regardless of the tested substance, reflecting the heterogeneity of the patient subgroups and the individual patient cancer tissue. A statistical comparison over all tested compounds was therefore not significant. It should be noted that except for EC treatment, the case number for all other treatment combinations was small. This could explain, why the applied treatment *in vitro* only explained 14% of the variance found for cell survival. Similar results were reported by other groups, heterogeneity in the cellular composition and the microenvironment observed in patient tumors were cited as the possible underlying cause [365, 366].

A clinical subgroup analysis of the study samples tested with EC *in vitro* demonstrated a differential response in regard to HER2 status, combined hormone and HER2 status, as well as the effected side. The finding that HER2 status as a single factor or combined with the HR status effects treatment *in vitro* was not surprising given that in general this tumor biology has shown higher rates of pCR as well as high chemosensitivity *in vitro* in other published studies, the underlying reasons for these findings were discussed above [49, 71, 367].

Interestingly, tumors originating from the right side also responded significantly better to EC compared to tumors of the left breast, even though tumors originating on the right side were more often smaller in size at diagnosis (cT1/2). The case numbers are small, however, other researchers have found a similar diversity between the left and the right mammary gland, both in regard to developmental markers as well as disease occurrence and progression [368, 369]. An asymmetry has also been re-

ported in other solid tumors such as kidney, lung, testicular, melanoma, colorectal, and ovarian cancer [370]. Differences in the expression patterns of the developmental markers may offer an explanation for this finding, although there are few publications on asymmetrical carcinogenesis. In a study on breast patient-derived tissue samples Campoy et al compared the methylation profile in regard to sidedness and found inherent differences [371]. It could also be speculated that side-specific influences from the microenvironment may effect tumor development and growth unequally. Behavioral patterns as well as growth factors were also cited as an underlying cause for increased frequency of cancer in the left breast [372].

On an individual patient basis, a total of six patients were identified where more than one treatment combination could be tested due to sufficient vital tumor tissue. The results of the breast cancer spheroid model correctly identified the efficacy of the clinical treatment combination by demonstrating lower cell survival with this drug combination over other treatments tested (data not shown). Interestingly at least two or more treatment combinations proved equally effective *in vitro*, implying that a decision between approved anthracyclines (namely doxorubicin or epirubicin) or taxanes (namely paclitaxel or docetaxel) could be made according to each patient's comorbidity or tolerability. In the study cohort a direct comparison was not possible due to small sample sizes. Two study samples were tested with anthracycline/taxane-based treatment combinations and one with trastuzumab-based combinations. All demonstrated a low response *in vitro,* corresponding to the unfavorable histological outcome. In one patient not achieving pCR under EC followed by Doc treatment (mean cell survival = 39%), a significantly more effective treatment result in the breast cancer spheroid model was seen with the addition of 5-FU (mean cell survival = 18%; p =.004). Among the tumor samples tested *in vitro* with trastuzumab-based therapy, a selective effect was seen. When the *in vitro* treatment with trastuzumab showed no additional benefit as compared to chemotherapy alone, the patients were histologically confirmed HER2 negative (N=5). These results indicate that the breast cancer spheroid model may be able to distinguish the efficacy of the treatment guideline directed anthracycline/taxane based regimen depending on the individual tumor biology. Low cell yield is currently the main limiting factor in determining the selective applicability of the assay. By maximizing the number of cells isolated from each study samples more treatment options can be tested

in the assay. Therefore, this represents a key factor in validating the ability of the breast cancer spheroid model to correctly identify the most efficient treatment combination out of several equivalent treatment options.

Study site and patient characteristics

The final study cohort for our study consisted of 78 patients. Although the sample size was small in comparison to large-scale clinical trials, distribution of clinical characteristics was comparable to the distribution of clinical and histological characteristics found in current literature [373, 374]. The overall dropout rate for screened patients was 65.15%. Although the rate is high compared to other prospective trials, reason for exclusion due to laboratory criteria was only 26.2% of all cases (53 out of 202). Patient exclusion due to clinical criteria was slightly higher with a total of 35.1% (71 out of 202). Comparison of the included and excluded patients showed that no specific clinical subgroup was selected, thereby limiting selection bias.

Further analysis of the exclusion according to clinical criteria showed that the timeline of the neoadjuvant protocol, more specifically, the timing of the biopsy procedure and the subsequent determination of the treatment regimen, were problematic for the parallel assay analysis. Even though there was a preference at each participating study site for one or two chemotherapy regimens as mentioned above, no final decision regarding the chemotherapy regimen had been made for the respective breast cancer patient at biopsy. This explains the number of patients switching to a different chemotherapy treatment combination (N=21), adjuvant treatment or surgery alone (N=30). However, obtaining biopsy samples at a later time-point, such as during port implantation or sentinel node biopsy, was ruled out, since no information is currently available how the tumor biology is altered by this additional intervention and thereby possibly effecting subsequent assay results [375].

Age of study participants also played an important factor in patient screening, since older patients were more likely to discontinue treatment or even refuse chemotherapy. Non-adherence was most frequent in older patients (median age treatment non-adherence 56.5 years at diagnosis vs 48 median age patients receiving treatment as planned). Reasons were, in part, side effects (N=8) but also patient preferences (N=2). Similarly, these reasons have previously been reported with non-adherence most often linked to side effects such as alopecia and fatigue [376]. For

example, in a study on patient preferences and treatment adherence by DiBonaventura in 2014, 34.8% of metastatic breast cancer patients did not fully adhere to the prescribed oral treatment. The decision for one clinical treatment combination is ultimately made in collaboration between patient and doctor, and many factors should be involved in this shared decision-making process. Existing comorbidities, medical conditions and also patient preferences are all considered alongside tumor stage and biology [377, 378]. Potentially a predictive tests such as the one studied here could motivate patients to adhere to the full treatment as planned by visualizing efficacy before treatment start.

Surprisingly, not many patients were excluded due to metastatic disease (N=7, 3.8% of all screened patients), especially considering the state of disease was often unclear at the time of biopsy. Similarly, only two patients were excluded due to benign neoplasia. Current data cites a rate of 10% of patients with breast cancer that are advanced or stage IV at initial diagnosis [379, 380]. This fact shows that current routine screening and staging methods, mainly mammography and ultrasound examinations are fairly accurate in determining the extent and malignancy of suspicious findings with a sensitivity ranging from 48-96% [351, 381].

A comparison across all study sites showed a similar distribution of baseline patient characteristics. Although the patients screened for study inclusion in Starnberg were slightly older (median 68 years at diagnosis, N=8) than those screened at the other study sites (overall median 52 years at diagnosis), however, no patient from this study site was included in the final analysis making this difference in age insignificant to the study outcome (exclusion criteria for all patients: no neoadjuvant chemotherapy). Another finding was the clinical tumor size at diagnosis; screened patients from the coordinating study site (University Hospital LMU) were more frequently cT3/4 (12 out of 19 patients, 63.2%) compared to the other sites (overall 48 out of 188, 25.5%). The main reason is that patients with more extensive disease are more likely to be referred to a university hospital rather than a smaller clinic. This finding was further corroborated by the fact that patients from Munich's other university hospital, "Rechts der Isar", were more frequently diagnosed with metastatic disease (4 out of all 9 patients diagnosed cM1, 44.4%). However, these minor differences that were found in the patient population showed no associations to laboratory results and were therefore not considered to have any influence on the analysis of the final study data.

Factors influencing assay methodology

Current biobanking standards recommend several steps in optimizing tissue quality for the isolation of cells from fresh tissue samples such as: 1) An initial examination by a qualified pathologist, 2) transportation of the tissue sample in culture medium immediately after excision 3) subsequent transportation at 4˚C to the processing laboratory, 4) storage of the sample upon arrival in an incubator at 37˚C and 4% CO_2 until further processing. All tissue samples should also be 5) identified through a distinct code to prevent any incorrect allocation to patient demographics [382] .

All these recommendations were supported by the study synopsis, and subsequently documented, and complied with in the course of the SpheroNEO Study. The time difference between tissue excision and the start of the cell isolation procedure was kept at a minimum with a mean of 26.3 h, only slightly surpassing the recommended ischemic time of 24 h [383, 384].

Tissue availability remained a key issue. The limiting factor was the minimal number of cells that are required for a valid assay outcome. As described in the results section, all factors influencing successful assay outcome were either directly or indirectly related to the number of cells isolated from the study samples. Quality of the study sample also played a major role in the success of the assay, as was evident in the wide range of isolated cell number per total sample weight. Some samples weighed less but yielded a higher number of vital cells, while others weighed more but consisted almost completely of fatty or connective tissue. The spheroid methodology used in this study had previously been established using surgical specimen from colorectal carcinoma patients, which were relatively homogenous in tissue composition and consistently yielded greater numbers of cells per study sample [299]. Adapting this method to the much smaller core needle biopsy samples from breast cancer patients proved difficult, especially since the anatomy of the breast is much more diverse in its tissue components. For this reason it is essential to determine the cellular composition of the study sample, for example using immunohistochemistry or fluorescent staining methods either following tissue excision and/or after cell isolation. Genetic analysis or next-generation sequencing may be considered as well [385, 386]. This would sort out unfit samples at an early stage thus reducing time and cost.

Since a specified tissue amount in mg is an impractical measure in clinical routine, a minimum requirement of four biopsy cylinders could be considered optimal. If surgical bulk specimens are analyzed in the assay on the other hand, a weight requirement is more pragmatic, since the pathological analysis requires the dissection of the tumor for a microscopic analysis. A minimum requirement for surgical tissues was previously published [387]. Results obtained in this study showed that a minimum of four biopsy cylinders with a mean total weight of 89.6 mg (range 10.5 – 353.4 mg), were required to test a minimum of three treatment combinations. This would cover the *in vitro* analysis of one exemplary compound from each treatment combination, i.e. 1) Anthracycline-taxane, 2) anti-HER2 treatment combination, as well as 3) a platinum-containing regimen. The specific compounds included in the treatment combination for example Doc vs Pac or epirubicin vs doxorubicin would need to be narrowed down by the physician beforehand; otherwise the number of combinations would exceed the potentially available amount of tissue. Since at the time of the initial biopsy the HER2 status was not yet available, an anti-HER2 treatment combination should be included in case of a potentially HER2+ tumor. If the final treatment decision has already been made, for example due to preexisting comorbidities, testing in the breast cancer spheroid model would only require a minimum amount of tissue. The likelihood of treatment efficacy or resistance to the pre-specified drugs can be tested; however the comparison of treatment efficacy to other compounds will be limited.

Anti-hormonal compounds were not tested in the current study, and more research is required to optimize the assay for anti-hormonal treatment *in vitro*. These lipophilic compounds are often only available in solid form, making their application *in vitro* difficult. Although adding endocrine treatment to the list of compounds, would be of great value. Other drugs such as bevacizumab cannot be applied in the *in vitro* treatment setting due to the lack of a functioning blood vessel system in the spheroid model. However, not just these types of compounds but also other targeted drugs, such as immune modulators and signal transduction inhibitors, are the main focus of current drug development and a growing number of patients will receive these drugs as part of their treatment. An individual evaluation of *in vitro* drug efficacy will determine the validity of each new drug in the model, thereby ruling out any confounding issues. Patients receiving these types of compounds should currently not be includ-

ed in the study population, since the clinical results cannot be fully compared to the laboratory data. Especially since only a small fraction of patients may receive the drug and lessen the effect size of the primary endpoint. The association between clinical and laboratory outcome should first be verified using a homogenous study population with similar drug treatment, subsequently the laboratory data may be assumed as a surrogate for clinical outcome.

All other laboratory factors documented during the assay procedure were consistent and had little impact on assay success or outcome. The only exception was the choice of readout method, ATP or MTS. In retrospect, the ATP proved to be the substantially more sensitive readout method compared to MTS. Individual differences in cell survival between treatment options *in vitro* were much more distinguishable using ATP, while results obtained using MTS showed a much smaller range in values. It appears that an ATP readout is the method of choice in this model and should be used for all future study samples to ensure comparability both between patients and applied treatment combinations *in vitro*.

Other than the above specified limitations, the breast cancer spheroid assay was very efficient. This was reflected by the high viability seen overall study samples and the low rate of contamination. Outside factors such as tissue logistics appeared to play only a minor role in both the success of the assay and outcome. The time interval from sample excision to beginning of cell isolation was slightly higher in samples not fulfilling laboratory criteria, however the difference was not significant. Based on prior experience and published data a limitation to 48 h total transportation time is essential considering tissue and enzymatic degradation, as well as possible changes in gene and protein expression profiles [388-392]. Considering the types of logistics available today, this time limitation is feasible for fresh tumor study samples. In this study, nine study samples exceeded this time span, however, of these four were included in the main analysis and of the other five only three were excluded according to laboratory criteria. The four samples were included despite protocol deviation since all documented factors such as viability and baseline metabolic activity were within the normal range.

The utilized transportation medium did have an effect as can be seen in the 7 samples that were shipped in isotonic sodium chloride solution (0.9% NaCl). Out of these

samples, only two could be tested in the assay and mean viability was 78.17% during cell isolation, compared to an overall mean of 86.06% for samples arriving in shipping medium.

Current and previous chemosensitivity testing

A tumor spheroid model, such as the one tested here, is able to replicate the heterogeneity of cell types, cell-cell interactions, and the microenvironment of the patient tumor more closely as compared to cell-based monolayer assays and other 3D assays [393-395]. In addition, through the 3D structure a penetration barrier is formed allowing different amounts of cytostatic agent to reach each tumor cell [396]. Comparable chemoresponse assays were either not validated in a prospective trial with adequately powered endpoints, did not achieve the high sensitivity/specificity as seen here, and/or do not resemble the original 3D structure of the patient tumor. Thus, this assay represents a model, which may improve treatment selection utilizing the tumor heterogeneity of breast cancer present in individual patients. This is based upon results obtained in a prospective study conducted according to high quality standards with a homogenous patient cohort. The current expert opinion should be reconsidered regarding the application of chemoresponse assays in clinical diagnostics.

Chemosensitivity/chemoresistence assays, collectively referred to as chemoresponse assays using patient tissue-derived cells have been tested since the early 1970s, and the results obtained from the first type, clonogenic assays, initially seemed promising [291, 295, 397, 398]. Although re-evaluation of the data led to the discredit of the method due to the failed association with clinical outcome [399]. Various approaches and laboratory methods, such as differential staining/dye exclusion, incorporation of radioactive precursors by macromolecules, colorimetric assays, chemoluminescence, and measurement of differential optical density, have been used not only for breast cancer patients but other solid tumors as well [295, 398, 400-404]. The methodological approaches for all of these assays are similar; initially the patient tumor sample is disassociated into a single-cell suspension with subsequent culture of the isolated cells. The length of this culture period depends on whether or not the cells are intended to proliferate or not, longer cell culture may prefer specific cell types such as fibroblasts and result in changes in cellular physiology [405]. A drug or combination of drugs is applied and the resulting change in cell sur-

vival is quantified and interpreted after a period of incubation. Results are analyzed in comparison to individual patient outcome, mainly survival endpoints. Currently there are several assays which are commercially available. Among them are the differential staining cytoxicity assay (Weisenthal Cancer Group, Huntington Beach, CA, USA), the EVA/PCD™ assay (Rational Therapeutics, Long Beach CA, USA), the fluorometric microculture cytoxicity assay, thymidine incorporation assay, the histoculture drug resistance assay (HDRA, AntiCancer Inc., San Diego, CA, USA, SRL Inc., Tokyo, JP), the adenosine triphosphate bioluminescence assay, ChemoFX® (Helomics Corp., Pittsburgh, PA, USA), CorrectChemo® (Diatech Oncology, Franklin, TN, USA), extreme drug resistance assay (EDR®, Exiqon Diagnostics, Tustin, CA, USA, TherapySelect, Heidelberg, DE), Oncogramm (Oncomedics, Limoges, FR) to name a few.

Only a limited number have been tested in a randomized prospective trial, such as the tritiated thymidine incorporation assay testing extreme drug resistance [406]. Using cells in monolayer or clusters, tritiated thymidine is added to the tumor cells and the radioactivity incorporated into the DNA is quantified to determine drug resistance *in vitro*. Drug resistance or sensitivity is quantified through a direct or inverse measurement of the incorporated radioactivity that is generated from viable cells that incorporate the compound into their DNA following cytostatic treatment *in vitro*. Analysis in several prospective randomized trials with ovarian cancer patients did not show any significant difference in overall response of the assay group compared to the control group, which received treatment according to standard clinical criteria [407]. In a similar retrospective analysis using breast cancer tissue, between patients with low drug resistance in the assay compared to those with intermediate/high drug resistance significant differences in prognosis were found. However, the identified patient subgroups showed initial differences in disease stage and nodal status which may be the underlying cause for the observed differences in survival. The grouping of the patients into drug resistance profiles is also unclear and may have been a result of an otherwise small sample size. Also no randomization procedure was included in the trial [408].

Using the ATP assay to measures luminescence of intracellular ATP as a surrogate marker for cell survival following cytostatic treatment, a randomized controlled study was conducted using cells in monolayer isolated from recurrent platinum-resistant

ovarian cancer patients [409]. In this study no significant differences in prognosis were seen in the assay-directed versus the control treatment group. However, another study including patients with metastatic melanoma did find significant differences in progression-free survival with patients receiving *in vitro* sensitive treatment [410]. The ChemoFX assay also utilizes an ATP readout system. Currently there are several published studies, which showed an increase in OS of 14 months using assay-directed treatment for ovarian cancer patients [411]. In a study using breast cancer biopsies, pCR correlated with assay outcome *in vitro* and was found to achieve a sensitivity of 83.3% and 71.4% specificity [291]. However, the patient sample size was 34 in this analysis and only conducted as a sub study; the main objective of the article was a general assessment of the validity of the assay methodology. It is unclear if the analysis is sufficiently powered to obtain a valid statistical result for this secondary endpoint. Although several prospective trials have been conducted using this assay, mainly in ovarian cancer with mixed results, health care providers have criticized the lack of a randomization procedure in the conducted studies. Without randomization any observed effect may be attributed to known or unknown confounding factors, therefore skewing results in one direction or another.

For the other listed assays, no randomized prospective trials are available, for example the differential staining cytotoxicity assay, which uses a counterstaining method using Fast Green Dye (dead cells) and hematoxylin-eosin (viable cells). The ratio of dead to viable cells is quantified following cytostatic treatment *in vitro*. The idea behind the approach is relatively simple. However, the methodology is complex and utilizes a difficult scoring system. The interpretation of the results should only be conducted by trained professionals. In a small study by Cortazar et al on small cell lung cancer patients, a significant improvement in patient survival was seen (38.5 assay-directed vs 19 mths standard chemotherapy selection). However, due to the methodological complexity, an implementation of this assay in the clinical routine is not feasible. This method is currently successfully used in hematologic malignancies where it is being used for *in vitro* drug assessment [412, 413].

The EVA/PCD™ assay is also based on a hematoxylin-eosin counterstaining method with Nigrosin and Fast Green Dye to quantify apoptotic cells in microspheroids (50-100 cells per spheroid). A study from 2012 showed an overall response rate of 64.5%, which was greater than twice the response rate (complete and partial re-

sponse) observed for historic controls [414]. Positive aspects included the use of a 3D cell culture model and the application of guideline recommended compounds in several concentrations. Limitations in the study design are a comparison to historic controls and no report of the examination that was used to determine clinical response to treatment.

A slightly different differential staining technique is used in the fluorometric microculture cytotoxicity assay, which associates higher levels of fluorescence - produced through the hydrolysis of fluorescein diacetate - with increased drug resistance [415]. A newer procedure was offered by the company Oncomedics (Limoges, FR), which is referred to as Oncogramm. Using patient cells and a triple staining procedure following treatment *in vitro*, live and dead cells are counted using a fluorescent microscope. Preliminary studies in metastasized colorectal cancer specimen reported a good sensitivity (84.6%) for the assay, although specificity was low at 33.3% [416]. This type of assay has not been analyzed in a prospective randomized study.

The histoculture drug resistance assay [417] grows small tissue particles in a 3D collagen matrix. The inhibition rate was determined using the MTT readout following treatment *in vitro* with applicable drugs. Although the 3D architecture of the patient tumor was preserved in this assay, the trials used a retrospective design. In more recent trials, no control group was included for this assay. In a recent publication using tissue from anaplastic thyroid cancer patients, the assay outcome (inhibition rate) was associated with prolonged patient survival, although the results are based on an individual patient comparison, and the patient cohort is very heterogeneous both in extent of disease and tissue excision site, making a direct correlation to clinical outcome potentially biased [418].

The colorimetric MTT readout method measures mitochondrial activity through the enzymes involved in the reduction of NADP(H) and has previously been used to study cytostatic efficacy. One such example is a retrospective study analyzing the benefit of assay-based chemotherapy in gastric cancer patients by Wu et al. [419]. The study included a control group which received chemotherapy according to the physician's choice and compared the documented toxicities in both arms. No benefit in OS was seen for the patients tested with this assay. Also the number of adverse

events was the same in both groups. A similar study was conducted on advanced breast cancer patients, which showed more favorable results in patients receiving assay-directed treatment, however no significant differences were observed in the median survival in a comparison to the control arm [420].

The Microculture-Kinetic (MiCK) assay (CorrectChemo®, Diatech Oncology, Franklin, TN) uses optical density measurement over a time span of 48 h to determine induced cell death following treatment *in vitro* [421, 422]. Data from randomized trials using this assay are not available and prospective data is only available for acute myeolocytic leukemia or a patient cohort diagnosed with a variety of solid tumor types [423].

Generally problematic in many of these studies are the inhomogeneous patient and/or tissue populations. Patients with prior treatment were oftentimes part of the study cohort, and in a few cases tissue samples were obtained from the solid tumor site or metastatic ascites/pleural effusions. These factors make overall data regarding treatment outcome or prognosis difficult to interpret and a direct association between *in vitro* assay results and clinical outcome potentially confounded. In some older studies, patients suffering from different solid tumors were included in the study and differences in patient prognosis and endpoint measurements (disease-free vs progression-free vs OS) were not adjusted according to baseline differences in prognosis.

Based on the described obstacles, implementation of these assays into the clinical routine has proven difficult, and so far none of these assays are used on a routine basis. The reason behind this as voiced by current guidelines and reviews are that in general chemosensitivity- and chemoresistence assays have not shown conclusive evidence obtained through prospective randomized clinical trials [295, 414, 424], which represent the gold standard in clinical research today. Currently the opinion voiced by many treating physicians is that results from *in vitro* chemoresponse assays are promising and interesting but the data remain inconclusive especially regarding a benefit in survival. This is why many doctors are reluctant to recommend such a test to their patients [72, 115].

Current evaluation of the validity of chemosensitivity testing

In light of the fact that the latest review dates from 2011 (ASCO) and the guidelines regarding the validation of potential biomarkers have been updated, the current position toward these assays should be reconsidered [295, 425-428]. Currently, national (AGO, DKFZ) and international professional organizations (ASCO, NCCN, NCI), as well as major healthcare providers do not recommend chemoresponse assays for routine clinical application outside of clinical trials [295, 341, 354, 429, 430]. Approval from regulatory authorities is not required since the test does not fall under the authorities' jurisdiction. In Germany, for example, this type of assay falls under the regulations covering *in vitro* diagnostics (IVD-Richtlinie 98/79/EG) and medical devices. A high level of evidence through randomized clinical trials is however required by healthcare providers, in order to subsidize the cost of such a test.

Other concerns, often listed by physicians, are the inability to include systematic effects, such as pharmacodynamics and pharmacokinetics, as well as blood supply to and from the tumor, all of which are difficult if not impossible to mimic *in vitro*. Some of these concerns may be resolved using simultaneous *in vitro* and *in vivo* testing, although the addition of a murine component brings a potentially confounding variable into the equation. The ideal decision-aiding tool should predict the most favorable balance of tolerability and efficacy for each patient while limiting confounding effects by the assay itself, however there is currently no assay available that addresses all these aspects simultaneously [431].

Several aspects should be considered in the search for the ideal model or assay, such as the type of material used in the assay, as well as the potential feasibility for clinical translation. Using patient cancer tissue obtained in routine diagnostic procedure or surgical tumor removal for *in vitro* analysis seems to be a logical step to treatment selection for individual patients. Current ethical considerations, as well as the continuous improvement in biobanking standards make this a feasible approach and a framework most representative of the heterogeneity found in patient tumors [432, 433]. Other options are animal models, mainly murine, as well as immortalized cell lines. The drawback to *in vivo* models using immunodeficient mice is the murine microenvironment which may have a confounding effect on tumor biology and treatment efficacy. Tumors often take weeks to develop, making this model setup ques-

tionable for clinical implementation due to the time delay until assay data is available. Experiments using knock-out models or xenograft transplants offer insight into the underlying disease pathways and are valuable preclinical models, but have not been able to predict patient treatment outcome in a prospective study [434, 435]. The subrenal capsule assay is a model system using immunocompromised or other mouse breeds where small amounts of tumor tissue or tumor cell lines was implanted in the subrenal capsule and subsequently studied for differential growth following treatment [436]. This model has been tested as a potential assay system to predict clinical outcome in ovarian cancer, however, there no improvement in patient survival has been demonstrated [437, 438]. A similar approach was developed by the National Cancer Institute using implantable biocompatible hollow fibers where tumor cells can grow *in vivo* [439]. Tumor efficacy can also be tested using this method, however there are no studies, where this approach was used in predicting clinical outcome using patient tumor tissue [440].

Cell lines in comparison are much more cost-efficient and less care-intensive while offering little information into the effects from the tumor microenvironment due to the limited cellular heterogeneity. Data obtained through genetic profiling and extensive characterization of established cell lines already allow mathematical modeling of potential treatment outcome, aiding in the preclinical drug testing process [441, 442].

Current research is mainly done using gene-based assays, which have shown to accurately predict the necessity of systemic therapy +/- endocrine maintenance therapy, although results are not applicable to all patient subgroups equally [443]. This aspect also applies to the newly developed immunohistochemistry assay as well as to the next generation sequencing approaches: both rely on breast cancer subtyping (luminal A, luminal B, HER2-like, basal-like) to determine the treatment strategy i.e. combination of endocrine treatment and chemotherapy with the most favorable prognosis [444]. Next generation sequencing by analyzing the genomic and proteomic characteristics of individual patient tumors may also identify new targets for more effective treatment strategies and targeted drugs [445]. However, these assays do not address the issue of which compounds should be included in the drug treatment and which would yield the most effective treatment outcome. An ideal assay would potentially combine *in vitro* testing with genomic subtyping. Also promising are the findings from studies on the immune profile of patient tumors, however several fac-

tors will still have to be investigated further, such as the contribution of the individual cell types and the contributing mechanisms [216, 446].

Interventional study using the breast cancer spheroid model

The data presented in this thesis indicates that the breast cancer spheroid model may not only be predictive of treatment outcome, but also potentially selective in discerning ineffective from effective treatment options. Additional data will need to be obtained to strengthen these findings in a larger, randomized trial with an endpoint measuring survival. In an interventional study this and other factors such as improved patient and tissue screening using in- and exclusion criteria based on data obtained in the SpheroNEO study. In addition to the information gathered already, an analysis of the health economics of this assay could provide further evidence for approval of the assay by health insurance providers.

Survival data is especially important for assay validation considering the scientific discussion concerning pCR as a surrogate endpoint on a trial-level for long-term treatment success [447]. Some argue that pCR only acts as a surrogate marker for HER2+ and triple negative disease and maintain the position that survival is the empirically strongest endpoint and should be used as a primary objective in clinical trials [71, 448, 449]. However, others consider pCR a valid marker for prognosis in all breast cancer subtypes [447, 450] and the drug approving agency in the United States (FDA) has approved the use of pCR as a surrogate marker in drug approval trials [451]. Proponents argue that this will enable drugs to be brought onto the market more quickly than waiting for survival data. Opponents cite the example of the anti-angiogenesis drug bevacizumab which was approved using a fast track approach. Later, the approval was revoked in the treatment for primary breast cancer due to insufficient evidence for treatment efficacy and potentially serious side effects in some patients [452].

Depending on the extent of the treatment, follow-up would be a minimum of five years following surgery, however guideline recommendations regarding this time span differ [355, 453]. According to current data the rate of recurrence has continually dropped due to improved treatment options with most cases being observed in the first two years following initial treatment. The OS after 5 years lies between 80-90%. However, a small percentage of patients have shown disease recurrence after up to

20 years following the initial diagnosis [454, 455]. Even patients with pCR have a small percentage of developing recurrent disease [456].

In order to validate the results from this explorative study an interventional randomized controlled study is essential to determine the independent predictive power of the spheroid model and such a confirmatory phase III study is planned. In addition an interventional study is required by German health insurance providers for the compensation assessment of this assay. The cutoff of 35% cell survival differentiating between complete pathological response and not, will be analyzed in regard to its validity and reliability in a larger cohort of patients. A total sample size of 318 patients with 1:1 randomization procedure (159 per arm) has already been determined. These numbers are based on the assumption of 70% assay success rate and an improved pCR rate through assay-directed chemotherapy selection of 44%, power and α are 0.80 and 0.05 respectively.

The study design incorporates both the treating physicians, as well as the local tumor board in the treatment decision-making process. Study samples will again be accrued during the initial diagnosis examination, following pathological examination and extraneous tissue (minimum 4 biopsy cylinders) will be shipped to the central laboratory for cell isolation and spheroid preparation. The in- and exclusion criteria will remain largely the same as for the SpheroNEO study, however in order to improve patient screening the minimum number of biopsy cylinders will be included (N=4). Patients will also be required to have an ECOG of 0 or 1 to maximize treatment adherence, similarly patients taking concurrent medication or suffering from a serious medical condition that would contraindicate chemotherapy or put the patient at an increased risk are excluded from study participation. By excluding patients with contralateral or inflammatory breast cancer the study population will be more homogenous in regard to outcome and recommended drug treatment.

Following successful screening, patients will be randomized to the assay-directed or the control arm. Patients in the control arm will be treated as determined by the tumor board, and data from the spheroid assay will not be forwarded to study site personnel. Patients in the assay-directed arm will also be discussed in the local tumor board, however the treating physicians will have access to the data obtained from the spheroid assay and may base their treatment decision on these results. If the

treatment is not applied according to the results of the spheroid assay, the reason must be stated by the physician such as patient preferences. This questionnaire was suggested by the ruling ethics committee and will be required for the interventional study. Following the completion of chemotherapy by all study patients, a comparison will be made between the control and study arm to determine if the data from the spheroid assay has led to an increase in successful treatment outcome. The study will again use patients eligible for neoadjuvant chemotherapy and the primary endpoint will progression-free survival. As became evident during the SpheroNEO study, the assistance of a contract research organization (CRO) would be highly advisable in the site feasibility and management, as well as data collection. The number of sites would also need to be increased to accrue the necessary number of patients in a sufficient time span, the addition of other countries beside Germany should be considered such as Austria and the Netherlands, the Netherlands currently rank 3rd among European countries in the age-standardized breast cancer incidence rate [457]. Given the patient numbers successfully included in the current study per month, an estimated number of 53 study sites would need to be initiated to obtain the patients needed in a time span of two years.

A cost-benefit analysis will be conducted as a secondary endpoint to examine the potential benefit of an assay-directed treatment compared to standard clinical based treatment decision-making. Health economics are becoming increasingly important for health insurance providers and pharmaceutical companies due to the enormous costs of targeted oncology drugs. Treatment-related costs for oncology drugs rank fifth in overall health care expenditures. For instance, the cost of one year of targeted anti-HER2 medication lies around 35 000 - 55 000 € for a single patient [458]. This illustrates the importance of distinguishing non-efficacious drugs early on in preclinical drug development and identifies patients who will be incorrectly treated. Thereby harmful toxicities may be avoided for the patient, as well as unnecessary costs for the insurance provider. Additional secondary endpoints are assessment of adherence to the assay results by the decision-making physician(s) and comparison of quality of life in the study and the control arm.

Conclusion

In the treatment of breast cancer, there are an abundance of drugs and targeted therapies available today, yet, not all patients respond equally well. The rate of pCR remains stable at 22%, with percentages varying according to disease subtypes (luminal A 6.4%, luminal B/HER2- 11.2%, basal-like 31.0%, HER2-like 32.9% [49, 459, 460]. The introduction of targeted therapy nearly two decades ago has vastly improved the prognosis for a subset (15-20%) of patients with HER2+ tumor biology, yet the remaining majority of patients lacking this trait continue to receive standard chemotherapy which is selected using empirical decision algorithms. Since time is of the essence in cancer treatment, the current trial and error selection of anti-cancer treatment mainly based on lengthy and costly clinical drug trials should be expanded to include preclinical *in vitro* models. Using a 3D cell culture technique on patient-derived cells, the breast cancer spheroid model reflects many aspects of the patient tumor biology. The data described in this manuscript show that the assay has achieved high levels of sensitivity and specificity regarding clinical outcome in a prospective study. Further research is warranted to validate this assay for the individual patient treatment selection and clinical implementation. A consolidation of laboratory and clinical data would not only speed up the process of preclinical drug testing but would also aid in identifying the treatment with the most favorable prognosis for individual breast cancer patients [461, 462].

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APPENDIX

INDEX OF FIGURES

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LIST OF ABBREVIATIONS

2D	2-Dimensional
3D	3-Dimensional
5-FU	5-Fluorouracil
ABC	ATP-binding cassette
AC	Anthracycline + Cyclophosphamide
AC -> T	Anthracycline + Cyclophosphamide -> Taxane
AC -> TCb	Anthracycline + Cyclophosphamide -> Taxane + Carboplatin
AC -> TH	Anthracycline + Cyclophosphamide -> Taxane + Trastuzumab
Akt	Protein kinase B
AUC	Area under curve
BCS	Breast conserving surgery
BMI	Body mass index
CA 15-3	Carcinoma Antigen 15-3
CAF	Cancer associated fibroblast
CEA	Carcinoembryonic antigen
cps	Counts per second
CRF	Case report form
DAPI	4', 6-Diamidino-2-phenylindole
DCIS	Ductal carcinoma *in situ*
DFS	Disease-free survival

DMBA	7, 12-Dimethylbenzo [*a*] anthracene
DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
Doc	Docetaxel
DocAC	Docetaxel + Doxorubicin + Cyclophosphamide
EC	Epirubicin + Cyclophosphamide
EDR	Extreme drug resistance
ER	Estrogen receptor
EGFR	Epidermal growth factor receptor
EtOH	Ethanol
Eve	Everolimus
FBS	Fetal bovine serum
FEC	5-Fluorouracil + Epirubicin + Cyclophosphamide
FISH	Fluorescence in situ hybridization
HDRA	Histoculture drug resistance assay
HER2	Human epidermal growth factor receptor 2
H	Trastuzumab
HR	Hormone receptor
IGFR1	Insulin-like growth factor 1 receptor
IHC	Immunohistochemistry
ISO	International Organization for Standardization
IVD	*In vitro* diagnostics

MAPK	Mitogen-activated protein kinase
MCTS	Multicellular tumor spheroids
MDR	Multi drug resistance
MDSC	Myeloid-derived suppressor cell
MEM	Minimum Essential Medium
MiCK	Microculture Kinetic
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
NADP	Nicotinamide adenine dinucleotide phosphate
NEAA	Non-essential amino acids
NMR	Nuclear magnetic resonance
OS	Overall survival
Pac	Paclitaxel
PAI-1	Plasminogen activator inhibitor-1
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
pCR	Pathological complete response

PET	Positron emission tomography
PFS	Progression-free survival
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
ppc	Peak plasma concentration
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
ROC	Receiver operating characteristic
SERM	Selective estrogen receptor modulator
STAT	Signal transducer and activator of transcription
TAM	Tumor-associated macrophages
TCbH	Docetaxel + Carboplatin + Trastuzumab
TDM-1	Trastuzumab emtansine
TNBC	Triple negative breast cancer
TM	Transport medium
TME	Tumor microenvironment
uPA	urokinase-type plasminogen activator
VEGF-A	Vascular endothelial growth factor A

Table 12: List of participating breast cancer centers

Site number	Study site	Samples received	Eligible patients	Patients enrolled per month	Drop-out rate (%)
01	Klinikum Landshut	26	18	0.94	45.45
03	Klinikum Starnberg	8	0	0.28	100.00
04	Frauenklinik KUM	22	9	0.69	64.00
05	Frauenklinik TUM	21	4	0.79	81.82
05	Radiologie TUM	4	1	0.17	75.00
07	Klinikum Harlaching	17	7	0.65	58.82
14	Klinikum St. Marien Amberg	15	8	1.67	46.67
15	Leopoldina Krankenhaus der Stadt Schweinfurt gGmbH	9	5	0.90	44.44
16	Klinikum Nürnberg	5	0	0.63	100.00
17	Städtisches Klinikum Karlsruhe	5	2	0.63	60.00
18	Markus Krankenhaus Frankfurt	15	1	1.67	93.33
19	Marienhospital Bottrop gGmbH	30	9	3.33	70.00
20	Evangelische Kliniken Gelsenkirchen	21	11	2.10	47.62
21	Klinikum Dritter Orden	4	3	0.80	25.00
	Total	**202**	**78**	**15.25**	**65.15**

Table 13: List of screened participants with reason for exclusion

Patient-ID	Year of Birth	Sample received	Comment	Included in final analysis	Reason for exclusion
A-008	1942	15.06.2010	-	No	Clinical treatment does not correspond to *in vitro* treatment
A-009	1963	30.06.2010	-	Yes	
A-010	1962	02.07.2010	-	Yes	
A-011	1954	02.07.2010	-	No	Clinical treatment does not correspond to *in vitro* treatment
A-013	1953	21.07.2010	cM1 (liver)	No	Metastatic disease
A-014	1968	26.08.2010	-	Yes	
A-015	1949	30.08.2010	-	No	Insufficient number of epithelial cells
A-016	1961	21.09.2010	-	Yes	
A-017	1967	27.10.2010	-	Yes	
A-018	1972	25.11.2010		Yes	
A-019	1972	15.12.2010	-	No	Technical difficulties
A-020	1979	16.02.2011	-	Yes	
A-021	1949	10.03.2011	-	Yes	
A-022	1986	24.05.2011	-	Yes	
A-023	1969	22.06.2011	-	Yes	
A-024	1967	30.06.2011	-	Yes	
A-025	1956	08.09.2011	-	Yes	
A-026	1951	11.10.2011	-	Yes	
A-027	1973	28.10.2011	-	Yes	
A-028	1938	15.12.2011	-	No	Clinical treatment does not correspond to *in vitro* treatment
A-029	1969	29.12.2011	Bilateral tumor	Yes	
A-030	Unknown	09.03.2012	-	No	Clinical data not available
A-031	1964	02.08.2012	-	Yes	

A-032	1969	22.08.2012	-	No	Cellular metabolic activity under threshold
A-033	1948	02.10.2012	-	Yes	
A-034	1948	21.11.2012	-	Yes	
B-002	1956	23.11.2010	cM1 (liver)	No	Metastatic disease
B-003	1939	30.11.2010	-	No	No neoadjuvant drug treatment
B-004	1946	21.01.2011	cM1 (bone), simultaneous renal cell carcinoma	No	No neoadjuvant drug treatment
B-005	1964	23.05.2011	-	No	No neoadjuvant drug treatment
B-006	1937	16.06.2011	-	No	No neoadjuvant drug treatment
B-007	1934	25.08.2011	-	No	Insufficient number of epithelial cells
B-008	1960	13.10.2011	-	No	No neoadjuvant drug treatment
B-009	1927	08.03.2012	-	No	No neoadjuvant drug treatment
C-004	1956	25.01.2011	Bilateral tumor	Yes	
C-005	1973	01.02.2011	Histologically no tumor	No	No malignant tumor
C-006	1953	09.02.2011	Loss to follow-up	No	Clinical data not available
C-007	1953	25.08.2011	-	No	No neoadjuvant drug treatment
C-008	1970	26.08.2011	-	No	Previous malignant disease
C-009	1935	30.08.2011	-	No	No neoadjuvant drug treatment
C-010	1937	02.09.2011	-	No	Previous malignant disease
C-011	1932	23.09.2011	-	No	No neoadjuvant drug treatment
C-012	1965	07.10.2011	Bilateral tumor	No	Previous malignant disease
C-013	1948	07.10.2011	-	No	Insufficient number of epithelial cells
C-016	1990	14.11.2011	-	Yes	
C-017	1949	17.11.2011	-	No	No neoadjuvant drug treatment
C-018	1950	20.12.2011	-	Yes	
C-019	1939	25.01.2012	End of chemotherapy after 4 cycles	Yes	
C-020	1937	17.02.2012	-	No	No neoadjuvant drug treatment
C-021	1947	27.03.2012	-	Yes	
C-022	1968	29.03.2012	-	Yes	
C-023	1948	05.04.2012	End of chemotherapy after 2 cycles due to side effects	Yes	
C-024	1969	13.04.2012	-	No	No neoadjuvant drug treatment
C-025	1974	21.06.2012	-	Yes	
C-026	1957	28.06.2012	-	No	Clinical treatment does not correspond to *in vitro* treatment
C-027	1983	08.08.2012	-	Yes	
D-002	1970	08.06.2010	-	Yes	
D-003	1958	17.06.2010	-	No	No neoadjuvant drug treatment

D-004	1961	18.06.2010	End of chemotherapy after 7 cycles due to progressive disease	Yes	
D-005	1959	25.06.2010	Bilateral tumor; M1(liver)	No	Previous malignant disease
D-006	1944	01.07.2010	Previous diagnosis of breast cancer in 2003	No	Previous malignant disease
D-007	1939	05.07.2010	Previous diagnosis of breast cancer in 1991	No	Previous malignant disease
D-008	1965	14.07.2010	-	No	No neoadjuvant drug treatment
D-009	1960	20.07.2010	-	No	No neoadjuvant drug treatment
D-010	1972	16.09.2010	-	No	No neoadjuvant drug treatment
D-011	1955	27.12.2010	Bilateral tumor	No	No neoadjuvant drug treatment
D-012	1966	01.09.2011	-	No	Insufficient number of epithelial cells
D-013	1955	05.09.2011	-	No	No neoadjuvant drug treatment
D-014	1968	08.09.2011	-	No	No neoadjuvant drug treatment
D-019	1951	23.12.2011	Bilateral tumor	No	No neoadjuvant drug treatment
D-020	1969	20.01.2012	M1 (central nervous system)	No	Metastatic disease
D-021	1969	15.02.2012	-	No	No neoadjuvant drug treatment
D-022	1938	06.03.2012	-	Yes	
D-023	1960	07.03.2012	-	Yes	
D-024	1971	25.04.2012	Treatment in Russia, loss to follow up	No	Clinical data not available
D-025	1969	26.04.2012	-	No	No neoadjuvant drug treatment
D-027	1948	18.06.2012	Bilateral tumor; M1 (Spleen, lung, liver, adrenal gland, bone)	No	Metastatic disease
D-015	1927	14.10.2011	-	No,	No neoadjuvant drug treatment
D-016	1959	14.10.2011	-	No	Clinical data not available
D-017	1938	28.10.2011	-	Yes	
D-018	1946	22.11.2011	cM1	No	Metastatic disease
E-001	Unknown	26.07.2010	Histologically no tumor	No	Clinical data not available
E-002	1964	29.10.2010	-	No	Insufficient number of epithelial cells
E-003	1941	10.11.2010	-	No	Yeast contamination
E-004	1966	08.12.2010	-	No	Cell viability < 50%
E-005	1965	15.12.2010	-	Yes	
E-006	1980	11.02.2011	-	No	Clinical treatment does not correspond to *in vitro* treatment
E-007	1965	11.03.2011	-	Yes	
E-008	1948	23.03.2011	-	No	Yeast contamination
E-009	1958	30.08.2011	-	Yes	
E-010	1954	04.01.2012	-	Yes	
E-011	1968	31.01.2012	-	No	Insufficient number of epithelial cells
E-012	1953	21.03.2012	-	No	Cellular metabolic activity under threshold

E-013	1952	30.03.2012	-	Yes	
E-014	1954	19.06.2012	-	No	Cellular metabolic activity under threshold
E-015	1947	01.08.2012	-	Yes	
E-016	1962	17.10.2012	-	No	Insufficient number of epithelial cells
E-017	1948	20.12.2012	-	Yes	
F-001	1970	17.12.2011	-	Yes	
F-002	1964	28.01.2012	-	No	Insufficient number of epithelial cells
F-003	1957	18.02.2012	-	Yes	
F-004	1980	18.02.2012	-	No	Cellular metabolic activity under threshold
F-005	1965	03.03.2012	-	Yes	
F-006	1956	08.03.2012	-	No	
F-007	1959	24.04.2012	-	No	Cellular metabolic activity under threshold
F-009	1965	14.06.2012	cM1	No	No neoadjuvant drug treatment
F-008	1981	15.06.2012	-	Yes	
F-010	1961	12.07.2012	-	Yes	
F-011	1961	26.07.2012	-	No	Cellular metabolic activity under threshold
F-012	1963	18.08.2012	-	Yes	
F-013	1956	18.08.2012	-	No	Cellular metabolic activity under threshold
F-014	1975	30.08.2012	-	Yes	
F-015	1970	07.09.2012	-	Yes	
G-001	1962	22.12.2011	-	Yes	
G-002	Unknown	29.12.2011	-	No	Cellular metabolic activity under threshold
G-003	1950	11.01.2012	-	Yes	
G-004	1964	28.01.2012	-	Yes	
G-005	1958	08.02.2012	Bilateral tumor; end of chemotherapy after 4 cycles	No	Clinical treatment does not correspond to *in vitro* treatment
G-006	1967	04.04.2012	-	Yes	
G-007	1955	18.04.2012	-	No	Cellular metabolic activity under threshold
G-008	1966	12.05.2012	-	No	Cellular metabolic activity under threshold
G-009	1948	05.07.2012	-	Yes	
H-001	1969	11.01.2012	-	No	No neoadjuvant drug treatment
H-002	1961	12.05.2012	-	No	Insufficient number of epithelial cells
H-003	1947	29.06.2012	-	No	Insufficient number of epithelial cells
H-004	1950	22.08.2012	-	No	Insufficient number of epithelial cells

H-005	1954	05.09.2012	-	No	Cellular metabolic activity under threshold
I-001	1948	10.05.2012	-	No	Clinical treatment does not correspond to *in vitro* treatment
I-002	1961	28.08.2012	-	Yes	
I-003	1950	29.08.2012	-	Yes	
I-004	1952	08.09.2012	-	No	Cellular metabolic activity under threshold
I-005	1965	15.09.2012	-	No	Cellular metabolic activity under threshold
J-001	1966	10.12.2011	-	No	No neoadjuvant drug treatment
J-002	1968	14.01.2012	-	No	Clinical treatment does not correspond to *in vitro* treatment
J-003	1954	14.01.2012	-	No	Clinical treatment does not correspond to *in vitro* treatment
J-004	1966	17.01.2012	-	No	Clinical treatment does not correspond to *in vitro* treatment
J-005	1931	07.02.2012	-	No	Clinical treatment does not correspond to *in vitro* treatment
J-006	1951	08.02.2012	-	No	No neoadjuvant drug treatment
J-007	1942	01.03.2012	-	No	Cellular metabolic activity under threshold
J-008	1944	06.03.2012	-	No	Clinical treatment does not correspond to *in vitro* treatment
J-009	1971	14.03.2012	-	No	Clinical data not available
J-010	1967	15.03.2012	-	No	Cellular metabolic activity under threshold
J-011	1932	29.03.2012	-	No	No neoadjuvant drug treatment
J-012	1970	04.04.2012	-	Yes	
J-013	1976	13.06.2012	-	No	Insufficient number of epithelial cells
J-014	1950	20.06.2012	-	No	Clinical treatment does not correspond to *in vitro* treatment
J-015	1928	01.09.2012	-	No	No neoadjuvant drug treatment
K-001	1930	17.12.2011	-	No	No neoadjuvant drug treatment
K-002	1974	04.01.2012	-	No	Cellular metabolic activity under threshold
K-003	1957	13.01.2012	-	No	Previous malignant disease
K-004	1941	10.02.2012	-	No	Clinical treatment does not correspond to *in vitro* treatment
K-005	1966	16.02.2012	-	No	Cellular metabolic activity under threshold

K-006	1960	21.02.2012	-	No	Clinical treatment does not correspond to *in vitro* treatment
K-007	1930	22.02.2012	-	No	Cellular metabolic activity under threshold
K-008	1966	31.03.2012	-	No	Cellular metabolic activity under threshold
K-009	1937	03.04.2012	-	No	Clinical treatment does not correspond to *in vitro* treatment
K-010	1958	05.04.2012	-	No	Insufficient number of epithelial cells
K-011	1966	20.04.2012	-	No	Clinical treatment does not correspond to *in vitro* treatment
K-012	1968	24.04.2012	-	No	Cellular metabolic activity under threshold
K-013 le	1936	27.04.2012	Bilateral tumor	No	Cellular metabolic activity under threshold
K-013 re	1936	27.04.2012	Bilateral tumor	Yes	
K-014	1944	01.05.2012	-	No	Cellular metabolic activity under threshold
K-015	1959	09.05.2012	-	No	Cell viability < 50%
K-016	1969	12.06.2012	-	Yes	
K-017	1972	20.06.2012	-	Yes	
K-018	1945	23.06.2012	-	No	Clinical treatment does not correspond to *in vitro* treatment
K-019	1966	06.07.2012	-	No	Clinical treatment does not correspond to *in vitro* treatment
K-020	1935	14.07.2012	-	No	Cellular metabolic activity under threshold
K-021	1956	19.07.2012	-	Yes	
K-022	1931	27.07.2012	-	No	Cellular metabolic activity under threshold
K-023	1942	17.08.2012	-	No	Cellular metabolic activity under threshold
K-024	1972	22.08.2012	-	No	Cellular metabolic activity under threshold
K-025	1934	05.09.2012	-	Yes	
K-026	1940	12.09.2012	-	Yes	
K-027	1936	15.09.2012	-	Yes	
K-028	1968	15.09.2012	-	Yes	
K-029	1971	19.09.2012	-	Yes	
L-001	1975	09.12.2011	Histologically no tumor	No	No malignant tumor
L-002	1963	22.12.2011	-	Yes	
L-003	1949	23.12.2011	-	No	Technical difficulties
L-004	1974	27.01.2012	-	Yes	
L-005	1966	07.03.2012	Switch of chemotherapy regimen after 4 cycles	No	Technical difficulties
L-006	1951	08.03.2012	-	No	Cell viability < 50%

L-007	1969	27.03.2012	-	Yes	
L-008	1967	04.04.2012	-	No	Cellular metabolic activity under threshold
L-009	1970	14.04.2012	-	Yes	
L-010	1953	19.04.2012	-	No	Cellular metabolic activity under threshold
L-011	1954	21.04.2012	-	Yes	
L-012	1958	25.05.2012	-	No	Cellular metabolic activity under threshold
L-013	1965	05.06.2012	-	Yes	
L-014	1955	19.06.2012	-	No	Cellular metabolic activity under threshold
L-015	1963	19.06.2012	-	No	Cellular metabolic activity under threshold
L-016	1956	19.06.2012	-	Yes	
L-017	1974	11.07.2012	-	Yes	
L-018	1968	20.07.2012	-	Yes	
L-019	1960	30.08.2012	-	Yes	
L-020	1964	31.08.2012	-	No	Clinical treatment does not correspond to *in vitro* treatment
L-021	1964	31.08.2012	-	Yes	
M-001	1956	15.05.2012	-	Yes	
M-002	1946	23.06.2012	-	Yes	
M-003	1949	12.07.2012	-	Yes	
M-004	1960	19.07.2012	-	No	Clinical treatment does not correspond to *in vitro* treatment

Table 14: Comparison of clinical criteria for included, enrolled, and excluded patient cohorts

	Enrolled			Excluded			All Patients		
Characteristic	**N**	**%**	**Mean**	**N**	**%**	**Mean**	**N**	**%**	**Mean**
Age at diagnosis	78		51	124		56	202		54
<= 40	13	16.7		9	7.3		22	10.90	
40 - 55	38	48.7		55	44.7		93	46.30	
> 55	27	34.6		59	48.0		86	42.80	
No data available	0	-		1	-		1	-	
Age grouped by median									
<= 50	43	55.1		46	37.4		89	44.30	
> 50	35	44.9		77	62.6		112	55.70	
No data available	0	-		1	-		1	-	
Grading at biopsy									
1	2	2.6		3	2.6		5	2.60	
2	39	50		61	53.5		100	52.60	
3	35	44.9		50	43.9		85	44.70	
No data available	2	2.6		10	-		12	-	

T-Stadium						
cT1a	0	-	2	1.8	2	1.10
cT1b	0	-	1	0.9	1	0.50
cT1c	11	14.1	14	12.6	25	13.30
cT2	45	57.7	68	61.3	113	60.10
cT3	17	21.8	17	15.3	34	18.10
cT4	4	5.1	9	8.1	13	6.90
No data available	1	1.3	13		9	
Nodal status						
cN+	42	53.8	51	45.9	93	49.50
cN-	35	44.9	60	54.1	95	50.50
No data available	1	1.3	13		14	
UICC						
IA	6	7.7	9	8.3	15	8.20
IIA	30	38.5	44	40.7	74	40.20
IIB	21	26.9	33	30.6	54	29.30
IIIA	15	19.2	7	6.5	22	12.00
IIIB	4	5.1	7	6.5	11	6.00
IIIC	0	-	1	0.9	1	0.50
IV	0	-	7	6.5	7	3.80
No data available	2	2.6	16		18	
Estrogen receptor (ER)						
Pos	49	62.8	75	72.1	124	69.30
Neg	26	33.3	29	27.9	55	30.70
No data available	3	3.8	20		23	
Progesterone receptor (PR)						
Pos	41	52.6	66	63.5	107	59.10
Neg	36	46.2	38	30.6	74	40.90
No data available	1	1.3	20		21	
HER2						
Pos	16	20.5	29	26.1	45	24.20
Neg	59	75.6	82	73.9	141	75.80
Unknown	3	3.8	13		16	
ER/PR Status Biopsy						
ER+/PR+	39	50	64	61.5	103	57.50
ER+/PR-/Unknown	10	12.8	11	10.6	21	11.70
ER-/unknown/PR+	2	2.6	2	1.6	4	2.20
ER-/PR-	24	30.8	27	26.0	51	28.50
No data available	3	3.8	20		23	
Histology						
Invasive ductal	53	67.9	76	65	129	66.20
Invasive ductal + DCIS	7	9.0	18	15.4	25	12.80
Invasive lobular	8	10.3	15	12.8	23	11.80
Invasive ductal/invasive lobular	1	1.3	0	-	1	0.50

Medullary	1	1.3		1	0.9		2	1.00	
Inflammatory	1	1.3		3	2.6		4	2.10	
Other	7	9.0		4	3.4		11	5.60	
No data available	0			7			7		
Ki67 Biopsy [%]	53		38.0						33.61
<14%	10	12.8		15	25.4	29.97	25	22.30	
>= 14%	43	55.1		44	74.6		87	77.70	
Unknown	25	32.1		65			90		
Clinical Tumor Size at Presentation, largest diameter [cm]									
MRI	36		3.62			3.5			3.56
Mammography	57		3.19			3.1			3.13
Sonography	69		3.01			2.94			2.97
Molecular Subtype Biopsy									
Luminal A	28	35.9		42	42.4		70	41.40	
Luminal B/HER2 neg	9	11.5		12	12.1		21	12.40	
Luminal B/HER pos	10	12.8		20	20.2		30	14.90	
HER2 enriched	5	6.4		6	6.1		11	5.40	
Triple neg	18	23.1		19	19.2		37	18.30	
Unknown	8	10.3		25			33		
Drug treatment									
Anthracycline -> Taxane	48	61.5					61	72.60	
TAC	8	10.3							
TCH	7	9.0					7	8.30	
Anthracycline -> Taxane + Other drug (Eve, H)	10	12.8					1	1.20	
Anthracycline -> Taxane + Carboplatin	3	3.9					3	3.60	
EC only	2	2.6					2	2.40	
pCR (ypT0/ypN0)		28.2							
Yes	22	28.2							
No	56	71.8							
Unknown	0	-							
Grade of regression									
0	2	2.6							
1	38	48.7							
2	6	7.7							
3	3	3.8							
4	21	26.9							
Unknown	8	10.3							

Surgical Therapy				
BCS Yes	54	69.2		
No	22	28.2		
Unknown	2	2.6		
Residual Tumor Size				
ypT0	19	24.4		
ypTis	4	5.1		
ypT1	26	33.3		
ypT2	16	20.5		
ypT3	8	10.3		
ypT4	1	1.3		
Unknown/ ypTx	4	5.1		

Baseline clinical data	Age at diagnosis	Age groups	BMI	BMI groups	Clinical tumor size at diagnosis, CT-Thorax	Clinical tumor size at diagnosis, MRT	Clinical tumor size at diagnosis, Mammography	Clinical tumor size at diagnosis, Ultrasound	Tumor stage	Tumor stage groups	Nodal status	UICC	Effected side	Centricity/focality	Histologic type	Grading	ER	PR	ER status	PR status	Hormone receptor status	HER2 score	HER2 status	HR/HER2	Ki67	Ki67 cutoff	CEA	CA 15-3	CEA status	CA 15-3 status
Age at diagnosis [y]	1.000	0.000	0.346	0.818	0.774	0.508	0.861	0.830	0.269	0.714	0.884	0.394	0.224	0.963	0.328	0.147	0.341	0.946	0.543	0.857	0.499	0.867	0.975	0.931	0.803	0.839	0.265	0.856	0.632	0.290
Age groups [≤50 vs >50]		1.000	0.085	0.549	0.463	0.194	0.806	0.180	0.656	0.608	0.652	0.749	0.235	0.739	0.456	1.000	0.821	0.189	0.147	0.498	0.142	0.652	0.780	0.662	0.813	0.782	0.079	0.812	0.149	0.469
BMI			1.000	0.000	0.537	0.007	0.079	0.352	0.649	0.720	0.048	0.098	0.535	0.982	0.222	0.438	0.242	0.044	0.063	0.192	0.013	0.445	0.201	0.053	0.652	0.245	0.296	0.455	0.475	0.521
BMI groups [Under/normal weight vs Overweight/adipose]				1.000	0.788	0.128	0.368	0.218	0.275	0.305	0.065	0.048	1.000	0.656	0.481	0.819	0.135	0.037	0.092	0.251	0.026	0.665	1.000	0.086	0.828	1.000	0.051	0.159	0.346	0.734
Clinical tumor size at diagnosis, CT-Thorax [cm]					1.000	NA	NA	0.790	0.391	NA	0.600	0.730	NA	0.018	NA	NA	0.221	0.228	0.266	0.266	0.266	0.732	NA	0.618	0.616	NA	NA	NA	NA	NA
Clinical tumor size at diagnosis, MRT [cm]						1.000	0.002	0.000	0.018	0.004	0.091	0.049	0.415	0.803	0.219	0.676	0.182	0.535	0.569	0.332	0.943	0.792	0.324	0.704	0.466	0.160	0.463	0.008	0.042	0.097
Clinical tumor size at diagnosis, Mammography [cm]							1.000	0.000	0.000	0.000	0.041	0.000	0.605	0.791	0.296	0.366	0.616	0.623	0.625	0.360	0.331	0.042	0.070	0.396	0.287	0.323	0.151	0.274	0.671	0.251
Clinical tumor size at diagnosis, Ultrasound [cm]								1.000	0.000	0.000	0.018	0.000	0.259	0.469	0.256	0.295	0.997	0.371	0.838	0.751	0.829	0.479	0.514	0.936	0.264	0.328	0.000	0.059	0.020	0.130
Tumor stage [cT1a, cT1b, cT1c, cT2, cT3, cT4]									1.000	0.000	0.011	0.000	0.038	0.465	0.353	0.522	0.023	0.126	0.162	0.101	0.258	0.866	0.340	0.582	0.160	0.466	0.095	0.011	0.248	0.512
Tumor stage groups [cT1/2 vs cT3/4]										1.000	0.002	0.000	0.181	0.371	0.203	0.432	0.255	0.941	0.785	1.000	1.000	0.298	0.132	0.478	0.302	0.517	0.152	0.103	0.121	0.411
Nodal status [cN+ vs cN-]											1.000	0.000	0.815	0.309	0.119	0.169	0.046	0.382	0.049	0.489	0.135	0.199	0.267	0.260	0.801	1.000	0.192	0.085	0.161	1.000
UICC												1.000	0.252	0.244	0.274	0.980	0.508	0.826	0.518	0.787	0.722	0.804	0.202	0.574	0.187	0.542	0.142	0.012	0.275	0.340
Effected side [left vs right]													1.000	0.314	0.473	1.000	0.007	0.026	0.129	0.154	0.128	0.121	0.090	0.022	0.438	0.264	0.173	0.055	0.637	0.473
Centricity/focality [Multifocal, multicentric, unicentric tumor]														1.000	0.433	0.267	0.904	0.254	0.829	0.682	0.959	0.127	0.002	0.053	0.347	0.481	0.350	0.595	0.203	0.366
Histologic type [Ductal/other vs lobular histology]															1.000	0.006	0.000	0.217	0.249	0.061	0.424	0.048	1.000	0.589	0.101	0.351	0.574	0.530	1.000	0.567
Grading [G1/2 vs G3]																1.000	0.000	0.000	0.000	0.000	0.000	0.690	0.576	0.000	0.005	0.054	0.503	0.893	1.000	0.736
ER [% positive stained cells]																	1.000	0.000	0.000	0.000	0.000	0.558	0.522	0.000	0.003	0.020	0.618	0.092	0.571	0.229
PR [% positive stained cells]																		1.000	0.000	0.000	0.000	0.858	0.237	0.000	0.028	0.033	0.570	0.176	0.645	0.222
ER status [ER+ vs ER-]																			1.000	0.000	0.000	0.292	1.000	0.000	0.071	0.132	0.461	0.453	1.000	1.000
PR status [PR+ vs PR-]																				1.000	0.000	0.783	0.163	0.000	0.003	0.013	0.522	0.121	1.000	0.168
Hormone receptor status [HR+ vs HR-]																					1.000	0.605	1.000	0.000	0.074	0.132	0.353	0.388	1.000	0.715
HER2 score [0-3]																						1.000	0.000	0.000	0.909	88.000	0.510	0.659	0.908	0.716
HER2 status [HER2+ vs HER2-]																							1.000	0.000	0.854	0.173	0.718	0.440	0.500	0.694
HR/HER2 [HR+/HER2+, HR+/HER2-, HR-/HER2+, HR-/HER2-]																								1.000	0.195	0.147	0.758	0.771	0.490	0.634
Ki67 [% positive stained cells]																									1.000	0.000	0.255	0.741	0.254	0.446
Ki67 cutoff [≤ 30% vs > 30%]																										1.000	0.542	0.462	1.000	1.000
CEA [µg/L]																											1.000	0.001	0.052	0.072
CA 15-3 [U/ml]																												1.000	0.159	0.004
CEA status [normal vs pathologic]																													1.000	0.149
CA 15-3 status [normal vs pathologic]																														1.000

Figure 11: Overview statistical analysis of all collected variables. P-values are shown and significant associations are highlighted in red with a p-value < 0.05. Correlations with two numerical variables were done using Spearman correlation, one numerical variable with a categorical variable was performed using t-test or ANOVA depending on the number of categories included. Distribution of two categorical variables was analyzed using Spearman's Chi-Square test.

Drug therapy

Baseline clinical data	Anthracycline/taxane-based chemotherapy	Anthracycline-based chemotherapy	Trastuzumab-based drug therapy	Treatment adherence	Reduced dose	Premature discontinuation of drug therapy	Change in drug regimen	Clinical tumor size midway chemotherapy, US	Change in tumor diameter, beginning - midway chemotherapy, US
Age at diagnosis [y]	0.844	0.909	0.942	0.022	0.414	0.001	0.756	0.116	0.014
Age groups [≤50 vs >50]	0.802	1.000	0.780	0.418	1.000	0.070	1.000	0.206	0.047
BMI	0.131	0.113	0.225	0.202	0.189	0.558	0.468	0.993	0.315
BMI groups [Under/normal weight vs Overweight/adipose]	1.000	0.802	1.000	0.421	0.154	0.737	0.358	0.713	0.787
Clinical tumor size at diagnosis, CT-Thorax [cm]	NA	NA	NA	NA	NA	NA	NA	NA	NA
Clinical tumor size at diagnosis, MRT [cm]	0.153	0.153	0.352	0.693	0.910	0.531	0.142	0.001	0.023
Clinical tumor size at diagnosis, Mammography [cm]	0.100	0.100	0.084	0.686	0.786	0.349	0.266	0.003	0.874
Clinical tumor size at diagnosis, Ultrasound [cm]	0.352	0.352	0.540	0.239	0.767	0.873	0.147	0.001	0.300
Tumor stage [cT1a, cT1b, cT1c, cT2, cT3, cT4]	0.252	0.444	0.410	0.077	0.592	0.059	0.440	0.605	0.121
Tumor stage groups [cT1/2 vs cT3/4]	0.155	0.396	0.209	0.235	0.698	0.057	0.610	0.224	0.025
Nodal status [cN+ vs cN-]	0.211	0.205	0.263	0.788	0.724	1.000	1.000	0.079	0.272
UICC	0.172	0.280	0.223	0.122	0.928	0.157	0.322	0.645	0.213
Effected side [left vs right]	0.017	0.036	0.148	1.000	1.000	0.727	1.000	0.104	0.635
Centricity/focality [Multifocal, multicentric, unicentric tumor]	0.042	0.067	0.001	0.131	0.316	0.459	0.693	0.617	0.842
Histologic type [Ductal/other vs lobular histology]	1.000	1.000	1.000	0.377	1.000	1.000	0.079	0.083	0.374
Grading [G1/2 vs G3]	0.800	1.000	0.575	0.282	0.166	1.000	1.000	0.645	0.029
ER [% positive stained cells]	0.405	0.405	0.676	0.406	0.206	0.773	0.690	0.033	0.111
PR [% positive stained cells]	0.331	0.319	0.315	0.172	0.131	0.939	0.576	0.375	0.778
ER status [ER+ vs ER-]	1.000	1.000	1.000	0.555	0.484	1.000	1.000	0.346	0.834
PR status [PR+ vs PR-]	0.128	0.079	0.173	0.591	0.490	0.726	1.000	0.174	0.092
Hormone receptor status [HR+ vs HR-]	0.783	0.783	1.000	0.562	0.710	0.705	1.000	0.346	0.834
HER2 score [0-3]	0.000	0.000	0.000	0.825	0.488	0.520	0.708	0.684	0.951
HER2 status [HER2+ vs HER2-]	0.000	0.000	0.000	0.336	0.674	1.000	0.578	0.503	0.586
HR/HER2 [HR+/HER2+, HR+/HER2-, HR-/HER2+, HR-/HER2-]	0.000	0.000	0.000	0.458	0.777	0.455	0.748	0.718	0.948
Ki67 [% positive stained cells]	0.808	0.587	0.926	0.857	0.724	0.242	0.622	0.207	0.106
Ki67 cutoff [≤ 30% vs > 30%]	0.119	0.224	0.099	0.745	1.000	0.669	1.000	0.094	0.030
CEA [µg/L]	0.515	0.439	0.718	0.198	0.271	NA	0.394	0.673	0.270
CA 15-3 [U/ml]	0.379	0.599	0.440	0.716	0.519	NA	0.949	0.377	0.880
CEA status [normal vs pathologic]	1.000	1.000	0.500	0.559	0.569	1.000	1.000	0.224	0.076
CA 15-3 status [normal vs pathologic]	0.457	1.000	0.694	1.000	1.000	1.000	1.000	0.167	0.301

Endpoints and data at surgery

Baseline clinical data	Breast conserving surgery	Clinical response	ypT groups	Grade of regression	pCR	Nodal status surgery	Lympathic invasion	PR status	PR	ER status	ER	Change in PR biopsy - surgical specimen	Change in ER biopsy - surgical specimen	Ki67 cutoff
Age at diagnosis [y]	0.105	0.384	0.057	0.214	0.045	0.008	0.012	0.082	0.787	0.179	0.228	0.198	0.439	0.156
Age groups [≤50 vs >50]	0.125	0.465	0.340	0.432	0.207	0.004	0.021	0.166	0.594	0.128	0.092	0.021	0.411	0.371
BMI	0.571	0.972	0.451	0.080	0.879	0.035	0.268	0.899	0.641	0.280	0.066	0.072	0.903	0.040
BMI groups [Under/normal weight vs Overweight/adipose]	0.310	0.622	0.295	0.137	0.620	0.346	0.301	1.000	0.336	0.707	0.738	0.279	0.871	0.074
Clinical tumor size at diagnosis, CT-Thorax [cm]	0.603	NA	0.589	0.441	0.463	NA	NA	NA	NA	NA	NA	NA	NA	NA
Clinical tumor size at diagnosis, MRT [cm]	0.001	0.482	0.547	0.653	0.517	0.075	0.322	0.706	0.935	0.817	0.343	0.077	0.315	0.656
Clinical tumor size at diagnosis, Mammography [cm]	0.005	0.791	0.352	0.189	0.182	0.400	0.067	0.768	0.907	0.429	0.526	0.993	0.657	0.055
Clinical tumor size at diagnosis, Ultrasound [cm]	0.035	0.771	0.728	0.835	0.948	0.155	0.249	0.554	0.772	0.297	0.409	0.490	0.303	0.455
Tumor stage [cT1a, cT1b, cT1c, cT2, cT3, cT4]	0.005	0.250	0.119	0.525	0.820	0.621	0.168	0.491	0.748	0.148	0.211	0.507	0.258	0.225
Tumor stage groups [cT1/2 vs cT3/4]	0.001	0.068	0.010	0.230	0.779	0.538	0.067	1.000	0.464	0.262	0.194	0.782	0.166	1.000
Nodal status [cN+ vs cN-]	0.020	0.123	0.100	0.957	1.000	0.011	0.051	0.735	0.230	0.275	0.045	0.536	0.922	1.000
UICC	0.006	0.238	0.229	0.664	0.740	0.527	0.107	0.822	0.717	0.400	0.146	0.869	0.482	0.135
Effected side [left vs right]	0.600	0.410	0.280	0.028	0.801	0.737	0.166	1.000	0.971	0.255	0.139	0.073	0.093	0.652
Centricity/focality [Multifocal, multicentric, unicentric tumor]	0.978	0.607	0.385	0.224	0.067	0.482	0.544	0.344	0.055	0.580	0.556	0.596	0.982	0.425
Histologic type [Ductal/other vs lobular histology]	0.006	0.413	0.870	0.447	0.429	0.075	0.099	0.319	0.296	0.269	0.002	0.901	0.928	0.257
Grading [G1/2 vs G3]	0.445	0.263	0.313	0.397	0.123	0.072	1.000	0.016	0.134	0.002	0.000	0.094	0.238	0.006
ER [% positive stained cells]	0.312	0.777	0.014	0.049	0.009	0.087	0.115	0.000	0.003	0.000	0.000	0.008	0.046	0.021
PR [% positive stained cells]	0.703	0.682	0.069	0.173	0.013	0.061	0.169	0.000	0.002	0.000	0.000	0.000	0.186	0.077
ER status [ER+ vs ER-]	0.284	0.957	0.109	0.372	0.180	1.000	0.470	0.000	0.004	0.000	0.000	0.005	0.065	0.071
PR status [PR+ vs PR-]	0.313	0.348	0.014	0.035	0.002	0.072	0.317	0.000	0.003	0.000	0.000	0.004	0.675	0.026
Hormone receptor status [HR+ vs HR-]	0.174	0.975	0.060	0.377	0.272	1.000	0.474	0.000	0.003	0.000	0.000	0.005	0.633	0.026
HER2 score [0-3]	0.203	0.730	0.103	0.061	0.048	0.192	0.428	0.457	0.737	0.480	0.707	0.140	0.145	0.423
HER2 status [HER2+ vs HER2-]	0.370	0.549	0.015	0.001	0.001	0.024	0.104	1.000	NA	1.000	NA	NA	NA	NA
HR/HER2 [HR+/HER2+, HR+/HER2-, HR-/HER2+, HR-/HER2-]	0.166	0.926	0.038	0.019	0.001	0.084	0.319	0.000	0.045	0.000	0.000	0.081	0.842	0.031
Ki67 [% positive stained cells]	0.591	0.591	0.806	0.694	0.601	0.043	0.407	0.071	0.348	0.005	0.008	0.264	0.701	0.033
Ki67 cutoff [≤ 30% vs > 30%]	0.324	0.156	0.388	0.444	0.148	0.022	0.356	0.387	0.262	0.032	0.008	0.496	0.449	0.129
CEA [µg/L]	0.583	0.050	0.846	0.850	0.420	0.084	0.471	0.961	0.481	0.100	0.412	0.189	0.454	0.589
CA 15-3 [U/ml]	0.211	0.897	0.012	0.432	0.311	0.061	0.733	0.656	0.514	0.173	0.140	0.188	0.750	0.190
CEA status [normal vs pathologic]	0.293	0.453	0.341	0.660	0.556	0.082	0.547	1.000	0.510	0.213	0.433	0.621	0.637	0.491
CA 15-3 status [normal vs pathologic]	0.704	0.923	0.166	0.159	0.452	0.172	1.000	0.321	0.474	0.622	0.308	0.799	0.460	1.000

Baseline clinical data	Ki67	Change in Ki67 biopsy - surgical specimen	CEA status	CA 15-3 status	CEA	CA 15-3	Change in CEA, beginning - end chemotherapy	Change in CA 15-3, beginning - end chemotherapy	Tumor diameter surgical specimen	Clinical tumor size end chemotherapy, MRT	Clinical tumor size end chemotherapy, Mammography	US	Change in tumor diameter, midway - end chemotherapy, US	Change in tumor diameter, beginning - end chemotherapy, US
Age at diagnosis [y]	0.150	0.857	0.650	0.342	0.205	0.771	0.590	0.612	0.255	0.604	0.231	0.992	0.235	0.668
Age groups [≤50 vs >50]	0.170	0.777	1.000	0.695	0.240	0.232	0.619	0.999	0.398	0.458	0.503	0.495	0.118	0.406
BMI	0.326	0.039	0.460	0.740	0.977	0.978	0.960	0.559	0.218	0.746	0.367	0.224	0.377	0.336
BMI groups [Under/normal weight vs Overweight/adipose]	0.210	0.243	1.000	1.000	0.210	0.346	0.537	0.620	0.173	0.302	0.753	0.047	0.213	0.592
Clinical tumor size at diagnosis, CT-Thorax [cm]	NA	NA	NA	NA	NA	NA	NA	NA	0.333	NA	NA	0.407	NA	0.797
Clinical tumor size at diagnosis, MRT [cm]	0.712	0.723	0.638	0.085	0.725	0.685	0.521	0.327	0.025	0.129	NA	0.287	0.647	0.400
Clinical tumor size at diagnosis, Mammography [cm]	0.353	0.748	0.147	0.262	0.264	0.016	0.509	0.992	0.209	0.143	0.564	0.120	0.010	0.000
Clinical tumor size at diagnosis, Ultrasound [cm]	0.687	0.341	0.178	0.168	0.086	0.375	0.367	0.335	0.314	0.051	0.981	0.001	0.025	0.000
Tumor stage [cT1a, cT1b, cT1c, cT2, cT3, cT4]	0.255	0.716	0.402	0.174	0.013	0.107	0.788	0.260	0.000	0.169	0.381	0.005	0.967	0.034
Tumor stage groups [cT1/2 vs cT3/4]	0.827	0.902	1.000	0.322	0.340	0.348	0.962	0.426	0.005	0.319	0.381	0.004	0.772	0.033
Nodal status [cN+ vs cN-]	0.580	0.223	0.620	0.111	0.584	0.112	0.104	0.626	0.005	0.166	NA	0.030	0.898	0.118
UICC	0.478	0.306	0.560	0.178	0.194	0.026	0.366	0.196	0.000	0.381	0.740	0.013	0.993	0.073
Effected side [left vs right]	0.933	0.554	0.615	0.688	0.322	0.752	0.935	0.094	0.054	0.433	NA	0.036	0.157	0.528
Centricity/focality [Multifocal, multicentric, unicentric tumor]	0.500	0.059	0.460	0.468	0.811	0.839	0.824	0.943	0.427	0.935	0.571	0.541	0.800	0.680
Histologic type [Ductal/other vs lobular histology]	0.001	0.817	1.000	1.000	NA	0.772	NA	0.128	0.291	0.643	NA	0.764	0.742	0.726
Grading [G1/2 vs G3]	0.004	0.154	1.000	0.097	0.952	0.168	0.690	0.293	0.510	0.797	0.918	0.648	0.719	0.455
ER [% positive stained cells]	0.009	0.886	0.608	0.015	0.560	0.204	0.196	0.264	0.058	0.840	0.251	0.200	0.267	0.621
PR [% positive stained cells]	0.032	0.913	0.982	0.096	0.956	0.514	0.459	0.246	0.100	0.707	0.290	0.132	0.265	0.451
ER status [ER+ vs ER-]	0.023	0.962	0.615	0.202	0.856	0.305	0.050	0.516	0.141	NA	NA	0.454	0.403	0.866
PR status [PR+ vs PR-]	0.017	0.962	1.000	0.047	0.673	0.094	0.131	0.375	0.098	0.275	NA	0.129	0.265	0.992
Hormone receptor status [HR+ vs HR-]	0.017	0.962	0.615	0.202	0.856	0.305	0.050	0.516	0.064	NA	NA	0.454	0.403	0.866
HER2 score [0-3]	0.397	0.972	0.741	0.648	0.313	0.593	0.656	0.915	0.105	0.819	0.684	0.640	0.622	0.192
HER2 status [HER2+ vs HER2-]	NA	NA	1.000	1.000	NA	0.435	NA	0.890	0.000	0.727	0.583	0.320	0.955	0.939
HR/HER2 [HR+/HER2+, HR+/HER2-, HR-/HER2+, HR-/HER2-]	0.003	0.891	0.529	0.406	0.446	0.589	0.165	0.940	0.046	0.692	0.583	0.656	0.867	1.000
Ki67 [% positive stained cells]	0.000	0.156	0.529	0.180	0.195	0.387	0.967	0.866	0.495	0.002	NA	0.021	0.258	0.123
Ki67 cutoff [≤ 30% vs > 30%]	0.043	0.112	1.000	0.396	0.381	0.476	0.362	0.503	0.358	0.116	NA	0.122	0.157	0.164
CEA [µg/L]	0.920	0.597	0.109	0.291	0.012	0.003	0.292	0.236	0.938	0.486	NA	0.019	0.157	0.021
CA 15-3 [U/ml]	0.496	0.692	0.846	0.129	0.185	0.009	0.077	0.066	0.002	0.392	0.094	0.044	0.063	0.984
CEA status [normal vs pathologic]	0.074	NA	0.491	1.000	0.248	0.453	0.086	0.447	0.651	NA	NA	0.035	0.426	0.340
CA 15-3 status [normal vs pathologic]	0.629	0.441	0.558	0.638	0.162	0.280	0.191	0.010	0.046	0.524	NA	0.040	0.207	0.657

Endpoints and data at surgery	Drug therapy	Endpoints and data at surgery
Anthracycline/taxane-based chemotherapy [Yes vs No]	1.000	
Anthracycline-based chemotherapy [Yes vs No]	0.000	1.000
Trastuzumab-based drug therapy [Yes vs No]	0.000	1.000
Treatment adherence [Yes vs No]	0.549	0.335
Reduced dose [Yes vs No]	1.000	0.676
Premature discontinuation of drug therapy [Yes vs No]	0.696	0.676
Change in drug regimen [Yes vs No]	0.807	0.133
Clinical tumor size midway chemotherapy, Ultrasound [cm]	0.380	0.982
Change in tumor diameter, beginning - midway chemotherapy, Ultrasound [cm]	0.562	0.562
Breast conserving surgery [Yes vs No]	0.778	0.580
Clinical response [Complete response, partial response, no response, stable disease, progress]	0.933	0.714
ypT groups [ypT0, ypTis, ypT1, ypT2, ypT3, ypT4]	0.021	0.034
Grade of regression [0-4]	0.009	0.022
pCR [Yes vs No]	0.009	0.012
Nodal status surgery [ypN+ vs ypN-]	0.291	0.495
Lymphatic invasion [L0 vs L1]	0.713	1.000
PR status [PR+ vs PR-]	0.603	0.338
PR [% positive stained cells]	0.021	0.021
ER status [ER+ vs ER-]	0.610	0.601
ER [% positive stained cells]	0.115	0.115
Change in PR biopsy - surgical specimen [% positive stained cells]	0.966	0.966
Change in ER biopsy - surgical specimen [% positive stained cells]	0.854	0.854
Ki67 cutoff [≤ 10% vs >10%]	1.000	1.000
Ki67 [% positive stained cells]	NA	NA
Change in Ki67 biopsy - surgical specimen [% positive stained cells]	NA	NA
CEA status [normal vs pathologic]	0.529	0.529
CA 15-3 status [normal vs pathologic]	1.000	1.000
CEA [µg/L]	0.093	0.093
CA 15-3 [U/ml]	0.480	0.480
Change in CEA, beginning - end chemotherapy [µg/L]	NA	NA
Change in CA 15-3, beginning - end chemotherapy [U/ml]	0.890	0.890
Tumor diameter surgical specimen [cm]	0.597	0.980
Clinical tumor size end chemotherapy, MRT [cm]	0.455	0.574
Clinical tumor size end chemotherapy, Mammography [cm]	0.583	0.583
Clinical tumor size end chemotherapy, Ultrasound [cm]	0.179	0.179
Change in tumor diameter, midway - end chemotherapy, Ultrasound [cm]	0.799	0.799
Change in tumor diameter, beginning - end chemotherapy, Ultrasound [cm]	0.944	0.944

Endpoints and data at surgery	Drug therapy	Endpoints and data at surgery
Anthracycline/taxane-based chemotherapy		
Anthracycline-based chemotherapy		
Trastuzumab-based drug therapy		
Treatment adherence		
Reduced dose		
Premature discontinuation of drug therapy		
Change in drug regimen		
Clinical tumor size midway chemotherapy, US		
Change in tumor diameter, beginning - midway chemotherapy, US		
Breast conserving surgery	1.000	
Clinical response	0.615	1.000
ypT groups	0.124	0.005
Grade of regression	0.041	0.000
pCR	0.136	0.000
Nodal status surgery	0.014	0.047
Lymphatic invasion	0.205	0.004
PR status	0.112	0.012
PR	0.889	0.077
ER status	0.818	1.000
ER	0.353	0.467
Change in PR biopsy - surgical specimen	0.754	0.275
Change in ER biopsy - surgical specimen	0.924	0.962
Ki67 cutoff	0.853	0.265
Ki67	0.941	0.798
Change in Ki67 biopsy - surgical specimen	0.752	0.657
CEA status	0.865	0.370
CA 15-3 status	0.710	0.610
CEA	0.434	0.615
CA 15-3	0.750	0.322
Change in CEA, beginning - end chemotherapy	0.886	0.874
Change in CA 15-3, beginning - end chemotherapy	0.461	0.333
Tumor diameter surgical specimen	0.934	0.047
Clinical tumor size end chemotherapy, MRT	0.717	0.008
Clinical tumor size end chemotherapy, Mammography	0.781	0.001
Clinical tumor size end chemotherapy, Ultrasound	0.386	0.001
Change in tumor diameter, midway - end chemotherapy, Ultrasound	0.155	0.764
Change in tumor diameter, beginning - end chemotherapy, Ultrasound	0.028	0.381
	0.586	0.554

Endpoints and data at surgery	Drug therapy	Endpoints and data at surgery
Anthracycline/taxane-based chemotherapy		
Anthracycline-based chemotherapy		
Trastuzumab-based drug therapy		
Treatment adherence		
Reduced dose		
Premature discontinuation of drug therapy		
Change in drug regimen		
Clinical tumor size midway chemotherapy, US		
Change in tumor diameter, beginning - midway chemotherapy, US		
Breast conserving surgery		
Clinical response		
ypT groups		
Grade of regression		
pCR		
Nodal status surgery		
Lymphatic invasion		
PR status		
PR		
ER status		
ER		
Change in PR biopsy - surgical specimen		
Change in ER biopsy - surgical specimen		
Ki67 cutoff		
Ki67		
Change in Ki67 biopsy - surgical specimen		
CEA status		
CA 15-3 status		
CEA		
CA 15-3		
Change in CEA, beginning - end chemotherapy		
Change in CA 15-3, beginning - end chemotherapy		
Tumor diameter surgical specimen		
Clinical tumor size end chemotherapy, MRT		
Clinical tumor size end chemotherapy, Mammography		
US		
Change in tumor diameter, midway - end chemotherapy, US		
Change in tumor diameter, beginning - end chemotherapy, US		

		Baseline laboratory data																				In vitro treatment									
		No. of biopsy cylinders received	**Weight tissue sample**	**Weight per biopsy cylinder**	**No. of cells per mg tissue received**	**No. of days TM expired**	**Time difference biopsy - begin lab procedure**	**Mechanical isolation**	**Enzymatic isolation**	**Total time isolation procedure**	**Total enzyme added**	**Total No. of vital cells after filtration**	**Cell viability**	**No. of cells per spheroid**	**No. of cells per spheroid groups**	**No. of spheroids generated**	**No. of technical replicates**	**Incubation of generated spheroids [h]**	**Mucous exterior lining [Yes vs No]**	**Cell density before treatment**	**Same-day preparation cytostatic compounds**	**Time between compounds preparation and in vitro treatment**	**No. of treatment options**	**Visible morphological changes after treatment**	**Mucous exterior lining following treatment**	**Readout method**	**Incubation with MTS reagent**	**Incubation with ATP reagent**	**Metabolic activity mean solvent control**	**Metabolic activity mean solvent control per cell**	**Cell survival**	**Cutoff cell survival**
Baseline laboratory data	**No. of biopsy cylinders received**	1.000	0.000	0.250	0.773	0.471	0.139	0.000	0.441	0.184	0.037	0.080	0.883	0.043	0.010	0.128	0.190	0.272	0.049	0.180	0.071	0.791	0.013	0.832	0.189	0.578	0.886	0.868	0.586	0.726	0.486	0.500
	Weight tissue sample [mg]		1.000	0.000	0.000	0.436	0.416	0.000	0.872	0.325	0.000	0.000	0.000	0.019	0.154	0.000	0.000	0.323	0.638	0.055	0.157	0.760	0.000	0.620	0.019	0.991	0.609	0.829	0.218	0.440	0.150	0.255
	Weight per biopsy cylinder [mg]			1.000	0.322	0.643	0.458	0.023	0.004	0.002	0.000	0.000	0.282	0.194	0.512	0.000	0.000	0.301	0.031	0.007	0.661	0.944	0.000	0.223	0.015	0.607	0.512	0.021	0.000	0.006	0.663	0.561
	No. of cells per mg tissue received				1.000	0.718	0.465	0.028	0.001	0.004	0.280	0.000	0.000	0.000	0.000	0.000	0.000	0.830	0.002	0.501	0.466	0.020	0.000	0.367	0.054	0.690	0.645	0.027	0.751	0.732	0.009	0.077
	No. of days TM expired [d]					1.000	0.256	0.372	0.326	0.424	0.441	0.822	0.928	0.758	0.607	0.752	NA	0.500	NA	0.195	0.533	NA	0.549	NA	NA	NA	NA	0.869	0.002	0.127	0.742	0.554
	Time difference biopsy - begin lab procedure [h]						1.000	0.261	0.192	0.134	0.359	0.717	0.561	0.606	0.665	0.235	0.118	0.556	0.067	0.868	0.161	0.803	0.324	0.178	0.910	0.718	0.735	0.968	0.869	0.879	0.526	0.676
	Mechanical isolation [min]							1.000	0.761	0.037	0.000	0.000	0.000	0.541	0.833	0.000	0.000	0.355	0.078	0.497	0.030	0.058	0.000	0.296	0.030	0.302	0.658	0.964	0.576	0.749	0.579	0.586
	Enzymatic isolation [min]								1.000	0.000	0.013	0.103	0.149	0.054	0.343	0.272	0.333	0.378	0.304	0.116	0.835	0.067	0.439	0.063	0.004	0.707	0.648	0.376	0.040	0.006	0.937	0.951
	Total time isolation procedure [min]									1.000	0.002	0.547	0.482	0.046	0.305	0.794	0.911	0.296	0.242	0.075	0.522	0.137	0.985	0.141	0.003	0.949	0.778	0.391	0.060	0.009	0.971	0.960
	Total enzyme added [units]										1.000	0.000	0.005	0.769	0.513	0.000	0.000	0.637	0.059	0.003	0.255	0.795	0.000	0.677	0.006	0.974	0.090	0.530	0.079	0.245	0.520	0.590
	Total No. of vital cells after filtration											1.000	0.000	0.196	0.317	0.000	0.000	0.723	0.020	0.590	0.349	0.019	0.000	0.999	0.284	0.634	0.500	0.820	0.788	0.971	0.102	0.254
	Cell viability [%]												1.000	0.828	0.683	0.000	0.000	0.395	0.069	0.372	0.540	0.878	0.001	0.485	0.283	0.073	0.811	0.290	0.757	0.581	0.121	0.322
	No. of cells per spheroid													1.000	0.000	0.045	0.125	0.157	0.557	0.006	0.603	0.320	0.053	0.021	0.619	0.042	0.585	0.532	0.503	0.934	0.827	0.404
	No. of cells per spheroid groups [< 10 000, 10 000 - 20 000, > 20 000]														1.000	0.007	0.023	0.391	0.134	0.008	0.007	0.791	0.023	0.043	0.356	0.024	0.425	0.117	0.112	0.445	0.841	0.846
	No. of spheroids generated															1.000	0.000	0.911	0.565	0.046	0.550	0.023	0.000	0.263	0.681	0.441	0.428	0.112	0.007	0.092	0.008	0.048
	No. of technical replicates																1.000	0.883	0.753	0.012	0.609	0.057	0.000	0.309	0.783	0.163	0.189	0.027	0.002	0.039	0.023	0.120
	Incubation of generated spheroids [h]																	1.000	0.256	0.831	0.559	0.998	0.943	0.827	0.391	0.024	0.962	0.020	0.018	0.015	0.010	0.086
	Mucous exterior lining [Yes vs No]																		1.000	0.575	0.705	NA	0.330	NA	0.003	NA	NA	0.256	0.446	0.965	0.774	1.000
	Cell density before treatment [very thin, slightly thin, moderately thin, dense]																			1.000	0.911	0.989	0.061	0.439	0.392	0.293	0.449	0.450	0.169	0.283	0.210	0.228
In vitro treatment	**Same-day preparation cytostatic compounds** [Yes vs No]																				1.000	NA	0.431	0.091	0.041	0.576	0.167	0.501	0.542	0.404	1.000	0.491
	Time between compounds preparation and in vitro treatment [d]																					1.000	0.083	NA	NA	NA	NA	0.463	0.917	0.513	0.649	0.687
	No. of treatment options																						1.000	0.153	0.882	0.443	0.441	0.137	0.015	0.094	0.002	0.015
	Visible morphological changes after treatment [Yes vs No]																							1.000	NA	0.124	0.550	0.661	0.212	0.310	0.775	0.651
	Mucous exterior lining following treatment [Yes vs No]																								1.000	NA	NA	0.169	0.373	0.404	0.971	1.000
	Readout method [MTS vs ATP]																									1.000	NA	NA	0.013	0.002	0.000	0.000
	Incubation with MTS reagent [h]																										1.000	NA	0.814	0.752	0.830	NA
	Incubation with ATP reagent [h]																											1.000	0.817	0.862	0.367	0.359
	Metabolic activity mean solvent control [cps]																												1.000	0.000	0.016	0.170
	Metabolic activity mean solvent control per cell [cps/cell]																													1.000	0.023	0.185
	Cell survival [% solvent control]																														1.000	0.000
	Cutoff cell survival [≤ 35 vs >35%]																															1.000

		In vitro treatment cont.															
		Mean cell survival EC	Stdv mean cell survival EC	Mean cell survival ECD	Stdv mean cell survival ECD	Mean cell survival EC-Trastuzumab	Stdv mean cell survival EC-Trastuzumab	Mean cell survival FEC	Stdv mean cell survival FEC	Mean cell survival DocAC	Stdv mean cell survival DocAC	Mean cell survival TCbH	Stdv mean cell survival TCbH	Mean cell survival Doc	Stdv mean cell survival Doc	Mean cell survival Pac	Stdv mean cell survival Pac
Baseline laboratory data	**No. of biopsy cylinders received**	0.203	0.582	0.589	0.466	0.867	0.506	0.953	0.756	0.708	0.605	0.712	0.977	0.390	0.815	0.693	0.337
	Weight tissue sample [mg]	0.258	0.792	0.684	0.037	0.381	0.571	0.641	0.222	0.204	0.537	0.226	0.359	0.852	0.836	0.172	0.602
	Weight per biopsy cylinder [mg]	0.295	0.087	0.913	0.030	0.171	0.811	0.788	0.197	0.239	0.714	0.363	0.424	0.752	0.324	0.364	0.617
	No. of cells per mg tissue received	0.013	0.024	0.784	0.102	0.045	0.241	0.836	0.611	0.102	0.445	0.344	0.278	0.180	0.782	0.082	0.756
	No. of days TM expired [d]	0.836	0.459	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.128	0.051	0.619	0.959
	Time difference biopsy - begin lab procedure [h]	0.172	0.601	0.589	0.237	0.765	0.753	0.252	0.641	0.473	0.403	0.322	0.702	0.797	0.376	0.439	0.673
	Mechanical isolation [min]	0.328	0.671	0.604	0.206	0.620	0.041	0.470	0.188	0.305	0.895	0.774	0.649	0.554	0.982	0.133	0.783
	Enzymatic isolation [min]	0.899	0.231	0.341	0.408	0.175	0.042	0.424	0.705	0.880	0.225	0.821	0.730	0.648	0.851	0.420	0.078
	Total time isolation procedure [min]	0.957	0.201	0.491	0.151	0.106	0.102	0.485	0.924	0.829	0.211	0.720	0.862	0.726	0.842	0.696	0.044
	Total enzyme added [units]	0.286	0.183	0.448	0.990	0.042	0.567	0.962	0.241	0.197	0.954	0.365	0.330	0.897	0.699	0.139	0.378
	Total No. of vital cells after filtration	0.146	0.373	0.337	0.269	0.811	0.048	0.615	0.324	0.221	0.522	0.311	0.449	0.809	0.993	0.192	0.638
	Cell viability [%]	0.297	0.156	0.408	0.071	0.669	0.258	0.910	0.848	0.454	0.875	0.196	0.501	0.891	0.931	0.127	0.496
	No. of cells per spheroid	0.895	0.033	0.771	0.293	0.120	0.931	0.567	0.299	0.600	0.475	0.707	0.133	0.942	0.282	0.502	0.623
	No. of cells per spheroid groups [< 10 000, 10 000 - 20 000, > 20 000]	0.558	0.021	0.495	0.436	NA	NA	0.100	0.989	0.149	0.206	0.661	0.095	0.567	0.884	0.670	0.354
	No. of spheroids generated	0.105	0.334	0.333	NA	0.488	0.018	0.697	0.321	0.054	0.513	0.245	0.263	0.549	0.806	0.052	0.871
	No. of technical replicates	0.488	0.784	0.333	NA	0.811	0.414	0.930	0.189	0.033	0.394	0.443	0.362	0.446	0.465	0.125	0.623
	Incubation of generated spheroids [h]	0.414	0.366	0.255	0.553	0.235	0.673	0.004	0.007	0.180	0.646	0.196	0.329	0.856	0.810	0.724	0.865
	Mucous exterior lining [Yes vs No]	0.538	0.823	NA	NA	NA	NA	NA	NA	0.963	0.372	0.993	0.373	NA	NA	NA	NA
	Cell density before treatment [very thin, slightly thin, moderately thin, dense]	0.206	0.562	0.530	0.819	0.603	0.532	0.926	0.179	0.319	0.892	0.013	0.710	0.701	0.926	0.658	0.756
In vitro treatment	**Same-day preparation cytostatic compounds** [Yes vs No]	0.792	0.453	0.609	NA	0.428	0.386	0.608	0.856	0.583	0.560	0.242	0.704	0.727	0.274	0.824	0.399
	Time between compounds preparation and in vitro treatment [d]	0.423	0.498	NA	NA	NA	NA	0.707	0.670	NA	NA	NA	NA	NA	NA	0.028	0.646
	No. of treatment options	0.023	0.153	NA	NA	0.364	0.000	0.344	0.603	0.068	0.666	0.301	0.256	0.628	0.755	0.024	0.812
	Visible morphological changes after treatment [Yes vs No]	0.762	0.614	NA	NA	NA	NA	0.044	0.784	NA	NA	NA	NA	0.528	0.078	NA	NA
	Mucous exterior lining following treatment [Yes vs No]	0.355	0.090	NA	NA	NA	NA	NA	NA	0.963	0.372	NA	NA	NA	NA	NA	NA
	Readout method [MTS vs ATP]	0.000	0.002	0.333	0.899	0.163	0.164	NA	NA	NA	NA	NA	NA	0.533	0.197	NA	NA
	Incubation with MTS reagent [h]	0.670	0.678	0.601	0.555	0.592	0.855	NA	NA	NA	NA	NA	NA	0.559	0.561	NA	NA
	Incubation with ATP reagent [h]	0.524	0.580	NA	NA	0.979	0.263	0.832	0.185	0.368	0.590	0.881	0.207	0.368	0.451	0.220	0.822
	Metabolic activity mean solvent control [cps]	0.334	0.791	0.600	0.581	0.973	0.805	0.147	0.312	0.232	0.408	0.657	0.102	0.845	0.344	0.729	0.319
	Metabolic activity mean solvent control per cell [cps/cell]	0.422	0.348	0.612	0.578	0.974	0.809	0.151	0.185	0.220	0.317	0.389	0.548	0.940	0.440	0.773	0.254
	Cell survival [% solvent control]	0.000	0.355	0.068	0.980	0.000	0.044	0.014	0.396	0.000	0.140	0.000	0.658	0.258	0.356	0.093	0.667
	Cutoff cell survival [≤ 35 vs >35%]	0.000	0.178	NA	NA	0.050	0.269	0.085	0.627	0.004	0.353	0.000	0.610	0.456	0.095	0.757	0.009

In vitro treatment cont.	Mean cell survival EC	Stdv mean cell survival EC	Mean cell survival ECD	Stdv mean cell survival ECD	Mean cell survival EC-Trastuzumab	Stdv mean cell survival EC-Trastuzumab	Mean cell survival FEC	Stdv mean cell survival FEC	Mean cell survival DocAC	Stdv mean cell survival DocAC	Mean cell survival TCbH	Stdv mean cell survival TCbH	Mean cell survival Doc	Stdv mean cell survival Doc	Mean cell survival Pac	Stdv mean cell survival Pac
Mean cell survival EC	1.000	0.318	NA	NA	0.001	0.083	0.192	0.450	0.676	0.888	NA	NA	0.335	0.309	0.193	0.388
Stdv mean cell survival EC		1.000	NA	NA	0.698	0.334	0.180	0.218	0.952	0.740	NA	NA	0.772	0.667	0.606	0.935
Mean cell survival ECD			1.000	0.500	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Stdv mean cell survival ECD				1.000	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Mean cell survival EC-Trastuzumab					1.000	0.148	NA	NA	NA	NA	NA	NA	0.159	0.697	NA	NA
Stdv mean cell survival EC-Trastuzumab						1.000	NA	NA	NA	NA	NA	NA	0.817	0.038	NA	NA
Mean cell survival FEC							1.000	0.047	0.424	0.635	NA	NA	0.534	0.768	0.325	0.697
Stdv mean cell survival FEC								1.000	0.178	0.034	NA	NA	0.958	0.805	0.808	0.420
Mean cell survival DocAC									1.000	0.154	NA	NA	0.995	0.537	0.491	0.288
Stdv mean cell survival DocAC										1.000	NA	NA	0.794	0.352	0.702	0.076
Mean cell survival TCbH											1.000	0.640	NA	NA	NA	NA
Stdv mean cell survival TCbH												1.000	NA	NA	NA	NA
Mean cell survival Doc													1.000	0.000	0.274	0.606
Stdv mean cell survival Doc														1.000	0.145	0.124
Mean cell survival Pac															1.000	0.900
Stdv mean cell survival Pac																1.000

		Baseline clinical data																														
		Age at diagnosis	Age groups	BMI	BMI groups	Clinical tumor size at diagnosis, CT-Thorax	Clinical tumor size at diagnosis, MRT	Clinical tumor size at diagnosis, Mammography	Clinical tumor size at diagnosis, Ultrasound	Tumor stage	Tumor stage groups	Nodal status	UICC	Effected side	Centricity/focality	Histologic type	Grading	ER	PR	ER status	PR status	Hormone receptor status	HER2 score	HER2 status	HR/HER2	Ki67	Ki67 cutoff	CEA	CA 15-3	CEA status	CA 15-3 status
Baseline laboratory data	**No. of biopsy cylinders received**	0.160	0.807	0.382	0.659	0.415	0.811	0.222	0.611	0.340	0.076	0.237	0.543	0.059	0.042	0.010	0.028	0.089	0.189	0.118	0.093	0.142	0.163	0.143	0.128	0.380	0.542	0.741	0.498	0.754	0.474
	Weight tissue sample [mg]	0.251	0.442	0.505	0.496	0.021	0.082	0.077	0.131	0.001	0.258	0.555	0.002	0.232	0.117	0.437	0.295	0.415	0.618	0.255	0.433	0.254	0.578	0.844	0.237	0.259	0.493	0.823	0.379	0.902	0.925
	Weight per biopsy cylinder [mg]	0.305	0.803	0.232	0.173	0.920	0.014	0.148	0.013	0.091	0.038	0.532	0.418	0.107	0.444	0.990	0.819	0.185	0.330	0.690	0.506	0.693	0.242	0.456	0.661	0.484	0.220	0.886	0.170	0.883	0.552
	No. of cells per mg tissue received	0.122	0.117	0.957	0.539	0.028	0.658	0.850	0.673	0.330	0.683	0.391	0.245	0.191	0.433	0.857	0.001	0.027	0.095	0.089	0.003	0.041	0.599	0.431	0.026	0.348	0.862	0.626	0.388	0.656	0.644
	No. of days TM expired [d]	0.236	0.354	0.496	0.374	NA	0.061	0.600	0.944	0.001	0.448	0.169	0.001	0.453	0.824	NA	0.289	0.294	0.381	0.555	0.554	0.555	0.279	0.354	0.000	0.165	0.743	0.064	0.013	NA	NA
	Time difference biopsy - begin lab procedure [h]	0.782	0.674	0.161	0.627	0.945	0.183	0.089	0.098	0.853	0.425	0.420	0.895	0.981	0.988	0.731	0.511	0.119	0.090	0.750	0.275	0.885	0.906	0.843	0.799	0.088	0.094	0.533	0.636	0.704	0.055
	Mechanical isolation [min]	0.750	0.511	0.993	0.545	0.099	0.772	0.233	0.093	0.038	0.175	0.087	0.058	0.683	0.621	0.561	0.801	0.701	0.524	0.733	0.937	0.666	0.661	0.466	0.710	0.846	0.680	0.180	0.079	0.192	0.450
	Enzymatic isolation [min]	0.516	0.825	0.270	0.246	0.203	0.206	0.646	0.329	0.655	0.413	0.568	0.470	0.128	0.198	0.128	0.021	0.045	0.138	0.137	0.046	0.071	0.681	0.833	0.362	0.933	0.819	0.953	0.171	0.534	0.015
	Total time isolation procedure [min]	0.571	0.728	0.284	0.315	0.459	0.196	0.477	0.198	0.563	0.284	0.829	0.481	0.171	0.175	0.174	0.022	0.064	0.194	0.174	0.051	0.101	0.581	0.956	0.479	0.906	0.764	0.837	0.100	0.258	0.013
	Total enzyme added [units]	0.132	0.678	0.455	0.361	0.112	0.011	0.046	0.060	0.085	0.224	0.228	0.206	0.442	0.714	0.918	0.444	0.774	0.482	0.254	0.949	0.413	0.145	0.140	0.265	0.502	0.980	0.421	0.868	0.885	0.504
	Total No. of vital cells after filtration	0.417	0.319	0.430	0.421	0.009	0.125	0.289	0.382	0.000	0.316	0.430	0.001	0.321	0.059	0.635	0.159	0.169	0.288	0.238	0.211	0.237	0.560	0.549	0.226	0.187	0.325	0.946	0.728	0.580	0.488
	Cell viability [%]	0.850	0.824	0.902	0.945	0.008	0.906	0.237	0.095	0.023	0.367	0.739	0.053	0.357	0.112	0.696	0.022	0.079	0.023	0.072	0.028	0.053	0.196	0.635	0.039	0.101	0.071	0.007	0.759	0.603	0.574
	No. of cells per spheroid	0.616	0.658	0.859	0.869	0.643	0.779	0.724	0.856	0.712	0.679	0.167	0.854	0.646	0.452	0.076	0.001	0.072	0.163	0.102	0.011	0.074	0.038	0.272	0.121	0.165	0.992	0.661	0.419	0.946	0.634
	No. of cells per spheroid groups [< 10 000, 10 000 - 20 000, > 20 000]	0.013	0.031	0.835	0.261	NA	0.046	0.393	0.430	0.851	0.673	0.244	0.648	0.836	0.730	0.052	0.000	0.207	0.701	0.449	0.054	0.420	0.218	0.691	0.819	0.163	0.639	0.440	0.627	0.934	0.558
	No. of spheroids generated	0.047	0.162	0.878	0.630	0.212	0.107	0.182	0.071	0.044	0.353	0.924	0.022	0.377	0.518	0.341	0.020	0.062	0.173	0.063	0.040	0.054	0.649	0.395	0.006	0.163	0.414	0.768	0.592	0.441	0.460
	No. of technical replicates	0.311	0.500	0.304	0.343	0.298	0.192	0.903	0.112	0.034	0.473	0.791	0.013	0.943	0.678	0.174	0.040	0.050	0.175	0.048	0.056	0.042	0.635	0.503	0.009	0.435	0.837	0.948	0.710	0.050	0.604
	Incubation of generated spheroids [h]	0.462	0.252	0.595	0.669	0.716	0.569	0.164	0.730	0.113	0.166	0.070	0.124	0.601	0.768	0.282	0.365	0.895	0.606	0.841	0.788	0.907	0.891	0.766	0.988	0.675	0.719	0.751	0.833	0.658	0.973
	Mucous exterior lining [Yes vs No]	0.308	0.715	0.303	0.721	NA	0.318	0.127	0.799	0.208	0.079	1.000	0.483	0.430	0.118	1.000	0.283	0.019	0.001	0.248	0.022	0.106	0.730	0.258	0.126	0.307	1.000	NA	NA	NA	0.300
	Cell density before treatment [very thin, slightly thin, moderately thin, dense]	0.341	0.433	0.655	0.632	NA	0.342	0.280	0.110	0.138	0.150	0.391	0.350	0.442	0.992	0.305	0.168	0.678	0.804	0.753	0.886	0.683	0.039	0.944	0.847	0.848	0.878	0.470	0.558	0.594	0.340
In vitro treatment	**Same-day preparation cytostatic compounds** [Yes vs No]	0.887	1.000	0.222	0.497	0.788	0.699	0.657	0.568	0.316	0.443	0.494	0.355	0.345	0.421	0.715	0.645	0.256	0.411	0.237	0.365	0.322	0.089	1.000	0.742	0.405	0.414	0.073	0.156	0.152	1.000
	Time between compounds preparation and in vitro treatment [d]	0.713	0.687	0.450	0.367	NA	0.349	0.771	0.764	0.607	0.857	0.776	0.784	0.695	0.584	NA	0.209	0.084	0.324	0.132	0.209	0.132	0.126	0.093	0.112	0.415	0.537	0.314	0.131	NA	NA
	No. of treatment options	0.026	0.149	0.937	0.527	0.258	0.166	0.133	0.114	0.187	0.290	0.743	0.067	0.213	0.694	0.324	0.012	0.128	0.253	0.069	0.040	0.051	0.669	0.573	0.012	0.038	0.121	0.530	0.625	0.302	0.611
	Visible morphological changes after treatment [Yes vs No]	0.011	0.191	0.395	0.386	NA	0.185	0.780	0.277	0.311	0.367	0.416	0.795	0.390	0.814	1.000	0.187	0.762	0.993	0.395	0.379	0.367	0.057	0.579	0.401	0.475	1.000	0.532	0.397	1.000	1.000
	Mucous exterior lining following treatment [Yes vs No]	0.527	0.596	0.927	0.608	NA	0.484	0.024	0.047	0.112	0.145	0.358	0.536	0.596	0.633	1.000	1.000	0.435	0.390	0.622	0.329	0.329	0.551	0.525	0.496	0.459	1.000	NA	NA	NA	NA
	Readout method [MTS vs ATP]	0.003	0.004	0.954	0.780	NA	0.573	0.153	0.609	0.319	0.350	0.390	0.242	0.770	0.712	1.000	0.782	0.731	0.191	0.555	0.575	0.360	0.025	0.017	0.091	0.287	0.175	0.729	0.232	1.000	0.730
	Incubation with MTS reagent [h]	0.102	0.369	0.203	0.141	NA	0.407	0.745	0.296	0.505	0.559	0.368	0.724	0.402	0.309	NA	0.311	0.352	0.387	0.517	0.339	0.517	0.757	NA	0.113	0.670	0.295	0.282	0.643	0.516	0.585
	Incubation with ATP reagent [h]	0.521	0.554	0.074	0.072	0.263	0.981	0.959	0.358	0.960	0.973	0.065	0.711	0.671	0.521	0.778	0.972	0.499	0.527	0.456	0.777	0.536	0.915	0.448	0.739	0.416	0.351	0.500	0.768	0.041	0.581
	Metabolic activity mean solvent control [cps]	0.075	0.063	0.622	0.678	0.794	0.637	0.870	0.501	0.964	0.771	0.082	0.769	0.418	0.115	0.501	0.740	0.821	0.506	0.528	0.944	0.437	0.572	0.425	0.700	0.076	0.132	0.755	0.552	0.419	0.729
	Metabolic activity mean solvent control per cell [cps/cell]	0.111	0.065	0.588	0.747	0.789	0.834	0.833	0.906	0.944	0.636	0.204	0.722	0.293	0.167	0.808	0.899	0.732	0.509	0.781	0.706	0.659	0.872	0.483	0.839	0.177	0.272	0.710	0.517	0.410	0.918
	Cell survival [% solvent control]	0.898	0.877	0.389	0.940	0.208	0.894	0.149	0.633	0.413	0.181	0.813	0.279	0.060	0.093	0.569	0.339	0.094	0.058	0.084	0.007	0.090	0.019	0.000	0.002	0.082	0.012	0.419	0.148	0.092	0.513
	Cutoff cell survival [≤ 35 vs >35%]	0.817	1.000	0.147	0.818	0.266	0.772	0.204	0.468	0.757	0.603	1.000	0.735	0.159	0.029	0.460	0.486	0.069	0.054	0.224	0.019	0.313	0.011	0.000	0.001	0.339	0.098	0.522	0.199	0.302	0.494

		Drug therapy									Endpoints and data at surgery																											
		Anthracycline/taxane-based chemotherapy	Anthracycline-based chemotherapy	Trastuzumab-based drug therapy	Treatment adherence	Reduced dose	Premature discontinuation of drug therapy	Change in drug regimen	Clinical tumor size midway chemotherapy, US	Change in tumor diameter, beginning - midway chemotherapy, US	Breast conserving surgery	Clinical response	ypT groups	Grade of regression	pCR	Nodal status surgery	Lympathic invasion	PR status	PR	ER status	ER	Change in PR biopsy - surgical specimen	Change in ER biopsy - surgical specimen	Ki67 cutoff	Ki67	Change in Ki67 biopsy - surgical specimen	CEA status	CA 15-3 status	CEA	CA 15-3	Change in CEA, beginning - end chemotherapy	Change in CA 15-3, beginning - end chemotherapy	Tumor diameter surgical specimen	Clinical tumor size end chemotherapy, MRT	Clinical tumor size end chemotherapy, Mammography	US	Change in tumor diameter, midway - end chemotherapy, US	Change in tumor diameter, beginning - end chemotherapy, US
Baseline laboratory data	**No. of biopsy cylinders received**	0.070	0.113	0.140	0.415	0.809	0.963	0.188	0.497	0.766	0.527	0.812	0.565	0.641	0.827	0.893	0.587	0.092	0.045	0.080	0.031	0.221	0.160	0.741	0.598	0.806	0.360	0.585	0.280	0.981	0.762	0.572	0.092	1.000	0.684	0.360	0.079	0.327
	Weight tissue sample [mg]	0.960	0.959	0.886	0.738	0.934	0.976	0.456	0.945	0.435	0.857	0.668	0.759	0.678	0.370	0.577	0.989	0.287	0.814	0.545	0.814	0.471	0.853	0.885	0.965	0.258	0.890	0.147	0.857	0.381	0.563	0.996	0.334	0.773	0.853	0.950	0.898	0.413
	Weight per biopsy cylinder [mg]	0.866	0.818	0.498	0.388	0.412	0.517	0.464	0.020	0.022	0.529	0.735	0.259	0.940	0.432	0.763	0.136	0.922	0.150	0.762	0.278	0.590	0.610	0.739	0.971	0.141	0.717	0.097	0.186	0.443	0.060	0.662	0.000	0.375	0.417	0.366	0.573	0.981
	No. of cells per mg tissue received	0.234	0.194	0.367	0.873	0.372	0.825	0.388	0.298	0.041	0.351	0.524	0.128	0.109	0.086	0.507	0.677	0.226	0.408	0.482	0.284	0.791	0.815	0.513	0.526	0.837	0.112	0.578	0.107	0.547	0.034	0.890	0.856	0.411	0.633	0.979	0.406	0.806
	No. of days TM expired [d]	0.354	0.354	0.354	0.365	NA	0.365	NA	0.203	0.118	0.206	0.729	0.797	0.470	0.302	0.850	0.982	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.488	0.484	0.482	NA	0.403	0.598	NA	NA	NA	NA	NA
	Time difference biopsy - begin lab procedure [h]	0.826	0.699	0.794	0.950	0.352	0.409	0.308	0.886	0.435	0.171	0.786	0.972	0.366	0.615	0.776	0.645	0.369	0.004	0.632	0.143	0.890	0.610	0.327	0.649	0.191	0.316	0.136	0.789	0.821	0.811	0.792	0.790	0.250	0.728	0.121	0.762	0.681
	Mechanical isolation [min]	0.147	0.141	0.480	0.951	0.401	0.683	0.568	0.543	0.283	0.221	0.839	0.107	0.308	0.471	0.238	0.314	0.656	0.391	0.540	0.537	0.764	0.873	0.512	0.550	0.613	0.579	0.707	0.920	0.024	0.166	0.454	0.156	0.154	0.513	0.566	0.848	0.387
	Enzymatic isolation [min]	0.343	0.311	0.928	0.219	0.022	0.764	0.628	0.409	0.141	0.271	0.173	0.244	0.390	0.205	0.998	0.520	0.828	0.571	0.302	0.376	0.985	0.577	0.961	0.861	0.334	0.775	0.280	0.547	0.858	0.469	0.222	0.308	0.563	0.835	0.817	0.585	0.639
	Total time isolation procedure [min]	0.529	0.492	0.959	0.237	0.040	0.708	0.558	0.325	0.214	0.183	0.187	0.403	0.535	0.278	0.784	0.686	0.903	0.466	0.258	0.461	0.966	0.567	0.968	0.931	0.396	0.867	0.272	0.555	0.585	0.342	0.185	0.214	0.438	0.634	0.702	0.616	0.844
	Total enzyme added [units]	0.109	0.072	0.153	0.441	0.944	0.944	0.107	0.946	0.775	0.939	0.210	0.745	0.616	0.439	0.230	0.494	0.844	0.980	0.517	0.563	0.121	0.435	0.936	0.686	0.799	0.122	0.461	0.177	0.952	0.099	0.986	0.930	0.481	0.808	0.463	0.980	0.728
	Total No. of vital cells after filtration	0.623	0.606	0.554	0.615	0.674	0.800	0.776	0.576	0.155	0.531	0.898	0.591	0.584	0.275	0.371	0.693	0.184	0.596	0.214	0.156	0.417	0.759	0.085	0.044	0.933	0.359	0.307	0.058	0.903	0.009	0.893	0.380	0.883	0.376	0.901	0.520	0.545
	Cell viability [%]	0.569	0.485	0.635	0.778	0.669	0.634	0.790	0.571	0.223	0.467	0.986	0.509	0.679	0.140	0.684	0.346	0.696	0.098	0.163	0.033	0.465	0.017	0.328	0.269	0.564	0.030	0.938	0.271	0.998	0.079	0.592	0.681	0.215	0.750	0.275	0.575	0.094
	No. of cells per spheroid	0.507	0.649	0.265	0.869	0.867	0.763	0.903	0.685	0.188	0.977	0.567	0.938	0.090	0.551	0.530	0.662	0.080	0.698	0.617	0.525	0.399	0.379	0.556	0.315	0.780	0.010	0.550	0.003	0.464	0.495	0.455	0.808	0.589	0.310	0.125	0.703	0.902
	No. of cells per spheroid groups [< 10 000, 10 000 - 20 000, > 20 000]	0.974	0.901	0.623	0.912	0.408	0.831	0.812	0.301	0.224	0.839	0.351	0.934	0.945	0.675	0.767	0.963	0.530	0.593	0.554	0.878	0.593	0.105	0.391	0.288	0.959	0.057	0.048	0.120	0.774	0.784	0.535	0.870	0.613	0.342	0.649	0.898	0.896
	No. of spheroids generated	0.637	0.710	0.376	0.911	0.479	0.576	0.601	0.446	0.096	0.472	0.625	0.001	0.086	0.103	0.423	0.759	0.360	0.792	0.266	0.241	0.493	0.890	0.045	0.038	0.732	0.311	0.275	0.159	0.832	0.009	0.778	0.208	0.870	0.646	0.884	0.631	0.528
	No. of technical replicates	0.325	0.373	0.224	0.962	0.562	0.521	0.854	0.447	0.110	0.421	0.884	0.025	0.170	0.060	0.783	0.531	0.567	0.865	0.395	0.404	0.447	0.758	0.109	0.108	0.691	0.346	0.190	0.220	0.931	0.006	0.793	0.132	0.927	NA	0.789	0.671	0.763
	Incubation of generated spheroids [h]	0.775	0.940	0.777	0.873	0.864	0.191	0.084	0.227	0.929	0.028	0.088	0.318	0.150	0.464	0.404	0.638	0.069	0.177	0.501	0.225	0.248	0.686	0.669	0.823	0.043	0.489	0.536	0.439	0.918	0.946	0.695	0.032	0.079	0.852	0.316	0.416	0.605
	Mucous exterior lining [Yes vs No]	0.465	0.465	0.271	0.388	0.089	1.000	1.000	0.378	0.274	1.000	0.135	0.198	0.790	1.000	0.656	0.330	0.167	0.205	0.167	0.164	0.326	0.514	1.000	0.971	0.302	1.000	1.000	NA	NA	NA	NA	0.217	NA	NA	0.949	0.454	0.668
	Cell density before treatment [very thin, slightly thin, moderately thin, dense]	0.841	0.841	0.963	0.737	0.184	0.510	0.890	0.735	0.983	0.220	0.580	0.830	0.942	0.718	0.220	0.804	0.521	0.663	0.141	0.524	0.752	0.825	0.068	0.087	0.664	0.364	0.453	0.720	0.920	0.609	0.936	0.558	0.678	0.326	0.027	0.627	0.144
In vitro treatment	**Same-day preparation cytostatic compounds** [Yes vs No]	0.201	0.130	0.786	0.435	1.000	0.729	0.362	0.056	0.160	0.800	0.402	0.360	0.897	0.461	1.000	0.017	1.000	0.717	0.716	0.805	0.660	0.871	0.379	0.396	0.690	1.000	0.111	0.527	0.484	0.847	0.965	0.990	0.285	NA	0.217	0.071	0.328
	Time between compounds preparation and in vitro treatment [d]	0.776	0.776	0.093	0.132	0.857	0.025	NA	0.640	0.694	0.132	0.055	0.037	0.091	0.239	0.643	0.297	0.069	0.184	NA	0.184	0.184	NA	NA	NA	NA	NA	NA	0.719	0.229	NA	NA	0.333	NA	NA	0.357	0.605	0.214
	No. of treatment options	0.343	0.417	0.556	0.844	0.665	0.358	0.403	0.529	0.105	0.517	0.348	0.000	0.032	0.118	0.522	0.644	0.313	0.682	0.262	0.281	0.549	0.913	0.055	0.055	0.886	0.788	0.437	0.488	0.675	0.035	0.782	0.268	0.932	0.602	0.799	0.864	0.397
	Visible morphological changes after treatment [Yes vs No]	0.320	0.320	1.000	0.313	1.000	1.000	1.000	0.451	0.187	1.000	0.792	0.873	0.743	0.631	0.576	1.000	1.000	0.534	1.000	0.958	0.017	0.972	1.000	0.779	NA	1.000	0.473	0.095	0.954	0.913	0.130	0.551	0.796	NA	0.012	0.062	0.430
	Mucous exterior lining following treatment [Yes vs No]	1.000	1.000	0.550	0.308	0.475	0.585	0.438	NA	NA	0.308	0.380	0.369	0.580	1.000	0.524	0.576	1.000	NA	1.000	NA	NA	NA	NA	NA	NA	0.250	1.000	NA	NA	NA	NA	0.177	NA	NA	0.665	NA	0.589
	Readout method [MTS vs ATP]	0.004	0.031	0.032	1.000	0.380	0.676	1.000	0.137	0.867	0.060	0.305	0.010	0.270	0.031	0.468	1.000	0.472	0.775	1.000	0.178	0.452	0.156	0.400	0.119	0.508	1.000	1.000	0.199	0.460	0.540	0.277	0.076	0.891	0.381	0.052	0.806	0.916
	Incubation with MTS reagent [h]	NA	NA	NA	0.757	0.885	NA	NA	0.819	0.367	0.139	0.988	0.131	0.993	NA	0.864	0.950	0.319	0.671	0.525	0.046	0.519	0.708	0.508	0.607	0.479	0.590	0.435	0.580	0.779	0.984	0.657	0.331	0.694	NA	0.235	0.948	0.624
	Incubation with ATP reagent [h]	0.080	0.080	0.601	0.092	0.272	0.549	0.683	0.948	0.540	0.642	0.764	0.755	0.683	0.243	0.888	0.572	0.119	0.954	0.028	0.149	0.564	0.891	0.236	0.192	0.358	0.277	0.286	0.060	0.911	0.010	0.957	0.211	0.283	0.989	0.221	0.175	0.132
	Metabolic activity mean solvent control [cps]	0.468	0.431	0.438	0.516	0.880	0.441	0.959	0.716	0.937	0.030	0.714	0.892	0.959	0.964	0.141	0.459	0.567	0.049	0.975	0.773	0.417	0.914	0.200	0.028	0.574	0.464	0.351	0.138	0.808	0.011	0.694	0.856	0.604	0.875	0.243	0.658	0.628
	Metabolic activity mean solvent control per cell [cps/cell]	0.410	0.367	0.504	0.833	0.900	0.407	0.758	0.820	0.955	0.054	0.713	0.889	0.829	0.617	0.348	0.372	0.500	0.068	0.965	0.708	0.548	0.972	0.168	0.102	0.653	0.761	0.769	0.493	0.710	0.019	0.511	0.979	0.534	0.885	0.580	0.690	0.490
	Cell survival [% solvent control]	0.001	0.004	0.000	0.929	0.761	0.641	0.025	0.039	0.135	0.021	0.000	0.000	0.000	0.000	0.002	0.435	0.978	0.935	0.521	0.498	0.457	0.957	0.099	0.110	0.506	0.502	0.470	0.324	0.223	0.201	0.292	0.046	0.257	0.471	0.023	0.583	0.849
	Cutoff cell survival [≤ 35 vs >35%]	0.002	0.004	0.000	0.789	1.000	0.475	0.074	0.009	0.089	0.198	0.001	0.000	0.000	0.000	0.001	0.305	1.000	0.775	0.676	0.475	0.633	0.991	0.103	0.170	0.833	0.600	0.238	0.454	0.151	0.159	0.735	0.244	0.932	0.583	0.032	0.543	0.890

In vitro treatment cont.	Baseline clinical data																													
	Age at diagnosis	Age groups	BMI	BMI groups	Clinical tumor size at diagnosis, CT-Thorax	Clinical tumor size at diagnosis, MRT	Clinical tumor size at diagnosis, Mammography	Clinical tumor size at diagnosis, Ultrasound	Tumor stage	Tumor stage groups	Nodal status	UICC	Effected side	Centricity/focality	Histologic type	Grading	ER	PR	ER status	PR status	Hormone receptor status	HER2 score	HER2 status	HR/HER2	Ki67	Ki67 cutoff	CEA	CA 15-3	CEA status	CA 15-3 status
Mean cell survival EC	0.582	0.724	0.031	0.264	0.045	0.487	0.727	0.541	0.555	0.248	0.808	0.274	0.007	0.158	0.588	0.327	0.172	0.114	0.304	0.014	0.304	0.008	0.000	0.019	0.256	0.105	0.441	0.148	0.004	0.368
Stdv mean cell survival EC	0.049	0.047	0.839	0.781	0.101	0.007	0.258	0.458	0.931	0.666	0.081	0.687	0.834	0.728	0.440	0.065	0.868	0.365	0.703	0.597	0.703	0.365	0.486	0.731	0.934	0.430	0.703	0.691	0.474	0.821
Mean cell survival ECD	0.162	0.333	0.324	NA	NA	0.357	0.353	0.581	0.572	0.513	0.223	0.473	0.641	0.613	NA	0.520	0.317	0.286	0.684	0.520	NA	0.333	NA	0.562	0.205	0.590	0.893	0.583	0.524	0.902
Stdv mean cell survival ECD	0.644	0.899	0.236	NA	NA	0.426	0.869	0.657	0.310	NA	0.666	0.116	0.844	NA	NA	NA	0.434	0.859	NA	NA	NA	0.416	NA	NA	0.103	NA	0.092	0.093	NA	0.133
Mean cell survival EC-Trastuzumab	0.837	0.839	0.242	0.365	NA	0.636	0.444	0.173	0.767	NA	0.1	0.262	0.327	0.694	NA	0.223	0.539	0.509	0.714	0.446	0.714	0.433	NA	0.999	0.792	NA	0.317	0.004	NA	NA
Stdv mean cell survival EC-Trastuzumab	0.356	0.594	0.952	0.526	NA	0.103	0.523	0.067	0.537	NA	0.174	0.443	0.144	0.498	NA	0.360	0.157	0.168	0.239	0.146	0.572	0.894	NA	0.510	1.000	NA	0.352	0.017	NA	NA
Mean cell survival FEC	0.061	0.074	0.740	0.852	0.959	0.622	0.476	0.514	0.052	0.084	0.02	0.092	0.860	0.863	NA	0.758	0.555	0.245	0.444	0.197	0.444	0.812	NA	0.588	0.853	0.499	0.725	0.031	NA	NA
Stdv mean cell survival FEC	0.903	0.407	0.802	0.652	0.665	0.168	0.042	0.011	0.004	0.000	0.232	0.009	0.687	0.989	NA	0.313	0.973	0.779	0.702	0.954	0.702	0.805	NA	0.861	0.766	0.670	0.649	0.616	NA	NA
Mean cell survival DocAC	0.505	0.507	0.652	0.925	NA	0.286	0.807	0.990	0.591	0.685	0.324	0.494	0.983	0.044	NA	NA	0.192	0.241	0.025	0.234	0.234	0.310	NA	0.111	0.192	NA	NA	NA	NA	NA
Stdv mean cell survival DocAC	0.194	0.141	0.374	0.536	NA	0.776	0.198	0.149	0.405	0.574	0.762	0.573	0.650	0.498	NA	NA	0.002	0.003	0.506	0.400	0.400	0.001	NA	0.216	0.833	NA	NA	NA	NA	NA
Mean cell survival TCbH	0.720	0.984	0.679	0.901	NA	0.593	0.281	0.268	0.449	NA	0.967	0.074	0.332	0.453	NA	0.661	0.275	0.402	0.371	0.359	0.371	0.452	0.217	0.483	0.124	NA	0.240	0.174	NA	NA
Stdv mean cell survival TCbH	0.077	0.224	0.698	0.706	NA	0.431	0.007	0.283	0.335	NA	0.255	0.868	0.518	0.583	NA	0.162	0.491	0.542	0.487	0.510	0.487	0.563	0.270	0.625	0.582	NA	0.054	0.467	NA	NA
Mean cell survival Doc	0.664	0.812	0.888	0.145	0.830	0.708	0.558	0.703	0.035	0.090	0.34	0.049	0.366	0.942	0.434	0.506	0.926	0.949	0.538	0.843	0.538	0.533	NA	0.921	0.211	0.246	0.770	0.334	NA	0.198
Stdv mean cell survival Doc	0.163	0.203	0.652	0.668	0.979	0.452	0.811	0.477	0.036	0.154	0.524	0.072	0.976	0.405	0.843	0.694	0.275	0.212	0.235	0.259	0.235	0.488	NA	0.250	0.434	0.494	0.287	0.501	NA	0.596
Mean cell survival Pac	0.672	0.456	0.869	0.562	NA	0.370	0.012	0.414	0.381	0.240	0.796	0.634	0.532	0.996	NA	0.670	0.887	0.347	0.552	0.635	0.552	0.228	0.034	0.097	0.340	0.508	0.306	0.346	NA	NA
Stdv mean cell survival Pac	0.370	0.072	0.209	0.206	NA	0.918	0.677	0.580	0.898	0.704	0.64	0.929	0.821	0.125	NA	0.354	0.166	0.109	0.606	0.004	0.606	0.433	0.277	0.676	0.281	0.027	0.761	0.232	NA	NA

In vitro treatment cont.

Group	Endpoint	Mean cell survival EC	Stdv mean cell survival EC	Mean cell survival ECD	Stdv mean cell survival ECD	Mean cell survival EC-Trastuzumab	Stdv mean cell survival EC-Trastuzumab	Mean cell survival FEC	Stdv mean cell survival FEC	Mean cell survival DocAC	Stdv mean cell survival DocAC	Mean cell survival TCbH	Stdv mean cell survival TCbH	Mean cell survival Doc	Stdv mean cell survival Doc	Mean cell survival Pac	Stdv mean cell survival Pac
Drug therapy	**Anthracycline/taxane-based chemotherapy**	0.007	0.829	NA	NA	0.163	0.164	0.898	0.848	NA	NA	NA	NA	0.485	0.502	0.007	0.132
	Anthracycline-based chemotherapy	0.083	0.746	NA	NA	0.163	0.164	0.898	0.848	NA	NA	NA	NA	0.485	0.502	0.007	0.132
	Trastuzumab-based drug therapy	0.000	0.496	NA	NA	NA	NA	NA	NA	NA	NA	0.217	0.270	NA	NA	0.034	0.277
	Treatment adherence	0.381	0.899	NA	NA	0.518	0.631	0.162	0.881	0.628	0.341	0.124	0.473	0.529	0.159	0.760	0.663
	Reduced dose	0.761	0.804	NA	NA	0.223	0.360	NA	NA	NA	NA	NA	NA	0.461	0.413	NA	NA
	Premature discontinuation of drug therapy	0.463	0.788	NA	NA	NA	NA	0.162	0.881	0.628	0.341	0.282	0.751	0.522	0.188	NA	NA
	Change in drug regimen	0.068	0.737	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Clinical tumor size midway chemotherapy, US	0.058	0.017	0.199	0.247	0.324	0.214	0.256	0.287	NA	NA	NA	NA	0.442	0.625	0.842	0.777
	Change in tumor diameter, beginning - midway chemotherapy, US	0.168	0.000	0.397	0.591	0.444	0.386	0.969	0.926	NA	NA	NA	NA	0.710	0.622	0.417	0.799
Endpoints and data at surgery	**Breast conserving surgery**	0.516	0.319	0.339	0.899	NA	NA	0.011	0.338	0.628	0.341	0.934	0.659	0.347	0.286	0.704	0.013
	Clinical response	0.019	0.353	0.732	NA	0.436	0.008	0.372	0.507	0.406	0.756	0.397	0.546	0.622	0.650	0.728	0.135
	ypT groups	0.001	0.626	0.726	NA	0.860	0.169	0.427	0.722	0.010	0.000	0.075	0.442	0.565	0.695	0.508	0.177
	Grade of regression	0.000	0.649	0.704	NA	0.860	0.169	0.606	0.543	0.000	0.475	0.004	0.137	0.317	0.704	0.760	0.110
	pCR	0.000	0.368	NA	NA	0.834	0.931	0.550	0.536	0.008	0.224	0.030	0.084	0.129	0.644	0.835	0.727
	Nodal status surgery	0.004	0.455	NA	NA	NA	NA	0.073	0.666	0.991	0.778	0.954	0.557	0.695	0.978	0.143	0.839
	Lympathic invasion	0.050	0.456	NA	NA	NA	NA	0.376	0.351	NA	NA	NA	NA	0.796	0.756	NA	NA
	PR status	0.455	0.949	0.015	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.465	0.181	NA	NA
	PR	0.136	0.715	0.082	1.000	0.640	0.681	0.134	0.477	0.616	0.134	NA	NA	0.726	0.408	NA	NA
	ER status	0.393	0.605	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.373	0.231	NA	NA
	ER	0.119	0.458	0.266	1.000	0.027	0.014	0.134	0.477	0.616	0.134	NA	NA	0.212	0.293	NA	NA
	Change in PR biopsy - surgical specimen	0.413	0.097	0.281	NA	0.693	0.653	NA	NA	0.616	0.134	NA	NA	0.320	0.436	NA	NA
	Change in ER biopsy - surgical specimen	0.993	0.679	0.500	0.260	NA	NA	NA	NA	0.616	0.134	NA	NA	0.396	0.493	NA	NA
	Ki67 cutoff	0.272	0.334	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.286	0.425	NA	NA
	Ki67	0.113	0.270	0.614	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.279	0.426	NA	NA
	Change in Ki67 biopsy - surgical specimen	0.524	0.045	0.229	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.730	0.815	NA	NA
	CEA status	0.865	0.452	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.702	0.469	NA	NA
	CA 15-3 status	0.471	0.963	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.739	0.406	NA	NA
	CEA	0.508	0.391	0.224	0.199	NA	NA	0.733	0.726	NA	NA	NA	NA	0.628	0.370	NA	NA
	CA 15-3	0.772	0.521	0.420	0.395	NA	NA	0.035	0.576	NA	NA	NA	NA	0.826	0.776	0.464	0.433
	Change in CEA, beginning - end chemotherapy	0.533	0.984	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.299	0.253	NA	NA
	Change in CA 15-3, beginning - end chemotherapy	0.266	0.500	0.846	0.871	NA	NA	NA	NA	NA	NA	NA	NA	0.247	0.347	0.211	0.779
	Tumor diameter surgical specimen	0.039	0.715	0.568	0.392	0.240	0.176	0.297	0.421	0.505	0.171	0.534	0.188	0.984	0.930	0.263	0.375
	Clinical tumor size end chemotherapy, MRT	0.765	0.569	NA	NA	NA	NA	0.134	0.095	NA	NA	NA	NA	0.996	0.404	NA	NA
	Clinical tumor size end chemotherapy, Mammography	0.201	0.238	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	US	0.043	0.238	0.959	NA	0.605	0.867	0.482	0.537	0.371	0.401	0.619	0.846	0.160	0.659	0.781	0.550
	Change in tumor diameter, midway - end chemotherapy, US	0.721	0.637	0.556	NA	NA	NA	0.564	0.592	NA	NA	NA	NA	0.839	0.781	NA	NA
	Change in tumor diameter, beginning - end chemotherapy, US	0.629	0.587	0.340	NA	NA	NA	0.617	0.406	0.192	0.377	0.547	0.200	0.463	0.644	0.010	0.075