The role of PTEN, PI3K-Akt-mTOR signaling and pseudogene PTENP1 in breast cancer

Synnøve Yndestad

Thesis for the Degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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Scientific environment

All laboratory work was performed at Mohn Cancer Research laboratory, Haukeland University Hospital, and at The Laboratory Animal Facility at the Department of Clinical Medicine, University of Bergen.

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"Fairy tales are more than true – not because they tell us dragons exist but because they tell us dragons can be beaten."

-Neil Gaiman, Coraline

To my fellow army of knights, weapon smiths, trainers and supporters:

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Abbrevations

ceRNA = competing endogenous RNA
CISH = Chromogenic in situ hybridization
DSB = Double strand break
DSS = Disease specific survival
ER = Estrogen receptor
GFP = Green fluorescence protein
HER2 = Human epidermal growth factor receptor 2
IHC = Immunohistochemistry
IRES = Internal ribosome entry site
LABC = Locally advanced breast cancer
LncRNA = Long non coding RNA
LTR = Long terminal repeat
MDM2 = Mouse double minute 2 homolog
miRNA = microRNA
MPS = Massive parallel sequencing
MRE = MicroRNA response element
mRNA = messenger RNA
OS = Overall Survival
PI3K = Phosphatidylinositol-4,5-bisphosphate 3-kinase
RISC = microRNA-induced silencing complex
PR = Progesterone Receptor
PTEN = Phosphatase and tensin homolog
PTENP1 = Phosphatase and tensin homolog pseudogene 1
RFS = Recurrence free survival
RPPA = Reverse phase protein array
RT-PCR = Reverse transcriptase PCR
SERM = Selective estrogen receptor modulator
SSB = Single strand break
TMA = Tissue micro array
TNBC = Triple negative breast cancer
TP53 = Tumor protein 53
qPCR = quantitative PCR
wt = Wild type

Abstract

Background:

Breast cancer is the most common type of cancer in women, and a major cause of premature death still today. The PI3K-Akt-mTOR signaling (in short: PI3K signaling) pathway is a growth and pro-survival pathway, and is frequently deregulated in breast cancer due to amplifications, mutations and deletions of the genes encoding these important signaling hubs. PTEN inhibits the activation of Akt, and acts as an endogenous tumor suppressor by negatively regulating PI3K signaling. Loss of PTEN protein expression is frequently observed in breast cancer, which could contribute to hyperactivation of the PI3K pathway. Whereas aberrations in the PI3K pathway are associated with resistance towards endocrine therapy, less is known of the role of this signaling pathway in resistance to chemotherapy.

Epigenetic alterations like DNA methylation, histone modifications and non-coding RNAs can also influence cancer progression and resistance to therapy. Non-coding RNAs are functional RNA transcripts, but are not translated into proteins. MicroRNAs (miRNAs) regulate protein expression post-transcriptionally by binding to complementary sequences on mRNA transcripts, which is then degraded. *PTEN* and the *PTEN* pseudogene *PTENP1* share several miRNA binding sites, and *PTENP1* acts as a competing endogenous RNA (ceRNA) by adsorbing miRNAs which would otherwise degrade various protein-coding mRNA transcripts. In prostate cancer it has been showed that *PTENP1* expression protects *PTEN* mRNA from degradation and increases PTEN protein expression through ceRNA interaction. The role of the non-coding RNA *PTENP1* in breast cancer has not been addressed previously.

Main objectives

The first two papers presented in this thesis focused on unraveling the potential relationship between PI3K signaling and PTEN status, and mechanisms of resistance to chemotherapy in breast cancer. In the third study we examined the potential role of *PTENP1* pseudogen in breast cancer on tumor growth.

Results

In Paper 1, we explored the relationship between PI3K signaling and clinical outcome using data from three clinical studies. By examining breast cancer biopsies using IHC staining for PTEN, phosphorylated Akt (pAkt), pS6K and p4EPB expression we found that neither of the protein staining's were associated with survival outcome, or predictive of response to chemotherapy. However, we found that patients with a high intratumoral *PTEN* mRNA expression had a worse prognosis than patients with a low *PTEN* mRNA expression. Interestingly, this was only significant for patients with *TP53* wild type tumors. *PTENP1* was expressed in most of the breast cancer biopsies examined and correlated to *PTEN* expression. However, there was no prognostic impact of *PTENP1* gene expression, and no predictive value was observed, with respect to response to chemotherapy.

In Paper 2, we created anthracycline-resistant breast cancer cell lines to compare how PI3K signaling was affected by an acute dose of anthracycline in anthracycline-resistant vs. naïve cells. After 24h anthracycline exposure we observed an increase in phosphorylated (activated) Akt in ER positive cell lines, while no change was seen in ER negative cell lines. Also, the resistant cell lines showed a higher baseline expression of phosphorylated Akt. Accordingly, we examined the effect of Akt inhibition with and without doxorubicin in concert both *in vitro* and *in vivo*. A combination treatment with Akt inhibitor and doxorubicin reduced tumor size more than Akt inhibitor or chemotherapy alone in the ER positive MCF7. In contrast, in the ER negative MDA-MB-231 Akt inhibition had no additive benefit to the anthracycline. To examine the relevance of Akt and anthracycline resistance in a clinical setting, we examined the mRNA level of *AKT1* expression in breast cancer biopsies from two clinical studies. We found that an initial increase in *AKT1* 24 hours after the first treatment characterized ER positive tumors that subsequently responded to doxorubicin treatment, although this was not observed after 16 weeks of treatment. Thus, analysis of biopsies after 24 hours, to dissect the acute response to anthracyclines, seems to be of value to predict whether the patient will subsequently respond to treatment.

The third paper addressed the question of whether the non-coding RNA *PTENP1* has a functional role in breast cancer through ceRNA interactions. We found a diverging role for *PTENP1* that was dependent on the tumor´s ER status. *PTENP1* overexpression increased both the *PTEN* transcript and PTEN protein in ER negative cell lines, while ER positive cell lines had decreased *PTEN* transcript and unchanged PTEN expression subsequent to pseudogene transduction. By implanting *PTENP1*-overexpressing breast cancer cells in mice, *PTENP1* reduced tumor growth in ER negative breast cancer, while it increased tumor growth in ER positive breast cancer. This effect seemed unrelated to PI3K signaling, but rather related to AP2 and ER-signaling. *PTENP1* overexpression increased expression of hsa-miR-26a and profoundly decreased the hsa-miR-26a target *ESR1* and thereby ER expression in the ER positive MCF7. There was also a clinical correlate to this finding, where data from the TCGA database demonstrated that breast tumors with upregulated *PTENP1* expression exhibited lower ER expression than breast tumors with normal or no *PTENP1* expression.

Conclusion and implications

We found a high *PTEN* gene expression to be associated with worse prognosis for patients with breast cancers harboring preserved p53 function. The finding that a high level of *PTEN* expression is a marker for bad prognosis in breast cancer is surprising, but indicates that *PTEN* transcripts may not only influence PTEN protein levels and PI3K signaling, but also interact with other biological mechanisms to promote tumor progression. The interaction between *PTEN* and *TP53* seems essential in this context. Also, we demonstrate that ceRNA interactions are implicated in *PTEN* regulation, via its pseudogene *PTENP1*, and this interaction is fundamentally dependent on the tumor´s estrogen receptor status. Furthermore, in estrogen receptor positive breast cancers, upregulated PI3K signaling, and in particular Akt activation, seems to play a key role in resistance to anthracycline treatment, and potentially Akt inhibitors could be introduced in this setting to target chemoresistance.

List of publications

Paper 1:

High PTEN gene expression is a negative prognostic marker in human primary breast cancers with preserved p53 function

Yndestad S, Austreid E, Knappskog S, Chrisanthar R, Lilleng PK, Lonning PE, Eikesdal HP

Breast Cancer Research and Treatment, February 2017

Paper 2:

Activation of Akt characterizes estrogen receptor positive human breast cancers which respond to anthracyclines

Yndestad S, Austreid E, Svanberg IR, Knappskog S, Lonning PE, Eikesdal HP

Oncotarget, April 2017

Paper 3:

Divergent activity of the pseudogene *PTENP1* in ER-positive and negative breast cancer

Yndestad S, Austreid E, Skaftnesmo KO, Lonning PE, Eikesdal HP

Molecular Cancer Research, October 2017

Contents

1. Introduction

1.1 Cancer

Cancer is a family of disease characterized by cells undergoing abnormal division resulting in malignant growth displacing normal cells in bone marrow or blood, or developing solid tumors that destroy surrounding normal tissues. Cancer is a family of disease characterized by cells undergoing abnormal division resulting in malignant growth displacing normal cells in bone marrow or blood, or developing solid tumors that destroy surrounding normal tissues. A major obstacle to successful systemic cancer therapy is the fact that cancer is not a foreign intruder like a bacteria or a virus, it is the body's own cells that change characteristics and become malignant. These characteristics are known as the "Hallmarks of cancer" (Figure 1) (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011). The hallmark characteristics include the ability to sustain proliferative signaling, evade growth suppression, the ability to evade immune destruction, replicative immortality, activate invasion and metastasis, induce angiogenesis, resisting cell death and deregulate metabolism. Cancer is a genetic disease, but the development of cancer is not considered to be a single mutational event, but rather an accumulation of events where the cells acquire hallmark characteristics (Hanahan and Weinberg 2011). Enabling characteristics that facilitates carcinogenesis and progression include genomic instability i.e. by inactivation of

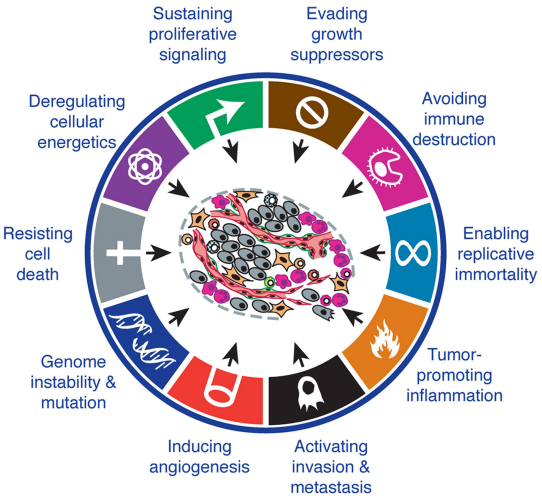

Figure 1. Hallmarks of cancer. Modified from (Hanahan and Weinberg 2011) 2001. Reprinted with permission.

genes involved in DNA repair or mitotic checkpoints like *TP53*, *ATM* or *BRCA1* (Negrini, Gorgoulis et al. 2010) and modification of the tumor niche by tumor-promoting inflammation. These characteristics span over a wide range, which reflects the diversity of the disease and the challenges one faces when designing effective cancer therapy. (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011, Vogelstein, Papadopoulos et al. 2013, Vogelstein and Kinzler 2015)

1.2 Epidemiology and cancer risk

Globally 8.8 million people died of cancer in 2015 making cancer the second leading cause of death worldwide (http://www.who.int/cancer/). The number of people diagnosed with cancer is expected to rise approximately 70% during the next twenty years (Ferlay, Soerjomataram et al. 2015) This increase is in part a reflection of our increased lifespan, since most malignancies occur after the age of 75, but also due to environmental factors, lifestyle and improved detection techniques (Cancer Registry of Norway 2016). With improved detection techniques one also introduce the risk of overdiagnosis. By identifying small non-aggressive and slow-growing malignant tumors that may never progress, overdiagnosis could contribute to an increase in incidence (Carter and Barratt 2017). Prostate cancer by prostate specific antigen (PSA) testing, breast cancer by mammography screening, thyroid cancer by ultrasound and lung cancer by computed tomography screening are the most likely cancers subjected to such overdiagnosis (Carter and Barratt 2017)

A predisposition to cancer can be caused by various hereditary factors. The number of known breast cancer susceptibility variants is increasing, which may lead to better risk prediction for individuals with a familial history of breast cancer in the future (Michailidou, Lindstrom et al. 2017, Milne, Kuchenbaecker et al. 2017). Specifically, germline mutations in *BRCA1/2*, *TP53* and *PTEN* are associated with a high lifetime risk of developing breast cancer. Germline mutations in the *BRCA1/2* gene predispose to breast and ovarian cancer. 45-75% of carriers of these mutations will develop

breast cancer, and 18-40% ovarian cancer (Baretta, Mocellin et al. 2016). Germline mutations in the *TP53* gene are responsible for the Li-Fraumeni syndrome, characterized by early onset of multiple cancer types, including breast cancer (Malkin, Li et al. 1990). Germline inactivating *PTEN* mutations cause Cowden syndrome, which is a relatively rare disorder and is characterized by frequent non-cancerous neoplasms, hamartomas, and an increased lifetime risk of developing breast, thyroid, endometrial and kidney cancer (Hollander, Blumenthal et al. 2011).

With respect to risk factors associated with increased risk of non-hereditary breast cancer, these are to a large extent hormone-related. The mammary glandular tissue is exposed to high and cycling levels of hormones which is also dependent on the length of each woman´s fertile period. An increased risk is correlated to the early onset of first menstruation, having few or no children and high age at first birth, a short time breast-feeding, a late menopause and the use of postmenopausal hormone substitution (Bray and Soerjomataram 2015).

Environmental factors can also increase the lifetime risk of developing breast cancer, such as high alcohol consumption and obesity (Lauby-Secretan, Scoccianti et al. 2016). However, only around 30% of all cancers are considered to be preventable by avoiding risk factors (Bray and Soerjomataram 2015). Furthermore, hereditary and environmental factors can not explain the development of all cases of breast cancer. Apart from this, somatic mutations occur randomly in healthy non-cancerous cells during replication throughout a person´s lifespan. The fidelity of DNA replication enzymes is around 1 error per $10^{9\text{-}10}$ nucleotides copied, after proofreading and mismatch repair (Ganai and Johansson 2016). Accordingly, cancer is also attributed to "bad luck" (Tomasetti and Vogelstein 2015). By mathematically correlating the lifetime risk of specific cancer types versus the number of stem cell divisions in the tissue wherein the cancer originates, Tomasetti and Vogelstein found a linear correlation between the number of stem cell divisions and the lifetime risk of cancer. They suggest that approximately 65% of the variance in cancer risk between different tissues could be a reflection of the total number of stem cell divisions in the specific tissues (Tomasetti and Vogelstein 2015). In breast cancer the proportion of driver

gene mutations attributed to environmental factors was found to be 15%, only 1,5% from hereditary factors and 83,4% from replicative "bad luck" (Tomasetti, Li et al. 2017). In contrast, driver mutations in melanoma were almost exclusively attributed to environmental factors (89%) and for lung cancer as high as 66%. Therefore, the potential for prevention by avoiding risk factors is fundamentally dependent on the type of cancer and its causative risk factors.

1.3 Breast Cancer

1.3.1 Incidence and mortality of breast cancer

Breast cancer is by far the most common cancer among females, and amount to 22% of all female cancers across all age groups in Norway (Cancer in Norway, 2015, The Norwegian Cancer Registry). In 2015, 3439 new cases of breast cancer were reported, whereof only 24 cases were diagnosed among males. The incidence has increased by 8% during the last reported 5-year period (from 2006-2010 to 2011-2015). During the same time interval, the 5-year relative survival has increased slightly from 87,8% to 89% and mortality has decreased moderately (Cancer in Norway 2015). The reduced mortality of breast cancer in Norway in recent years despite an increased incidence is probably due to earlier detection of breast cancer as part of mammography screening, and the improved survival could be explained by the mammography screening program in Norway and/or the development of better treatment (Narod, Iqbal et al. 2015). However, the influence of mammography screening on breast cancer-specific survival is debated (van den Ende, Oordt-Speets et al. 2017). However, the mortality of breast cancer is strongly related to disease stage (Cancer in Norway 2015). Five-year survival for patients with stage II disease (2011-15) was 92,3%, while patients with stage IV experienced a 25,5% five-year survival. With respect to long-term outcome, the survival was 77% after 15 years for all stages combined (Cancer in Norway 2015). For stage III disease or locally

advanced breast cancer (LABC), the prognosis is still poor, where 30% experience disease recurrence within 5 years after primary treatment (Cancer in Norway 2015).

1.3.2 Breast cancer treatment

The primary curative treatment for early breast cancer is surgery, which is in itself enough to avoid recurrence in cases where the disease is localized to the breast alone, and with low-risk tumor characteristics. The risk of breast cancer recurrence and cancer death increases substantially if regional lymph node metastases have developed (Cancer in Norway 2015). Despite axillary dissection, adjuvant radiotherapy and systemic cancer treatment are required in these cases to increase the chance of cancer cure.

Treatment of locally advanced tumors warrants a more aggressive approach than localized tumors, where neoadjuvant chemotherapy or endocrine therapy is administered prior to surgery and radiotherapy to shrink the tumor. Initial surgery in this patient category is strongly advised against, due to a high risk of local disease recurrence, either on the chest wall or the axilla, if performed prior to effective systemic therapy. In stead, effective neoadjuvant treatment is indicated, while observing prospectively that the tumor shrinks - pinpointing an effective regimen.

Radiation therapy is frequently used in the treatment of breast cancer. Ionizing radiation is used in order to shrink the size of the tumor, or it can be used after surgery to reduce the risk of recurrence. Ionizing radiation causes double stranded breaks in DNA, particularly in dividing cells. Radiation also causes generation of ROS, which also induces further DNA damage and single strand breaks (Borrego-Soto, Ortiz-Lopez et al. 2015). Cell death and growth arrest is then induced by the combined effect of the ionizing radiation-induced DNA damage and ROS generation.

Endocrine therapy is based upon the dependence of hormones as growth stimulants in hormone receptor positive breast cancer tissue. The influence of estrogen on breast cancer has been known for almost 100 years (DeVita and Chu 2008). The estrogen receptor was discovered in 1961, and the first targeted drug in breast cancer,

tamoxifen, was discovered six years later (DeVita and Rosenberg 2012). Tamoxifen and other SERMs (Selective Estrogen Receptor Modulators) bind to the estrogen receptor, blocking the proliferative effect of estradiol on the mammary epithelium. The estrogen-blocking property of tamoxifen makes this drug a fundamentally important adjuvant therapy in hormone receptor positive breast cancer, next to aromatase inhibitors, to reduce the risk of breast cancer recurrence (Early Breast Cancer Trialists' Collaborative, Davies et al. 2011). Aromatase inhibitors have a different mechanism than SERM. They block the enzyme aromatase and thereby stops the transformation of androgens to estrogens, this reduce estrogen production and hormone stimulation in hormone receptor positive breast cancer (Lonning and Eikesdal 2013).

Chemotherapy is a denomination for drugs that are cytotoxic to cells. The therapeutic agent interferes with DNA or destroys cellular compartments involved with cell division causing cellular stress leading to apoptosis and cell death. In general, cancer cells divide more rapidly than normal cells, and chemotherapy will therefore induce relatively more damage to cancer cells than normal cells. Modern chemotherapy regimens reduce breast cancer mortality by 30-35% over 10-years, and accumulating evidence indicates that patients with ER-negative tumors benefit more from chemotherapy than patients with ER-positive tumors (Berry, Cirrincione et al. 2006, Albain, Barlow et al. 2010, Albain, Anderson et al. 2012). The different therapeutic agents have different mechanisms of action, and modern adjuvant chemotherapy consists of combination regimens to increase the efficacy and reduce the risk of therapy resistance. In the adjuvant and neoadjuvant setting, one typically uses two or more drugs in combination. This includes in most cases anthracyclines (doxorubicin or epirubicin), and cyclophosphamide, followed by taxanes (paclitaxel or docetaxel), which is combined with the anti-HER2 antibody trastuzumab for HER2 amplified breast cancer. In advanced/metastatic disease several other drugs are also included.

The chemotherapeutics given to patients in the neoadjuvant clinical trials used in this work are: anthracyclines (doxorubicin and epirubicin), 5-fluorouracil (5-FU), mitomycin and taxanes (paclitaxel and docetaxel).

Anthracyclines have multiple and complex mechanisms of action. One is the inhibition of topoisomerase II, which prevents relaxation of supercoiled DNA making the DNA inaccessible for DNA polymerase for further replication. It also halts replication by binding to and intercalating between the nucleotides in the DNA. Furthermore, anthracyclines cause ROS generation which leads to DNA breaks and fragmentation, apoptosis and cell death (Minotti, Menna et al. 2004). **5-FU** is a uracil analogue, with a fluorine atom attached. It incorporates into RNA and DNA during transcription, and also inhibits thymidylase synthase causing imbalance in the dNTP pool (specifically dATP/dTTP ratio) resulting in disruption in DNA synthesis and DNA repair, and further, DNA damage and cell death (Longley, Harkin et al. 2003). **Mitomycin** alkylates and crosslinks DNA, causing permanent growth arrest due to DNA damage, and double-strand breaks (Tomasz 1995). **Taxanes**, such as paclitaxel arrest dividing cells in G2/M phase by disrupting the function of microtubules by inhibiting depolymerization, accordingly, the cell will not be able to proceed with metaphase (Baker and Dorr 2001).

Targeted therapy

The concept of targeted therapy stems from the idea that cancer cells express different biomarkers relative to normal cells. There might be excessive expression of receptors initiating proliferation pathways, or activating mutations/aberrations in pathways that induce cell growth. In theory, this makes it possible to create drugs that operate as "magic bullets", targeting only the malignant cells, and leaving the benign tissue unharmed based on the higher expression of these molecules in malignant cells. There is an immense effort being made to find the right targets and combination of targets, but there are still only a few therapies that have proven to be beneficial and implemented in the clinic for breast cancer treatment. The oldest type of targeted breast cancer therapy is endocrine therapy, including SERMs and aromatase

inhibitors as described above, and these are targeting the hormone receptor positive tumor cells to improve survival both in the adjuvant and metastatic setting still today. The HER2 targeted antibody trastuzumab provides a survival benefit to patients with HER2 positive breast cancer, by binding to the HER2 receptor that is profoundly overexpressed in these tumors. Another antibody, pertuzumab, was recently introduced and prevents HER2-HER3 dimerization and downstream intracellular signaling (Baselga, Cortes et al. 2012). By conjugating the tubulin inhibitor emtansine to trastuzumab a new drug was generated which delivers emtansine exclusively to HER2 expressing cells, which has proven beneficial to patients with HER2 positive breast cancer (Welslau, Dieras et al. 2014).

The PI3K-Akt-mTOR pathway is commonly activated in hormone receptor positive breast cancer, and upregulation of this pathway is involved in the development of resistance to endocrine treatment. The mTOR inhibitor everolimus is currently used in the treatment of postmenopausal women after first line endocrine therapy with a non-steroidal aromatase inhibitor has failed. Everolimus is used to avoid endocrine resistance and re-sensitize the tumor to further endocrine treatment, in combination with a steroidal aromatase inhibitor (Baselga, Campone et al. 2012).

Cyclin Dependent Kinases (CDKs) have an important function in regulation of the cell cycle. CDKs bind to various cyclins, and the cyclin-CDK complexes regulate the timing of the progression through the cell cycle. Cyclin D partners with CDK4 or CDK6, and the complex phosphorylates and inactivates Rb, which initiates DNA replication and the transition from G1 to S phase (Asghar, Witkiewicz et al. 2015). The **CDK4/6 inhibitors** palbociclib and ribociclib were recently approved by the US Food and Drug administration (Clark, Karasic et al. 2016, Hortobagyi, Stemmer et al. 2016) as well as European (EMA) and Norwegian regulatory authorities, for use in combination with endocrine therapy, in patients with hormone receptor positive metastatic breast cancer.

Immunotherapy

Immunotherapy is based on the principle of enhancing or inducing a patient's immune response to treat cancer. There are three main approaches undergoing extensive research at the moment; cell transfer therapy (Rosenberg and Restifo 2015), cancer vaccination (Melief, van Hall et al. 2015) and immune checkpoint inhibition (Francis and Thomas 2017). Checkpoint inhibition has gained a lot of attention the last couple of years, and has proven to be beneficial and been implemented in clinical use for several solid cancer types, such as melanoma, renal cancer, non-small cell lung cancer (NSLC), clear-cell renal carcinoma, bladder cancer and prostate cancer (D'Errico, Machado et al. 2017). Although, after a great number of clinical trials in breast cancer, response to checkpoint inhibition are reported in only a minority of breast cancer patients thus far (Spellman and Tang 2016). Among these the subgroup of triple negative breast cancer (TNBC) seems to display a better response to checkpoint inhibitors. Specifically, in PD-L1 expressing, metastatic TNBC, a response rate of 18,5% to the PD-1 inhibitor pembrolizumab was observed (Nanda, Chow et al. 2016). Multiple clinical trials are currently ongoing to decipher the clinical potential of such therapy further in breast cancer.

1.3.3 Prognostic and predictive factors in breast cancer

A Predictive marker will indicate whether a specific treatment A will be more effective than treatment B for a specific disease, and can be used to direct which treatment that should be chosen. A **prognostic marker** will indicate the expected clinical outcome of the disease, pointing to the risk of recurrence (i.e. recurrence-free survival; RFS) and/or death from the disease (disease-specific survival; DSS). A prognostic marker can not be used to pinpoint whether a given patient will benefit or not from a particular treatment. By performing a biopsy one can characterize and grade the tumor histologically, and examine known biomarkers. Describing the subtype of breast cancer is essential to estimate the risk of recurrence. Important prognostic factors in breast cancer are primary tumor size, lymph node status, infiltration to skin or chest wall, stage (I-IV), as well as hormone receptor, HER2 and proliferation markers like Ki67 status. A prognostic marker can establish the severity of the disease, but predictive markers are needed to provide the right therapy to the

right patient, i.e. personalized therapy. Currently there are few predictive biomarkers for breast cancer, but hormone receptor expression and HER2 overexpression are predictive markers for response to endocrine therapy and HER2 targeted treatment, respectively. However, not all patients will benefit from endocrine or HER2 targeted treatment despite harboring hormone or HER2 overexpressing tumors, since the biomarkers are not 100% positively predictive of response.

1.3.4 Breast cancer subtypes

Breast cancer consists of several subtypes with distinct pathological features where each subtype has different survival outcomes and direct different treatment options with respect to therapy sensitivity. The traditional histopathological-based phenotypic breast cancer subgroups divide breast cancer into hormone receptor positive breast cancer (ER+/PR+/HER2-), hormone receptor positive and HER2 positive (triple positive, ER+/PR+/HER2+), HER2 positive (ER-/PR-/HER2+) or triple negative (TNBC) (ER-/PR-/HER2-) based on immunohistochemistry staining (Figure 2).

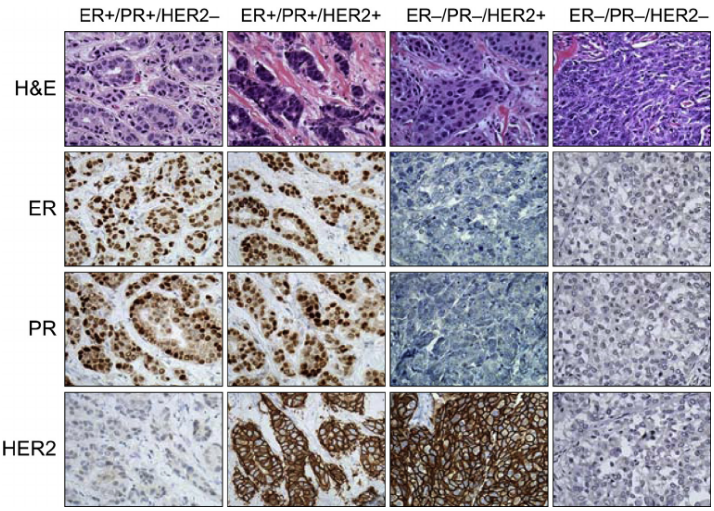

Figure 2. Histopathological Classification of Breast Cancer. Representative histology staining of invasive breast cancer by H&E, ER, PR and HER2. (Serrano 2010, Rivenbark, O'Connor et al. 2013) Reprinted with permission from American Society for Investigative Pathology, Elsevier Inc Copyright © 2013 by https://creativecommons.org/licenses/by-nc-nd/4.0/

Ki67 staining differentiates hormone receptor positive tumors further into Luminal A (Ki67 low) and Luminal B (Ki67 high) subtypes (Cheang, Chia et al. 2009, Goldhirsch, Wood et al. 2011, Soliman and Yussif 2016).

Measuring gene expression levels by mRNA and comparing the expression levels in various panels of genes is known as gene expression profiling. Gene expression profiling divides breast cancer into five intrinsic molecular subtypes similar to the histopathological based groups; Luminal A, Luminal B, HER2 enriched, Basal-like and Normal-like subtypes (Perou, Sorlie et al. 2000, Sorlie, Perou et al. 2001, Sorlie, Tibshirani et al. 2003, Cancer Genome Atlas 2012). There are several prognostic tests based on gene expression profiling, and both the 21-gene expression array (Sparano, Gray et al. 2015) and the 70-gene array Mammaprint (van 't Veer, Dai et al. 2002, Cardoso, van't Veer et al. 2016) can be used to identify patients with good or poor prognosis to aid the physician when deciding whether adjuvant chemotherapy should be given or not.

Given the recent advances in molecular subtyping, the hope is that multiple novel predictive markers will be identified in the years to come. However, still the only biomarkers to guide breast cancer treatment recommended by the ASCO guidelines besides disease stage are the estrogen and progesterone receptor status, HER2 status, and Ki67 staining. Of notice, the use of Ki67 is under debate due to lab-to-lab reproducibility issues and its purely prognostic value (Harris, Ismaila et al. 2016, Harris, Ismaila et al. 2016).

The most frequently mutated genes detected in human breast cancer are *PIK3CA* (27%), followed by *TP53* (23%), representing one oncogene and one tumor suppressor gene (The-Cancer-Genome-Atlas-Network 2012). Furthermore, *CDH1* (11%), *ESR1* (7%), *PTEN* (4%), *RB1* (3%) and *AKT1* (3%) are frequently mutated genes in breast cancer (Cosmic Catalogue of Somatic mutations; www.cancer.sanger.ac.uk)(Forbes, Beare et al. 2017). By combining histopathology with gene expression profiling and mutational analyses of key driver genes, a comprehensive understanding of the mechanisms driving the different subtypes can

be acquired. This provides an opportunity to design better treatment, but still today, there is no clear indication that such advanced analyses add anything with respect to treatment guidance, beyond simple IHC staining and CISH for ER, PGR, HER2 and Ki67.

1.4 The PI3K-Akt-mTOR signaling pathway and therapy resistance

1.4.1 PI3K-Akt-mTOR signaling

The PI3K-Akt-mTOR signaling pathway (Figure 3) is an important growth promoting pathway and regulates several critical functions in a cell, including protein synthesis, survival, migration, proliferation and glucose metabolism (Manning and Toker 2017). Through its crosstalk with the estrogen-, MAPK-, p53- and STAT3- signaling pathways, PI3K-Akt-mTOR signaling (in short: PI3K signaling) deregulation can influence a wide range of cellular processes (Toss, Venturelli et al. 2017).

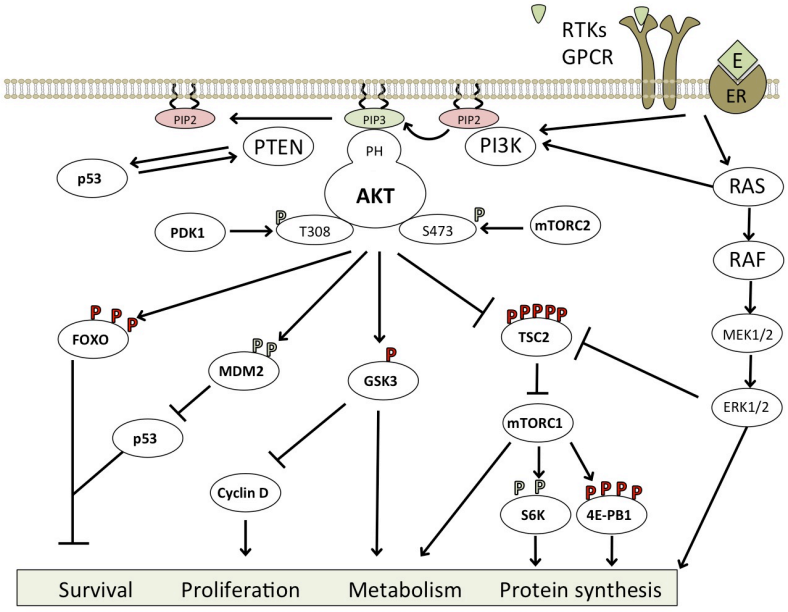

Figure 3. PI3K-Akt-mTOR signaling. P indicates phosphorylation, red color indicates that the phosphorylation has an inhibitory effect, whereas green indicates an activating effect of phosphorylation. ER: estrogen receptor, E: estradiol. Figure modified from (Manning and Toker 2017, Toss, Venturelli et al. 2017)

There are three classes of PI3Ks, Class I PI3Ks are activated by the approximately 20 classes of receptor tyrosine kinases (RTKs) like EGFR- (HER family), insulin-, VEGF- and AXL-receptors, and also by G-coupled receptors. Class I is the most studied PI3K and is also the one referred to in this work. Class I PI3Ks are responsible for the canonical PI3K-Akt-mTOR signaling by converting Phosphatidylinositol 4,5-bisphosphate (PIP2) to Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) on the cell membrane. While class II and III PI3Ks converts phosphatidylinositol (PI) as a substrate to generate PIP3 and is involved in growth-regulatory processes, only class I PI3Ks have a central role in cancer through its downstream signaling (Chalhoub and Baker 2009).

PIP2 and PIP3 are phospholipid components of the cell membrane, and PIP3 interacts with proteins containing a pleckstrin homology (PH) domain, like Akt kinase. Akt localizes to PIP3 on the cell membrane where PDK1 and mTORC2 activate Akt by phosphorylating Akt on the T308 and S473 phosphorylation sites respectively (Carracedo and Pandolfi 2008). PI3K is also activated by direct binding of RAS to the p110α subunit of Class I PI3K, (Shaw and Cantley 2006). Also, there are 21 small GTPase homologs belonging to the Ras or Rho family GTPases which are able to activate PI3K (Yang, Shin et al. 2012) demonstrating crosstalk with MAPK signaling.

Deregulation of PI3K-Akt-mTOR signaling is involved in type II diabetes, and pathway hyperactivation is known to induce tumorigenesis (Cantley 2002, Shaw and Cantley 2006). Sustained proliferative signaling, represented here as persistent activation of PI3K-Akt-mTOR signaling, is a hallmark of cancer (Hanahan and Weinberg 2011) and more than 70% of breast cancers harbor mutations or other alterations in this pathway (Miller, Rexer et al. 2011), in particular among hormone receptor positive breast cancers. The high prevalence of alterations makes the pathway highly relevant as a therapeutic target both in a first line setting and as secondary treatment after progression on first line endocrine treatment.

1.4.2 PI3K-Akt-mTOR signaling and drug resistance

Endocrine resistance can be caused by different molecular aberrations of signaling

pathways that are involved in crosstalk with ER signaling (Austreid, Lonning et al. 2014). Acquired resistance to endocrine therapy may be induced by alterations in the RAS-RAF-MAPK pathway, PI3K-Akt-mTOR pathway or the cyclin D/CDK4/6/Rb (Finn, Aleshin et al. 2016, Gluck 2017). In an effort to combat endocrine resistance, several trials have targeted the PI3K-Akt-mTOR pathway to increase the treatment efficacy, and also to resensitize the tumor to endocrine treatment. In the randomized phase 3 BOLERO-2 trial, the mTOR inhibitor everolimus and the steroidal aromatase inhibitor (AI) exemestane were compared to placebo and exemestane. By adding everolimus to exemestane treatment in patients with advanced ER+ and HER2 negative breast cancer that had progressed on non-steroidal AIs, a 6 months increase in progression-free survival was achieved (Baselga, Campone et al. 2012), demonstrating that inhibiting mTOR can sensitize tumors to treatment or delay the development of therapy resistance. Everolimus has since been implemented in routine clinical use, in combination with exemestane, as second line treatment for patients with ER positive advanced disease that progress on non-steroidal AIs in first line. Multiple clinical trials are now focusing on other compounds targeting the PI3K-Akt-mTOR pathway to further improve endocrine therapy and overcome endocrine resistance (Maurer, Martel et al. 2017).

Activation of RTKs like HER2 and proliferative survival pathways such as PI3K-Akt-mTOR, enable malignant cells to divide rapidly, resist apoptosis and promote survival, and are also implicated in resistance to chemotherapy (Austreid, Lonning et al. 2014, Martin, Smith et al. 2014, Avan, Narayan et al. 2016). Resistance towards chemotherapy has been studied extensively in the past, but the biological mechanisms of resistance are to a large extent still unknown (Austreid, Lonning et al. 2014).

1.4.3 PI3K - *PIK3CA*

The *PIK3CA* gene encodes the catalytic p110α subunit of phosphatidylinositol 3-kinase (PI3K), and oncogenic activating mutations in *PIK3CA* cause constitutive activation of PI3K, causing activation of Akt and downstream signaling molecules to promote cell proliferation and growth (Miller, Rexer et al. 2011). According to

Cosmic (Catalogue of somatic mutations in cancer) by the Wellcome Trust Sanger Institute, mutations in *PIK3CA* encompass the majority of somatic mutations in breast cancer with a frequency of 27%. Activating mutations in *PIK3CA* are present in more than 30% of hormone receptor positive tumors and are predominantly located to "hot spots" in exon 10 and 21, previously annotated to exons 9 and 20 (Samuels, Wang et al. 2004, Baselga 2011). The prevalence of oncogenic *PIK3CA* mutations in breast cancer makes drugs specific to the p110 subunit of PI3K an attractive candidate to improve cancer therapy in these patients. For instance, PI3K inhibition with the p110α specific inhibitor BYL719 sensitizes both cell lines and tumors to fulvestrant (Bosch, Li et al. 2015). Combining endocrine therapy with PI3K targeted treatment is therefore a rational approach, although side effects of PI3K inhibitors is a definite problem clinically (Austreid, Lonning et al. 2014).

1.4.4 Akt

Akt, also known as protein kinase B (PKB/AKT), belongs to a family of serine/threonine-specific protein kinases. Three isoforms of Akt; Akt1, Akt2 and Akt3 have been identified, which are transcribed from separate genes but with approximately 80% sequence homology (Brown and Banerji 2017). All isoforms contain three conserved domains; a lipid-binding PH-domain, a hydrophobic motif, and the central catalytic domain (Brown and Banerji 2017). The different isoforms can activate many of the same substrates, but there are also some subtype-specific substrates. *AKT1* knockout mice display growth retardation and perinatal lethality, *AKT2* knockout mice develop insulin-resistance while *AKT3* knockout mice suffer from decreased brain size (Toker and Marmiroli 2014). *AKT1* is mutated in 3% of breast cancers, whereas *AKT2* mutations are less frequent (Forbes, Beare et al. 2017). *AKT1* and *AKT2* are among the verified "cancer genes" listed by the Cosmic Cancer Gene Census, while *AKT3* is not (Futreal, Coin et al. 2004). Due to *AKT1* having a more important role in breast cancer than the other isoforms, we have focused on the *AKT1* isoform, and when mentioning Akt it is assumed Akt1 unless specified otherwise.

Akt is activated by extracellular stimuli by RTKs or G-coupled receptors through activation of PI3K and its conversion of PIP2 to PIP3. PI3K and the lipid PIP3 product is the rate-limiting step in PI3K signaling, and for Akt phosphorylation (Manning and Toker 2017). When un-phosphorylated Akt re-localize to the plasma membrane, its PH domain binds to PIP3 and Akt is phosphorylated by PDK1 on Thr308 and mTORC2 on Ser473 (See Figure 3, PI3K-Akt-mTOR signaling).

Akt influences more than a hundred downstream targets, including FoxO, MDM2, GSK3 and mTORC1 to regulate survival, proliferation, metabolism, protein synthesis, as well as angiogenesis and invasion (Manning and Toker 2017). Accordingly, Akt is a key signaling hub involved in sustaining proliferative and pro-survival signaling, and it also interacts with several other pathways including the RAS-ERK pathway (Manning and Toker 2017). This makes Akt an attractive target in the development of targeted cancer drugs.

Importantly, constitutive Akt activation disrupts the ability to induce senescence after doxorubicin treatment (Taylor, Lehmann et al. 2011), and constitutive Akt activation applies a selective pressure for the loss of p53 function to avoid oncogene-induced senescence in this setting (Astle, Hannan et al. 2012).

Increased Akt signaling is associated with resistance to chemotherapy, HER2 targeted therapy (trastuzumab) as well as tamoxifen treatment (Clark, West et al. 2002). A meta-analysis covering 3370 papers concludes that Akt signaling is activated by and involved in resistance to DNA damaging treatment such as platinum, taxanes, antimetabolites (including 5-FU) and radiation (Avan, Narayan et al. 2016). In addition, preclinical studies indicate that dominant negative Akt mutations increase doxorubicin sensitivity in breast cancer cell lines. (Clark, West et al. 2002). This implies that inhibiting Akt in cells with an activated PI3K-Akt-mTOR pathway might sensitize cells to treatment when a cytotoxic drug is combined with an Akt inhibitor.

1.4.5 P53

Germline mutations in the *TP53* gene are responsible for the hereditary disorder Li-Fraumeni syndrome characterized by predisposition to a broad spectrum of cancers. 50% of heterozygous carriers will develop cancer before 30 years of age, and at age 70, 90% will have developed a malignancy (Malkin, Li et al. 1990, Srivastava, Zou et al. 1990).

Regarding somatic *TP53* mutations, they are considered to be an early event in carcinogenesis; accordingly *TP53* mutations have also been reported in premalignant ductal carcinoma in situ (DCIS) (Rivlin, Brosh et al. 2011). Genomic rearrangements, amplifications and deletions commonly occur after *TP53* is mutated, due to the loss of DNA damage response mechanism, further destabilizing the genomic integrity (Hanel and Moll 2012, Zhang, Zhuang et al. 2017). Somatic mutations of *TP53* are among the most frequent alterations in human cancers all over, occurring at rates of up to 50% in ovarian cancer, and up to 85% in subtypes of aggressive breast cancers such as the basal-like subtype, and in the advanced disease setting (Olivier, Hollstein et al. 2010, Cancer Genome Atlas 2012).

The p53 protein is considered the most important tumor suppressor in the human genome and has acquired the nickname "guardian of the genome" (Lane 1992). p53 is important to maintain genomic stability and acts as a "master switch" that is rapidly upregulated in response to cellular stress such as DNA damage, hypoxia, shortened telomeres, ribosomal stress, oncogene activation and ROS accumulation. When p53 is upregulated it can induce temporary cell cycle arrest (quiescence), permanent cell cycle arrest (senescence) or activate programmed cell death (apoptosis) if the DNA damage is irreparable (Vousden and Lane 2007). The dose and dynamics of p53 activation controls what happens to the cell after DNA damage has occurred (Meek 2015). Low level stress induces a low levels of p53 upregulation and temporary cell cycle arrest, which promotes cell survival during DNA repair, while high levels of stress or persistent stress induces high levels of p53 and apoptosis (Vousden and Lane 2007). Although it appears to be a dose-response to stress-induced p53 activation,

more is not necessarily better in a therapeutic setting. High doses of DNA damaging drugs in *TP53* wt cells induces very high levels of p53 which mediates cell cycle arrest and mTOR inhibition, causing reversible quiescence and cell survival. In contrast, lower doses of DNA damaging drugs induces less p53 expression which causes cell cycle arrest but without inhibiting mTOR, and the cell becomes senescent or apoptotic (Figure 4) (Leontieva, Gudkov et al. 2010, Olga V. Leontieva 2010).

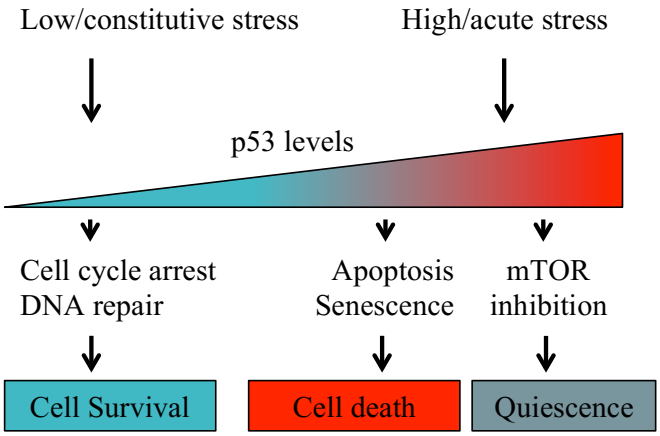

Figure 4. High and low degree stress induce different levels of p53 upregulation, resulting in different cellular outcome. Modified after (Vousden and Lane 2007, Leontieva, Gudkov et al. 2010, Olga V. Leontieva 2010)

The regulation of p53 level is mostly regulated through blocking its degradation. One example of this is how PTEN promotes MDM2 degradation by restricting MDM2 to the cytoplasm and thus protects p53 from ubiquitynilation by MDM2 (Mayo, Dixon et al. 2002). Ubiquitylation of p53 leads to its degradation by the proteasome, and other post-translational mechanisms like acetylation, SUMOylation and phospohorylation which influences its activity, localization and protein-associations demonstrating p53s highly dynamic and context-dependent function (Meek 2015).

1.4.6 P53 as a prognostic or predictive factor in breast cancer

The p53 protein is a transcription factor that binds to the consensus sequence RRRCWWGYYY to activate the transcription of several hundred genes (Fischer 2017). Thus, one would expect that *TP53* mutations would influence cancer progression to a major extent and possibly affect survival outcome and response to therapy. However, despite the prevalence of *TP53* mutations in cancer, there has been conflicting evidence as to the value of *TP53* mutations as a prognostic and predictive factor. This discrepancy might be due to the lack of standardization in methodology and which mutations that are included or excluded in the various studies (Soussi and Beroud 2001). In particular, the use of IHC to detect aberrant p53 proteins in the tumor tissue as a surrogate for *TP53* mutations is prone to methodological errors (Wynford-Thomas 1992, Hall and Lane 1994, Hurlimann, Chaubert et al. 1994, Geisler, Lonning et al. 2001, Ando, Oki et al. 2015).

TP53 has been the focus of the research conducted by the Bergen Breast Cancer Group for several decades. Our group, as well as others (Bergh, Norberg et al. 1995, Andersson, Larsson et al. 2005, Olivier, Langerod et al. 2006, Bonnefoi, Piccart et al. 2011, Eikesdal, Knappskog et al. 2014), have established a role for *TP53* mutations as a negative prognostic factor in breast cancer, in particular *TP53* mutations in the zink-binding domain L2 and L3 which binds to DNA and influences its role as a transcription factor (Bergh, Norberg et al. 1995, Geisler, Lonning et al. 2001, Chrisanthar, Knappskog et al. 2008). The prognostic impact of *TP53* was not only evident in the analysis of short-term survival after neoadjuvant chemotherapy, but also for long-term survival, extending more than 10 years (Eikesdal, Knappskog et al. 2014). In a large, pooled analysis of almost 2000 patients it was reported that mutations causing a truncated/non-functional protein or a complete lack of the p53 protein had the worst prognosis, followed by patients with mutations in the L2/L3 domain (Olivier, Langerod et al. 2006). This was independent of node status, tumor size, and ER status.

Furthermore, the Bergen Breast Cancer Group, as well as others, have established *TP53* as a predictive factor, demonstrating that *TP53* mutations are associated with resistance to anthracyclines (Aas, Borresen et al. 1996, Kandioler-Eckersberger, Ludwig et al. 2000, Geisler, Lonning et al. 2001, Chrisanthar, Knappskog et al. 2008, Lehmann-Che, Andre et al. 2010) and mitomycin-based chemotherapy (Geisler, Borresen-Dale et al. 2003). However, there is some controversy regarding the predictive value of *TP53,* and others have not found any association between a functional p53 and sensitivity to anthracycline or taxane-based therapy (Bonnefoi, Piccart et al. 2011).

While inactivating *TP53* mutations predict resistance to anthracyclines, they may at the same time be predictive of response to high dose epirubicin-cyclophosphamide in ER negative breast cancer (Lehmann-Che, Andre et al. 2010). In patients with ER negative status and *TP53* mutations, 15 out of 28 patients had a pathological complete response (pCR) subsequent to high dose epirubicin-cyclophosphamide, whereas none of the patients harboring breast cancers with wild type *TP53* experienced pCR (Bertheau, Turpin et al. 2007). Still, the number of studies finding a predictive value of *TP53* mutations towards treatment resistance or sensitivity are limited and this issue should be explored further in larger patient cohorts.

Patients with poor survival outcome despite preserved tumor p53 function

Although *TP53* inactivating mutations may predict a lack of response to anthracycline and mitomycin-based chemotherapy in patients with locally advanced breast cancer (Geisler, Lonning et al. 2001, Geisler, Borresen-Dale et al. 2003, Andersson, Larsson et al. 2005, Olivier, Langerod et al. 2006, Eikesdal, Knappskog et al. 2014), some patients with *TP53* wt status still progress on neoadjuvant treatment. Importantly, there are other ways to inactivate p53 apart from inactivating *TP53* mutations. For instance, MDM2 overexpression caused by amplifications of *MDM2* promotes polyubiquitylation and degradation of p53 (Haupt, Maya et al. 1997, Meek 2015, Zhang, Zhuang et al. 2017). Further upstream in the p53 pathway CHEK2 and ATM both influences p53 activity. ATM phosphorylates and activates

p53 in response to genotoxic stress, and *ATM* mutations precludes p53 induction (Rotman and Shiloh 1999). Whereas ATM activates CHEK2, and both ATM and CHEK2 activates p53, a low expression level of *ATM* mRNA will complement *CHK2* and *TP53* mutations in predicting response to treatment (Knappskog, Chrisanthar et al. 2012). Accordingly, among tumors without *TP53* mutations, loss of function in ATM or CHEK2 in the p53 pathway or aberrations in the Rb pathways are strong predictors of reduced sensitivity to DNA damaging drugs and may substitute for loss of p53 function (Knappskog, Chrisanthar et al. 2012, Lonning and Knappskog 2013). This points to the importance of considering pathway activation/inactivation, instead of focusing on single gene dysfunction to evaluate mechanisms of therapy resistance. Also, by combining alterations detected in multiple pathways simultaneously one can predict with higher precision resistance to chemotherapy. Of notice, simultaneous inactivation of both the RB and p53 pathway is a better predictor of resistance to DNA damaging drugs than any single genetic aberrations or pathway alone (Knappskog, Berge et al. 2015).

1.5 PTEN

Phosphatase and tensin homolog (PTEN), is an important tumor suppressor and acts as a negative regulator of PI3K-Akt-mTOR signaling. By acting as a lipid phosphatase, PTEN dephosphorylates the 3-inositol ring of PIP3 to PIP2. This stops activation of Akt by PIP3 and halts downstream Akt signaling, thereby suppressing proliferation, protein synthesis, glucose metabolism, and pro-survival signaling (Figure 3) (Carracedo and Pandolfi 2008, Manning and Toker 2017). The 403 amino acid long PTEN protein has 5 functional domains; The PIP2 binding domain (PDB), the phosphatase domain, C2 domain, carboxy-terminal tail and PDZ binding domain (Song, Salmena et al. 2012). Additionally, a 576 amino acid translational variant of PTEN has been identified which is translated from an alternate start codon, and is designated as PTEN Long. This protein is secreted, and as a membrane-permeable protein it has the ability to enter other cells to inhibit PI3K signaling in a paracrine

fashion (Hopkins, Fine et al. 2013).

Besides dephosphorylating PIP3 to PIP2 at the cell membrane, PTEN has lipid-phosphatase independent activity. PTEN can dephosphorylate Focal Adhesion Kinase, thereby repressing the cell´s ability to survive after detachment from the extracellular matrix (Tamura, Gu et al. 1999). Additionally, nuclear PTEN exerts phosphatase-independent functions by interacting with APC/C to promote the formation of an APC-CDH1 tumor-suppressive complex (Song, Carracedo et al. 2011). Furthermore, PTEN is important in maintaining genomic stability by interacting with centromeres, and lack of PTEN leads to centromere breakage (Shen, Balajee et al. 2007). Also, PTEN protects the cells DNA through Rad51, which is involved in DSB repair (Shen, Balajee et al. 2007). Moreover, PTEN influences cell cycle regulation by influencing multiple cell cycle checkpoints (Gupta, Yang et al. 2009, Brandmaier, Hou et al. 2017). Nuclear PTEN is also involved in ATM regulated DNA repair, making PTEN-proficient cells less sensitive to DNA damage, while cells lacking nuclear PTEN has been shown to be hypersensitive to DNA damage (Bassi, Ho et al. 2013). Based on all these important functions, PTEN has been proposed as "a new guardian of the genome" (Yin and Shen 2008).

1.5.1 PTEN aberrations and loss

Germline mutations of *PTEN* give rise to the PTEN hamartoma tumor syndrome which also includes Cowden syndrome, and manifests itself with the appearance of benign neoplasms and a high risk of developing, breast, endometrium and thyroid cancer (Hollander, Blumenthal et al. 2011). It is estimated that approximately 1 in 200 000 are heterozygous carriers of a germline *PTEN* mutation, and their lifetime risk of breast cancer is estimated to be 67-85% in females, which is close to the risk of breast cancer in patients with germline *BRCA1/2* mutations (Ngeow, Sesock et al. 2017).

PTEN was originally named Mutated in Multiple Advanced Cancers *MMAC1*, and is located at chromosome 10q23-24. While *PTEN* is deleted in more than 90% of glioblastoma (Steck, Pershouse et al. 1997), the Cosmic database of somatic

mutations (Forbes, Beare et al. 2017) reports *PTEN* mutations to be present in endometrium cancer in 38% of cases, while primary breast cancers are mutated in only 3,6% of cases. Although other genes, such as *ESR1*, are more frequently mutated in metastatic breast cancer than in primary breast cancer, the prevalence of *PTEN* mutations are currently not known to be more or less frequent in metastatic breast cancer than in early breast cancer (Lefebvre, Bachelot et al. 2016). However, acquired loss of PTEN is common in metastatic lesions, indicating a possible role for *PTEN* mutations as a metastatic event (Wikman, Lamszus et al. 2012, Juric, Castel et al. 2015).

Although a minority of sporadic breast cancers contain *PTEN* mutations, loss of PTEN protein expression is reported in 34% of breast cancer (Millis, Ikeda et al. 2016). Importantly, the presence or absence of PTEN in cancer is not a dichotomy. Even small changes in *PTEN* levels has an impact on cancer susceptibility, and in *PTEN* hypomorphic mice there is a inverted dose - response relationship regarding tumor incidence where a modest decrease in *PTEN* dose increases the susceptibility to develop cancer, in particular breast cancer (Alimonti, Carracedo et al. 2010). Although lower *PTEN* levels increases growth, germline homozygous loss of *PTEN* is embryonically lethal (Suzuki, de la Pompa et al. 1998). Importantly, complete loss of PTEN might be less oncogenic than a partial loss due to PTEN loss-induced senescence. However, PTEN loss-induced senescence is dependent on functional p53, and by concomitantly inactivating p53 together with complete PTEN loss, the cell can escape senescence and induce tumorigenesis (Chen, Trotman et al. 2005).

1.5.2 Mechanisms of non-genomic PTEN loss

There are several non-genomic mechanisms controlling PTEN expression. The *PTEN* gene can be epigenetically silenced by hypermethylation of the CpG islands in the promoter region, or by histone acetylation rendering gene transcription temporarily inaccessible (Correia, Girio et al. 2014). Also, gene expression levels can be transcriptional regulated by several factors; for instance p53 induces *PTEN* transcription by binding to the *PTEN* promoter region (Stambolic, MacPherson et al.

2001, Pappas, Xu et al. 2017). Post-transcriptional regulation of the *PTEN* mRNA transcript also affects PTEN expression. Various microRNAs, such as miR-21 and the miR17-92 cluster, are known to bind to the 3´UTR region of the *PTEN* transcript, causing it to be degraded and thus halts further translation and protein expression (Correia, Girio et al. 2014).

PTEN plasticity is common, and modifications of the PTEN protein cause transient suppression, or nuclear translocalization. In addition, PTEN protein levels can decrease in cancer tissue compared to normal tissue despite normal RNA levels due to post-translational mechanisms causing loss of PTEN expression (Leslie and Foti 2011, Naguib and Trotman 2013). Also, post-translational modifications may modify PTENs activity, stability, localization and conformation by phosphorylation, acetylation, oxidation, SUMOylation and ubiquitination, and defects in any of these mechanisms has the ability to influence the cells PTEN status and its interaction with other proteins (Hopkins, Hodakoski et al. 2014, Collaud, Tischler et al. 2015).

1.5.3 PTEN as a prognostic or predictive marker in breast cancer

There is conflicting evidence as to the value of PTEN as a useful prognostic marker in breast cancer. *In vitro* experiments show that suppressing PTEN function elevates activated Akt and increases drug resistance towards certain chemotherapeutics, and also sensitizes cancer cells to mTOR inhibition (Grunwald, DeGraffenried et al. 2002, Steelman, Navolanic et al. 2008). Clinically, loss of PTEN protein expression in metastatic breast cancer was associated with inferior survival compared to patients with PTEN positive tumors when treated with cyclophosphamide/methotrexate/5-FU or cyclophosphamide/doxorubicin/5-fluoracil (Wang, Hao et al. 2017). In another trial in patients with early breast cancer, lack of PTEN IHC staining was associated with higher tumor grade and earlier recurrence, but no difference in survival (Bose, Chandran et al. 2006). In yet another trial, PTEN IHC staining level was not associated with any survival impact among patients with early breast cancer (Panigrahi, Pinder et al. 2004).

While PTEN loss is associated with worse prognosis and higher tumor grade, PTEN

loss is not commonly found to be predictive of response to breast cancer treatment. In several clinical trials, PTEN loss was not predictive of response to breast cancer treatment in early breast cancer (Panigrahi, Pinder et al. 2004, Saal, Johansson et al. 2007, Lazaridis, Lambaki et al. 2014). In contrast, in HER2 positive breast cancer, PTEN loss predicted lack of response to trastuzumab-based therapy (Nagata, Lan et al. 2004, Esteva, Guo et al. 2010, Rimawi, De Angelis et al. 2017), whereas another trial found no prognostic or predictive value of PTEN loss with respect to the benefit of HER2 targeted treatment (Perez, Dueck et al. 2013).

PTEN loss may be predictive of response to specific targeted inhibitors involving PI3K-Akt-mTOR signaling. In a panel of human cancers, loss of PTEN or *PIK3CA* mutations predicted response to the mTOR inhibitor everolimus (in the absence of concomitant KRAS/BRAF mutations) (Di Nicolantonio, Arena et al. 2010). In an elegant example of how PTEN loss leads to therapy resistance Juric *et.al.* treated a patient with a *PIK3CA* mutated metastatic breast cancer with BYL719, a PI3Kα inhibitor. Initially the malignant disease responded well to treatment, but eventually resistant metastases developed. All metastases had acquired *ESR1* and *BRCA2* mutations and a single copy loss of *PTEN*, but in addition the metastases showed a parallel evolution of complete PTEN loss, and the metastases presented six different types of *PTEN* deletions on the remaining allele. Metastases with complete PTEN loss were not sensitive to PI3Kα inhibition, while the metastases showing PTEN expression were sensitive (Juric, Castel et al. 2015).

It is clear that the utility of PTEN expression as a biomarker warrants further investigations, particularly in the future testing of drugs targeting the PI3K-Akt-mTOR pathway.

1.6 Noncoding RNA

After the completion of the human genome project (McPherson, Marra et al. 2001) it became evident that the numbers of protein-coding genes are far less then expected. It is estimated that up to 90% of the genome is transcribed (Djebali, Davis et al. 2012), but still less than 2% of the genome encodes the ~22 000 protein-coding mRNAs (Collins, Lander et al. 2004). "Noncoding RNA" is mRNA that is transcribed from the DNA, but not expressed as proteins, and it was therefore assumed that they had no function. Noncoding RNA was thought of as evolutionary leftovers and previously labeled as "junk RNA" (Palazzo and Lee 2015). The majority of genomic research effort thus far has focused on the protein-coding part, and much less is known regarding the function of non-coding genes. Thus, the function of these non-coding genes represents a huge gap in our knowledge, and there is much left to discover.

Although not protein-coding, it has become clear that non-coding RNAs have many important functions. The functional importance of non-coding RNAs can be demonstrated by *Xist* RNA. *Xist* initiates the chromosomal X inactivation in females after fertilization, to avoid overexpression of X-chromosome genes. Females have two X-chromosomes and males only one, and to avoid overexpression of X-chromosome genes in females, one of the chromosomes is silenced. *Xist* is transcribed from the inactive X-chromosome and coats its chromatin, repressing expression of the entire chromosome (Wutz, Rasmussen et al. 2002, Lee and Bartolomei 2013).

Non-coding RNA is grouped in different classes according to size and function, such as microRNAs (~22 nt), small RNAs (<200nt), long RNAs or lncRNAs (>200nt), small interfering RNAs (siRNA), pseudogenes, circular RNAs and piRNAs to name a few (Palazzo and Lee 2015). They are involved in multiple genomic processes such as splicing, regulation of gene expression through antisense base pairing, guiding DNA synthesis, and in regulating methylation (Cech and Steitz 2014). LncRNA can also function as "address codes", by directing protein complexes, genes and chromosomes to specific localizations (Batista and Chang 2013). A more thorough

description of different mechanisms of non-coding RNA functions can be found in a review by Asgrand *et.al* (Angrand, Vennin et al. 2015)

1.6.1 Non-coding RNAs and their role in cancer

While there are around 20 000 protein-coding mRNAs, there are approximately 8 000 pseudogenes, around 60 000 lncRNAs (Iyer, Niknafs et al. 2015), 200 000 circular RNAs (Liu, Li et al. 2016) and almost 2 000 human microRNAs that have been reported (mirbase.org). LncRNAs are not as highly expressed as protein-coding mRNAs, but they are more tissue-specific, indicating highly specialized functions (Gloss and Dinger 2016). It is estimated that in the human genome there are at least 19 000 functional lncRNAs (Hon, Ramilowski et al. 2017), many of which are deregulated in cancer. Deregulation of ncRNA networks is common in cancer, and there has been identified ncRNAs acting as both tumor suppressors and oncogenic drivers in the majority of cancer types (Anastasiadou, Jacob et al. 2017). Overexpression of lncRNAs like *HOTAIR* (Gupta, Shah et al. 2010) and *MALAT1* (Tian and Xu 2015) have a negative prognostic impact in cancer. The non-coding RNA *BRCAT* is overexpressed in ER positive breast cancer, and is implicated in tamoxifen resistance in breast cancer cell lines (Iyer, Niknafs et al. 2015). Since the spectrum of non-coding RNAs may vary between different types of cancer and each non-coding RNA may function differently depending on the biological process which is studied, it is indeed a huge challenge to decipher if and what role each non-coding RNA has in any particular cancer (Fu 2014). However, the field of non-coding RNAs is rapidly growing and will shed further light on to the regulatory mechanisms governing cancer, which could lead to the discovery of novel non-coding RNA-based therapies (Fang and Fullwood 2016).

1.6.2 MicroRNA

MicroRNAs are evolutionary conserved short nucleotides approximately 22 nucleotides long single-stranded RNA. They can influence protein expression by binding to complementary regions of mRNA transcripts on sites known as "MicroRNA response Elements" (MRE), resulting in translational repression or

mRNA decay (Macfarlane and Murphy 2010). Around half of mammalian microRNA genes are located within introns of non-coding or protein-coding genes, and some overlap exons of non-coding RNAs. Their expression may be transcriptionally linked to these genes, while the other microRNAs have dedicated gene loci (Rodriguez, Griffiths-Jones et al. 2004). MicroRNAs are transcribed by RNA polymerase II as primary miRNAs (pri-miRNAs), which are cleaved and processed by Microprocessor (consisting of i.a. DROSHA and DGCR8) into pre-miRNAs. Pre-miRNAs can then be exported from the nucleus for further processing by DICER1, which results in mature miRNAs. The guide strand gets incorporated into the microRNA-induced silencing complex (RISC) which consists of i.a. Argonaute and DICER1, and the miRISC complex binds to target mRNAs based on various degrees of sequence complementarities (Lin and Gregory 2015). MicroRNA deregulation in cancer is common, and low expression of the miRNA processing genes *DROSHA* and *DICER1* are correlated to poor clinical outcome in ovarian cancer (Merritt, Lin et al. 2008). The expression of microRNAs are generally lower in tumor tissue than in normal tissue (Lu, Getz et al. 2005). However, the effect of specific microRNAs are highly context-dependent, and a microRNA can act as a tumor suppressor in one cancer type, while it promotes tumor progression in another (Svoronos, Engelman et al. 2016). Global microRNA expression differs between different cancer types (Lu, Getz et al. 2005, Rosenfeld, Aharonov et al. 2008) and the pattern of global microRNA expression can also be used to classify breast cancer intrinsic subtypes (Kurozumi, Yamaguchi et al. 2017). MicroRNAs that are associated with or induce cancer are considered to be "oncomiRs". Overexpression of microRNAs targeting tumor suppressors (such as miR-21a and miR-20 targeting *PTEN*) act as oncomiRs, while loss of expression of microRNAs targeting oncogenes will also promote cancer progression (Kurozumi, Yamaguchi et al. 2017). However, it is complicated to determine if a specific microRNA generally acts a tumor suppressor or an oncogene since several microRNAs have been reported as both (Svoronos, Engelman et al. 2016). The specificity of microRNA expression will induce different downstream effects and can act as a oncogene in one system, while it acts as a tumor suppressor in another (Svoronos, Engelman et al. 2016).

1.6.3 Pseudogenes and the ceRNA hypothesis

Pseudogenes are non-coding RNAs that show sequence similarities of more than 75% to a protein-coding gene. Due to a lack of functional start-codons, frame shifts or gain of internal stop-codons, pseudogenes produce no protein when transcribed, and they are considered as evolutionary remnants of their parental functional genes (Harrison and Gerstein 2002). Pseudogenes can evolve in two ways. First, through DNA duplication of the DNA sequence of a gene where modifications like mutations, deletions, insertions or frame shift renders the transcript non-coding. This is the duplicated or non-processed pseudogenes. Second, pseudogenes can evolve through retrotransposition, where a transcribed and spliced mRNA is reintegrated in the genome including its poly-AAA tail, but without a proper regulatory promoter region. This is the processed pseudogenes (Harrison and Gerstein 2002). The sequence similarities and the lower transcription levels than their parental genes make pseudogenes very hard to study, but massive parallel sequencing has revealed that more than 7000 pseudogenes are expressed in humans (Iyer, Niknafs et al. 2015). An indication of whether a gene is functional or not is their degree of phylogenetic preservation, and in many cases pseudogenes are preserved across species. Around 80% of the processed pseudogenes are specific for primates, pointing to them being a recent event in evolution. One functional role for pseudogenes described so far is regulation of gene expression of its parental gene by acting as an anti-sense transcript which blocks translation (Muro, Mah et al. 2011, Groen, Capraro et al. 2014).

In 2010, Poliseno *et.al*. published the pivotal paper "A coding-independent function of gene and pseudogene mRNAs regulate tumour biology." where they described a new mechanism for gene regulation, by which mRNAs which share binding sites for specific microRNAs or MicroRNA Response Elements (MREs) compete for the same pool of microRNAs (Poliseno, Salmena et al. 2010). By overexpressing the MREs containing the 3`UTR end of the *PTEN* pseudogene, entitled *PTENP1,* or overexpressing the *KRAS* pseudogene *KRAS1P*, they found increased *PTEN* and *KRAS* mRNA levels respectively in prostate cancer. This means that both the non-

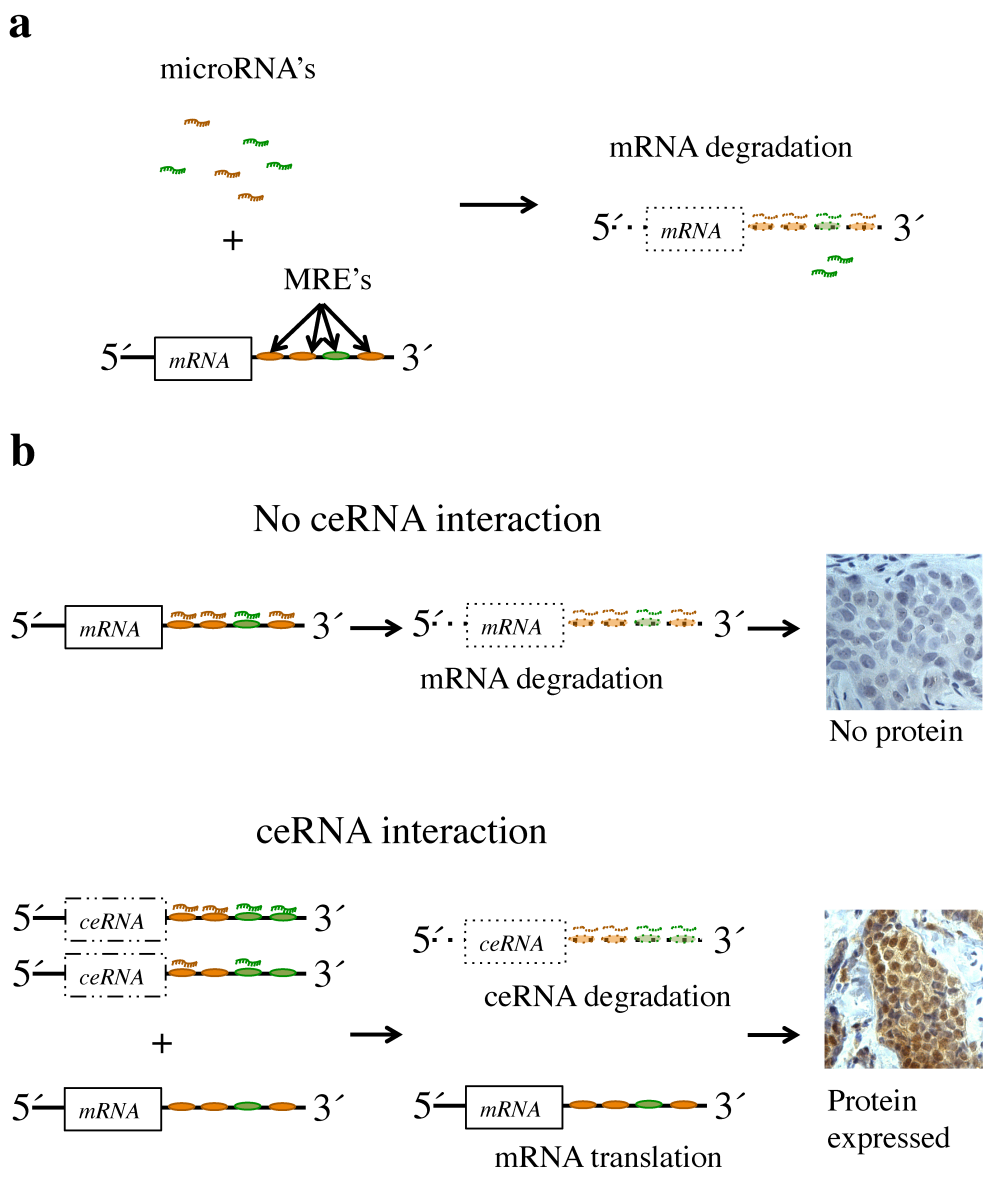

Figure 5. a) MicroRNAs bind to MicroRNA Response Elements (MREs) on the 3'UTR part of mRNAs causing mRNA translational repression and degradation. b) ceRNAs harboring shared MRE competes for microRNAs, which promote mRNA translation and protein expression.

coding pseudogenes and the protein-coding *PTEN*/*KRAS* mRNA transcripts exerted biological functions through competing endogenous RNA (ceRNA) networks. One such network is the *PTEN* ceRNA network where *PTEN* mRNA transcripts regulate the expression of seemingly unrelated genes through ceRNA interactions, including the mRNAs of *VAPA*, *CNOTL*, *RB1*, *RUNX1*, *PDGFRA* and *STAT3*. (Salmena, Poliseno et al. 2011, Tay, Rinn et al. 2014). This implicates that ceRNA networks can influence cancer genes by regulating tumor suppressors or oncogenes in *trans* (Figure 5) (Poliseno and Pandolfi 2015).

The extent at which a specific transcript will be affected by ceRNA interactions are highly context-dependent, where the level of mRNAs vs. microRNAs determine the outcome (Bosson, Zamudio et al. 2014). Also, a specific ceRNA will exert a different outcome depending on the microRNA and mRNA target pool available in the specific cell (Figure 6). The potential for downregulation by a microRNA is a function of microRNA and target-mRNA abundance. Also, microRNAs with multiple target mRNAs downregulate any particular mRNA less than a microRNA with few target mRNAs (Arvey, Larsson et al. 2010, Bosson, Zamudio et al. 2014)

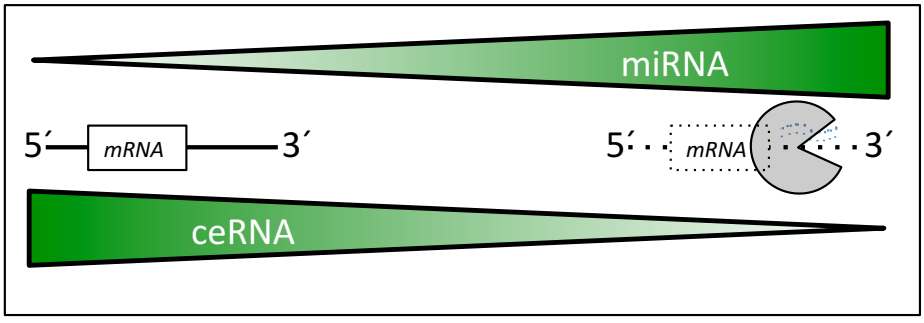

Figure 6. The relative abundance of microRNA and the number of MREs at each mRNA influence the potential for ceRNA interaction. High microRNA and low ceRNA concentration promote mRNA translational repression and degradation, while low microRNA and high ceRNA expression promotes protein translation.

The concept of ceRNAs have been disputed (Thomson and Dinger 2016), and some have argued that for ceRNA interactions to occur, there must be similar abundance of the target mRNA and microRNA (Denzler, Agarwal et al. 2014). Also, the microRNAs need to be expressed in high quantity, and experimentally *in vivo* overexpression of a particular ceRNA did not influence ceRNA targets or mediate downstream physiological effects in the liver (Denzler, Agarwal et al. 2014). For ceRNA interactions to occur *in vivo*, the ceRNA must be highly expressed or contain dozens of binding sites for any particular microRNA. However, microRNA and lncRNA expression levels are highly deregulated in cancer, which could make malignant cells more receptive to ceRNA interactions. Also, by computational identification of ceRNA-ceRNA networks in normal and breast invasive carcinoma, it was revealed that the "sponge activity" of lcnRNAs is disrupted in carcinoma compared to normal tissue due to altered microRNA:mRNA ratios (Paci, Colombo et al. 2014, Conte, Fiscon et al. 2017). This indicates that this biological mechanism may be important for cancer development and progression.

1.6.4 PTENP1

While the *PTEN* gene is located on chromosome 10q23, the pseudogene *PTENP1* is located on chromosome 9p21.3, and 1.2kb of its 3.9kb long genomic sequence has a 98% sequence homology to the coding region of *PTEN* (Whang, Wu et al. 1998, Juehui Liu 1999). *PTENP1* is a processed pseudogene which is transcribed in all normal tissues, but it lacks a start codon and thus far no PTENP1 protein has been identified (Juehui Liu 1999, Karro, Yan et al. 2007). Variants of *PTENP1* are present in several species, but only in a minority of mammals, 48 out of 65 mammals lack the pseudogene (Tang, Ning et al. 2016). Species that contain variants of *PTENP1* include some primates, a few rodents (not mice), pigs, but not goat, sheep or bovines, and no carnivores. This demonstrates that *PTENP1* have originated through several gene duplications and have a "birth and death" evolution pattern (Nei and Rooney 2005). Intriguingly, the naked mole rat that is known for its resistance to developing cancer has 17 copies of *PTENP1*. ceRNA crosstalk between *PTEN* and *PTENP1*was demonstrated by Poliseno *et.al* through the shared MREs of miR-20a, 19b, 21, 26a

and 214 (Figure 7). Across different species there are conserved binding sites for miR-19b, 26a and 20 at the 3´UTR part of *PTENP1*, indicating that ceRNA crosstalk influencing PTEN expression also exists in other species (Tang, Ning et al. 2016).

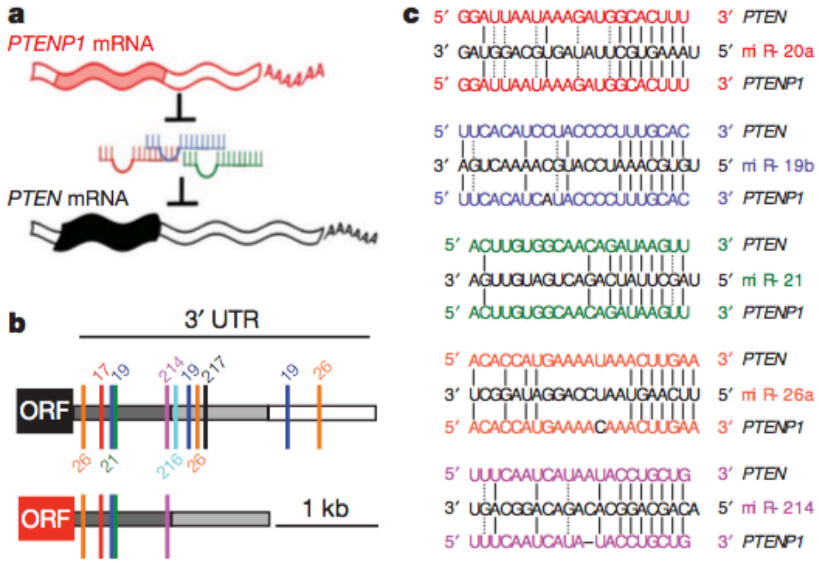

Figure 7. Shared binding sites/MREs on PTEN and PTENP1 mRNA transcript. (Poliseno, Salmena et al. 2010) Reprinted with permission from Nature Publishing group. (Poliseno, 2010) © 2010.

PTENP1 can also be transcribed in antisense as isoform α and β. The β isoform pairs with the *PTENP1* transcript to form RNA:RNA complexes, thereby affecting its stability and ceRNA function (Johnsson, Ackley et al. 2013). The *PTENP1* antisense α transcript can pair with the promoter-associated RNA *PTEN* 5′ UTR-containing transcripts, and guide DNMT3a to the *PTEN* promoter which epigenetically regulate PTEN transcription (Lister, Shevchenko et al. 2017). Both sequence and secondary structure of noncoding RNA and its target gene can influence regulation by the noncoding RNA (Lister, Shevchenko et al. 2017). This adds yet another layer of complexity to the function of *PTENP1*.

Discovery of *PTENP1* and analytical pitfalls

Historically, Whang et.al (Whang, Wu et al. 1998) examined the frequency of *PTEN*

mutations in prostate cancer by RT-PCR and sequencing of a panel of cell lines. Surprisingly, in all cell lines tested, including the *PTEN* null PC3 cell line, a RT-PCR product with the correct size for *PTEN* appeared. A more thorough examination revealed that this product was not *PTEN*, but rather genomic *PTENP1*. Also, 10 out of 11 sequence variants previously reported as *PTEN* mutations were in fact base substitutions present in the pseudogene DNA sequence. This stresses the need for both DNAse treatment before cDNA synthesis, and a strict selection of primers to avoid detecting the pseudogene when reporting the mutational status of the *PTEN* gene (Whang, Wu et al. 1998).

There has also been some controversy regarding *PTEN* promoter hypermethylation, given the sequence homology between *PTEN* and *PTENP1*. This became evident in our group in earlier work on *PTEN* methylation (Vidar Staalesen and Ranjan Chrisanthar, unpublished data). While assessing methylation profiles of the *PTEN* promoter in breast cancer patients, there was conflicting evidence as to its methylation status. *PTENP1* share 91% sequence identity with a 921 bp CpG island in the *PTEN* promoter, and it became evident that the assay used to assess *PTEN* promoter methylation was undistinguishable from the *PTENP1* promoter. By using an assay able to distinguish between the two they found the *PTENP1* promoter to be methylated rather than the *PTEN* promoter in several tumor biopsies, prompting the question of *PTENP1´*s biological relevance in breast cancer. Previously, studies have been published wherein *PTENP1* promoter methylation might be mistaken for *PTEN* methylation (Salvesen, MacDonald et al. 2001, Zysman, Chapman et al. 2002), rendering the clinical relevance of *PTEN* promoter methylation status unclear (Hesson, Packham et al. 2012). After a reexamination, Hesson *et.al*. found the *PTENP1* promoter and not the *PTEN* promoter to be frequently methylated in cancer cell lines. However, the relevance of *PTENP1* promoter methylations remains to be answered. Of notice, due to sequence similarities it is recommended to use allelic bisulphite sequencing to correctly identify methylation status of the gene/pseudogene in regions with a high degree of homology (Hesson, Packham et al. 2012).

2. Aims of Study

Loss of PTEN protein expression is frequently observed in human breast cancer, but the mechanisms governing PTEN expression remains to be elucidated. In particular the role of pseudogene *PTENP1* to regulate PTEN expression in *breast cancer* has not been addressed previously. Furthermore, the role of PTEN and *PTENP1* to regulate PI3K-Akt-mTOR signaling and its importance to chemoresistance in breast cancer is unknown. The overall aim of this thesis was to examine the role of *PTENP1* and *PTEN* gene expression, PTEN protein expression and PI3K-Akt-mTOR signaling towards treatment response and survival outcome in patients with locally advanced breast cancer. Further, we wanted to examine the role of PI3K-Akt-mTOR signaling in anthracyline-based chemotherapy resistance, and evaluate if specific inhibitors targeting the PI3K-Akt-mTOR pathway could be used therapeutically to potentiate treatment or counteract chemoresistance. Finally, we wanted to assess the biological impact of *PTENP1* by up- and downregulating its expression in preclinical models of breast cancer.

2.1 Paper 1

It has been established previously by our research group as well as by others, that patients with locally advanced breast cancers harboring *TP53* mutations are at increased risk of resistance to anthracyclines and mitomycin-based chemotherapy and have a worse survival outcome. However, not all patients with *TP53* mutated tumors progress on treatment, and not all patients experience breast cancer recurrence, despite *TP53* tumor mutations.

On the protein level, the tumor suppressor p53 promotes the expression of the PTEN tumor suppressor (Stambolic, MacPherson et al. 2001). In addition, PTEN protects p53 from degradation (Mayo, Dixon et al. 2002). Furthermore, PTEN is an important tumor suppressor and inhibitor of the growth-promoting PIK3-Akt-mTOR pathway.

Loss of PTEN expression is frequently observed in human breast cancer (Millis, Ikeda et al. 2016) and the negative prognostic impact of PTEN loss has been demonstrated (Wang, Hao et al. 2017). However, staining for PTEN expression by IHC is prone to interobserver variation, and alternative methods to assess PTEN expression objectively, such as *PTEN* gene expression by qPCR, could be a better method. Further, the expression of *PTENP1* and its role in treatment response and survival in human breast cancer has not been established previously.

The aims of this study were;

1. To determine the prognostic and predictive value of *PTEN* gene expression in patients with locally advanced breast cancer treated with chemotherapy.
2. To assess how *TP53* mutation status influences the prognostic and predictive impact of *PTEN* gene expression.
3. To examine protein expression of PTEN and PI3K-Akt-mTOR signaling by IHC and correlate the findings to treatment outcome.
4. To examine the correlation between PTEN protein and *PTEN* mRNA expression in breast cancer.
5. To examine the expression of *PTENP1* in breast cancer, and correlate the expression levels to *PTEN* expression level, as well as treatment outcome and survival.

2.2 Paper 2

Upregulated PI3K-Akt-mTOR signaling is observed more frequently in hormone receptor positive breast cancers, and inhibition of this pathway augments the response to endocrine therapy clinically (Baselga, Campone et al. 2012). However, upregulated PI3K-Akt-mTOR signaling is also observed when resistance to chemotherapy develops (Avan, Narayan et al. 2016). Accordingly, in this preclinical study we wanted to examine how PI3K-Akt-mTOR signaling is affected by short-term and long-term anthracycline treatment in ER positive and negative human breast cancer.

Then we wanted to assess the potential of PI3K-Akt-mTOR inhibitors to counteract chemoresistance. In particular, we wanted to examine the response to acute doxorubicin exposure in doxorubicin-naïve and doxorubicin-resistant cell lines. Furthermore, we wanted to examine whether an Akt inhibitor could be used to augment the response to doxorubicin.

The following aims were defined:

1. Create in-house doxorubicin-resistant breast cancer cell lines
2. Explore how PI3K-Akt-mTOR signaling is influenced by an acute exposure to doxorubicin in doxorubicin-naïve and resistant cell lines.
3. Design and perform a preclinical trial using agents targeting PI3K-Akt-mTOR signaling alone and combined with doxorubicin. This is in order to examine if inhibition of PI3K-Akt-mTOR signaling is an effective therapy by it self, and also if it can be utilized to augment the response to chemotherapy.
4. Compare PI3K-Akt-mTOR signaling in biopsies taken before and after neoadjuvant anthracycline treatment in patients with locally advanced breast cancer and examine if differences in mRNA levels is related to treatment outcome in two patient subgroups; 1-Short term response; biopsies collected before and 24 hours after the first anthracycline exposure. 2-Long term response; biopsies collected before and after 16 weeks of doxorubicin treatment.

2.3 Paper 3

Previously *PTENP1* was shown to adsorb miRNAs targeting *PTEN* for degradation, thereby increasing *PTEN* mRNA and protein expression in prostate cancer cell lines (Poliseno, Salmena et al. 2010). However, the role of *PTENP1* in breast cancer has not been defined.

In this paper we wanted to examine whether *PTENP1* exhibits a functional role in

breast cancer. Further, we wanted to assess whether overexpression of *PTENP1* in breast cancer affects PTEN and downstream AKT-mTOR signaling in breast cancer *in vivo* and *in vitro*. Also, we wanted to examine whether *PTENP1* up-or downregulation influences doxorubicin sensitivity.

To address these questions the following aims were defined:

1. Create a lentiviral vector containing *PTENP1* to make breast cancer cell lines stably overexpress *PTENP1* in order to examine the ceRNA effect of *PTENP1* overexpression on PTEN, PI3K-Akt-mTOR signaling and proliferation both *in vitro* and *in vivo*.
2. Evaluate whether *PTENP1* overexpression affects the expression levels of known microRNAs which are shared between *PTEN* and *PTENP1*.
3. Examine the effect of *PTENP1* downregulation by siRNA on PI3K-Akt-mTOR signaling
4. Examine if *PTENP1* overexpression or downregulation by siRNA affects doxorubicin sensitivity

3. Methodological considerations

3.1 Patient material

The Bergen Breast Cancer Group has systematically collected tumor biopsies from patients with locally advanced breast cancer included in neoadjuvant clinical studies since the early 1990´s. The tumor biopsies were taken directly from open surgery or in the outpatient clinic, snap-frozen in nitrogen, and subsequently stored at N_2 until DNA/RNA extraction, or embedded in paraffin for IHC analyses. High quality samples combined with long-term follow up data for all patients included in the trials makes this biobank a valuable resource for breast cancer research. In addition, we have close to 100% follow-up regarding patient data and survival outcome, which is unique compared to many international studies where follow-up is hampered since patients commonly switch hospitals, as well as regulations causing loss of insight into patient data for the researcher. Another advantage with our studies is the fact that the clinical trials have been conducted in the same defined patient population, providing homogenous data throughout the last three decades with respect to patient and tumor characteristics, which makes it easier to compare results between patient cohorts using our own historical controls. Importantly though, neoadjuvant treatment has changed extensively during these 27 years.

One disadvantage with these studies is the fact that the treatment is not compared to a control arm with standard treatment of care. This is due to the relatively small number of patients with locally advanced breast cancer diagnosed each year in Norway, making it very difficult to conduct such a randomized trial within a reasonable time frame. Even so, the purpose of this thesis was not to compare the efficacy of the different treatment regimens, but rather to identify mechanisms contributing to therapy resistance. Another limitation in our trials is the amount of tumor tissue available from biopsies. In many cases we only have access to True-Cut/needle biopsies. This makes it difficult to obtain enough sample material for all

the parameters one might want to analyze, and information from DNA and RNA analyses have been prioritized, whereas available tissue for proteinanalysis was in many cases limited.

The Doxorubicin cohort consists of patients with locally advanced breast cancer, recruited to a clinical trial running from 1991-1997 to evaluate the efficacy of low-dose doxorubicin 14 mg/m^2 body surface as first line monotherapy, administrated weekly for 16 weeks (Aas, Borresen et al. 1996, Geisler, Lonning et al. 2001, Eikesdal, Knappskog et al. 2014). Tumor biopsies were collected before starting therapy, and after 16 weeks of therapy. The Doxorubicin trial was a single-armed phase 2 trial including patients at one cancer center, Haukeland University Hospital.

The FUMI cohort consists of patients with locally advanced breast cancer, enrolled in a clinical trial running from 1993 to 2001 to evaluate a combined 5-fluorouracil (5-FU) and mitomycin treatment regimen (Geisler, Borresen-Dale et al. 2003). Primary treatment consisted of 5-FU 1000mg/m^2 on day 1 and 2, and mitomycin C 6mg/m^2 administered on day 2 in a three-week interval for four cycles. The FUMI trial was a single-armed phase 2 trial including patients at one cancer center, Haukeland University Hospital.

The Epi Tax cohort consists of patients with locally advanced breast cancer enrolled in a clinical trial running from 1997 to 2003 to identify biomarkers of drug resistance towards epirubicin or taxanes. Patients were randomized to one of two treatment arms and given either epirubicin 90 mg/m^2 or paclitaxel 200 mg/m^2 every three weeks (Chrisanthar, Knappskog et al. 2008, Chrisanthar, Knappskog et al. 2011). If the disease progressed on the first treatment, the patients were switched to the opposite treatment regimen. The EpiTax trial was a national phase 2 trial, including patients from all health regions of Norway.

The **Dose Dense cohort** consists of patients with locally advanced breast cancer, enrolled in a clinical trial running from 2007 to 2016, where patients received dose dense epirubicin 60 mg/m^2 for four cycles followed by docetaxel 100 mg/m^2 for four cycles, administered every two weeks. The Dose Dense trial was a single-armed

phase 2 trial including patients at one cancer center, Haukeland University Hospital. Tumor biopsies were collected before and after starting epirubicin and docetaxel treatment, and at surgery. The results from the trial have not been published thus far.

3.1.1 Statistics

To perform survival analysis we used date at diagnosis as the start point and last follow-up date as the end point. An "event" was defined as the time of recurrence of disease (to calculate recurrence-free survival; RFS), death due to breast cancer (to calculate disease-specific survival; DSS) or death regardless of cause (to calculate overall survival; OS) if exact cause of death was not available. Censoring was used when a patient was lost to follow-up and outcome was unknown, or when a patient was withdrawn from the study. The last registered patient contact was used as the censoring data point. Kaplan Meyer plots were calculated in order to visualize survival for patient groups with specific characteristics i.e. patients with high *PTEN* expression versus low *PTEN* expression. These plots display survival probability, i.e. the cumulative probability of an individual to not have experienced the "event" up to a specific time point.

To compare survival or "time to event" between groups, we have used both the log-rank test (Kaplan and Meier 1958) and Cox regression (Cox 1972). Both methods produce similar results, but with small differences. Log-rank tests the null hypothesis that there is no difference in survival times between groups at all times in the Kaplan Meyer plot based on a Chi square statistics. Cox regression describes the effect of a variable on survival by comparing the hazard ratio between the two groups, where the hazard is the risk of experiencing the event within a specific time point. Unlike log-rank, Cox regression also allows for adjustment for multiple variables.

One element for discussion is the fact that we transformed a continuous variable (gene expression) into a categorical variable (*PTEN* high or low), where the choice of cut-off was the median gene expression in each cohort. This transformation of the data was performed to make our comparisons between groups more robust, and to avoid overinterpretation of differences between patient subgroups. By selecting a

different cut-off value calculating hazard ratios using a continuous variable, the results could be different. Particularly, *PTENP1* gene expression was much lower than *PTEN* expression, and by comparing high/low gene expression based on the median expression instead of for instance the top 10% versus the low/non-expressing tumors, one might miss potential correlations between *PTENP1* expression and survival.

3.2 *In vitro* and *in vivo* models of disease

It is important to realize the potential and limitations of our model systems, and the statistician George E. P. Box stated it eloquently as; "All models are wrong, but some are useful" (Box and Norman 1987). Breast cancer cell lines are fast growing and there is easy access to a wide variety of well characterized cell lines to be purchased, making them highly accessible. Also, a large number of experiments can be performed within a short time frame. Importantly, cell lines derived and expanded from human tumors are subjected to a huge selective pressure, and the fastest growing cells *in vitro* may not represent the cells with the greatest malignant potential in their native environment. Over time as cell lines are passaged, they display genetic drift and changes in phenotype. Molecular portraits of breast cancer cell lines and breast tumors reveal that the expression levels of proteins in cell lines do not necessarily represent the protein expression in breast tumors (Cifani, Kirik et al. 2015). They may also become cross-contaminated by other cell lines, and thereby produce misleading results (Liscovitch and Ravid 2007, Capes-Davis, Theodosopoulos et al. 2010, Torsvik, Rosland et al. 2010, Weifeng Ke 2011). Because of the risk of misidentified and cross-contaminated cell lines resistant to anthracyclines we created our own doxorubicin-resistant cell lines (Liscovitch and Ravid 2007, Nardone 2008, Capes-Davis, Theodosopoulos et al. 2010).

When growing tumor cells *in vitro* you have a system with a low level of complexity, enabling you to focus on a single cell type in an artificial but controllable

environment. This is very useful when you want to examine for instance the effect of up- and downregulation of specific genes on growth or sensitivity to treatment. By growing tumor cells *in vivo*, you add several layers of complexity to the system, where the tumor cells grow in between the host´s vascular system, immune cells, extracellular matrix, fibroblasts and adipocytes. The tumor and host cells can interact through cell-cell contact and through cytokines, growth factors, hormones, matrix metalloproteinases and other secreted proteins (Ungefroren, Sebens et al. 2011, Balkwill, Capasso et al. 2012). All of this can influence the growth and survival of the tumor cells, and can cause discrepancy between *in vitro* and *in vivo* experiments. Also, therapeutic effects on cell growth or drug sensitivity can be profoundly influenced when the experiment is transferred from an *in vitro* setting to an *in vivo* setting.

Before any drug can be utilized in patients, it has to be tested preclinically in animals to assess safety and potential effects. Mice have a short life span, are easy to breed, handle and house. By using mice one can test and screen multiple drugs within a relatively short time frame and this is particularly useful for early stage drug screening to evaluate antitumor effects in a more biologically relevant manner, as compared to *in vitro* cell culture experiments (Ruggeri, Camp et al. 2014). When *in vivo* xenograft models are used, wherein cancer cells are injected to initiate tumors, one lacks the intratumoral heterogeneity of spontaneously arising malignancies. Another alternative is to utilize Patient Derived Xenografts (PDX) where tissue pieces from the patient are implanted in mice to encompass both the heterogeneity within a solid tumor and to implant at the same time both the malignant and non-malignant components of the tumor that cooperate during cancer progression. But also PDX models are subjected to selective pressure when transplanted to a new host, and only a subset of tumors are able to grow successfully in mice.

In this work we used both C3H mice and NOD/SCID mice as tumor hosts, and breast cancer cell lines were orthopically implanted in the mammary fat pad. All experiments where approved by and conducted according to guidelines by the Norwegian State Commission for Laboratory Animals. For the murine breast cancer

cell line C3HBA, it was possible to transplant tumor cells to syngeneic C3H mice living in a non-sterile environment. In contrast, the MDA-MB-231 and MCF7 cell lines are derived from human breast cancers, and the immunodeficient NOD/SCID mouse strain had to be used to avoid cross-species anti-tumor immunity (Ito, Hiramatsu et al. 2002). In contrast to C3H, the NOD/SCID mice are bred and kept in an aseptic environment.

3.3 Other methodological considerations

Analysis of mRNA vs. protein expression: While small variations in *PTEN* mRNA expression can influence the susceptibility to develop tumors (Alimonti, Carracedo et al. 2010), protein levels measured by IHC may not be accurate enough to pinpoint small variations in protein expression caused by gene expression changes. Many factors can influence IHC staining, all the way from tissue acquisition, handling, time and concentration of fixation and antigen retrieval, all or which can impact the accessibility and presence of epitopes (Leandro Luongo de Matos). The choice and validation of antibody, presence of background staining as well as interobserver variability between researchers when staining results are interpreted can also influence IHC staining quantification.

***PTEN-PTENP1* mRNA similarities**: The *PTEN* mRNA and *PTENP1* mRNA transcripts are encoded by a 1.2kb region which have a 98% sequence homology, and only a few nucleotides differentiate between the transcripts in this region (Whang, Wu et al. 1998). This makes it challenging to separate the transcripts by RT-PCR. Thus, PCR primer pairs need to be selected carefully (Juehui Liu 1999). We included a cell line with a known *PTEN* deletion, but preserved *PTENP1* in the assessment of *PTENP1* and *PTEN* primer pairs. Further, all primers were quality controlled by Sanger sequencing of the PCR products using a cell line known to express both *PTEN* and *PTENP1* transcripts.

Cell lines, mice and methods to examine the impact of *PTENP1* deregulation:

Mice lack the *PTENP1* gene (Tang, Ning et al. 2016), and by introducing *PTENP1* in a murine breast cancer cell line, we hypothesized that biological changes caused by upregulating the pseudogene *PTENP1* would be more profound than in a human cell line with prior *PTENP1* baseline expression. We first tried to create a lentiviral vector that contained the entire *PTENP1* transcript, but this produced a very low virus titer, probably due to the size of the plasmid being too large. The packaging-limit for lentiviral particles are around 11-12 kb insert between the long terminal repeat (LTR) retroviral promoter regions, and there is a drop in titer of 1 log for every 2 kb of insert (Kumar, Keller et al. 2001). The 11 kb pWPI plasmid already contains 6 kb between the LTR regions, which includes the GFP- and the translational promoting IRES-sequence. By reducing the size of the cloned fragment to only include the 3´UTR part of *PTENP1*, we got a high titer of functional virus capable of transducing our cell lines with acceptable efficiency. Although we did not transduce the complete *PTENP1* gene, the transduced *PTENP1* 3´UTR contained the MREs, enabling us to study the ceRNA function of *PTENP1* (Poliseno, Salmena et al. 2010).

Similar to other non-coding RNAs, *PTENP1* is not as highly expressed as protein-coding mRNAs (Gloss and Dinger 2016). This makes knockdown experiments difficult. To be able to study downregulation of *PTENP1* compared to downregulation of *PTEN*, one must also find cell lines expressing both. After an initial screen of several different breast cancer cell lines we found that MDA-MB-231 and MCF7 expressed both wt *PTEN* and *PTENP1*. These cell lines were therefore initially chosen based on this, and because they are known to be tumorigenic in mice.

4. Summary of Results

4.1 Paper 1

In this paper, *PTEN* and *PTENP1* expression levels in locally advanced breast cancer were examined in tumor biopsies from the Doxorubicin, FUMI and Epi/Tax neoadjuvant clinical trials. The correlations between gene expression levels and treatment response or survival were assessed in the Doxorubicin, FUMI and Epi/Tax patient cohorts. The mutational status of *TP53* and *PIK3CA* were established, together with ER status and HER2 status where tissue was available for such analyses. In the EpiTax cohort, TMA sections were available for immunostaining, and the expression of PTEN, phosphorylated AKT (Ser473), phosphorylated S6K (Ser371, Thr389) and phosphorylated 4EPB1 (Thr70) were examined, and the correlation to recurrence-free (RFS) and disease specific survival (DSS) was established by Cox regression.

There was a weak positive correlation between *PTEN* mRNA expression and PTEN staining in the 166 tumors where both RNA and TMA sections were available. Despite this, there was no correlation between low *PTEN* mRNA expression and lack of PTEN staining. Also, there was no correlation between low *PTEN* mRNA expression and increased downstream signaling staining of pAkt or pS6K. Furthermore, absence of PTEN staining did not correlate to increased staining of downstream signaling of phosphorylated Akt or S6K. Akt phosphorylation was however significantly more prevalent in tumors with *PI3KCA* mutations.

We then evaluated whether high or low intratumoral expression of *PTEN* or *PTENP1* mRNA influenced survival and response to treatment. We found that a high intratumoral *PTEN* expression level was associated with shorter disease-specific and recurrence-free survival for all cohorts combined. However, high *PTEN* expression was only associated with worse survival in patients with *TP53* wt tumors, and not for

patients with *TP53* mutated tumors.

There was a significant correlation between *PTEN* and *PTENP1* gene expression, indicating a co-regulation, but high or low *PTENP1* expression was not associated with survival as an independent biomarker.

Also, none of the IHC protein staining's performed was associated with survival outcome.

With regards to prediction of treatment response, we found no association between pretreatment *PTEN* or *PTENP1* mRNA expression levels and response to therapy. This was regardless of *TP53*, ER, *PI3KCA* or HER2 status. Activating *PIK3CA* mutations alone also did not influence response to doxorubicin, 5-FU/mitomycin, epirubicin or paclitaxel treatment. Furthermore, protein staining for PTEN, pAKT, pS6K and p4EPB1 did not predict response to any of the treatment regimens.

In summary, Paper 1 demonstrates that high intratumoral gene expression of *PTEN* in locally advanced breast cancer is associated with inferior survival outcome, but only for patients with tumors harboring *TP53* wt status.

4.2 Paper 2

Here we examined the role of Akt with respect to anthracycline resistance in breast cancer. We also evaluated whether Akt inhibition using the small molecule inhibitor A-443654 could be used to increase the efficacy of doxorubicin in doxorubicin-naïve and doxorubicin-resistant breast cancer cell lines, followed by a preclinical trial.

In the ER positive cell lines MCF7 and T47D, increased Akt phosphorylation (pAkt) was observed after a single dose of doxorubicin in doxorubicin-naïve cells, while no pAkt alteration was observed in the ER negative MDA-MB-231 cell line. Increased baseline pAkt was seen after long-term doxorubicin treatment in both the MCF7 and MDA-MB-231 cell lines. However, no further pAkt increase was induced if an

additional high, single dose of doxorubicin was given to the doxorubicin-resistant MCF7, whereas pAKT increased in the doxorubicin-resistant MDA-MB-231. PTEN expression was not affected by doxorubicin treatment.

The increased Akt phosphorylation in doxorubicin-resistant cells suggests that adding an Akt inhibitor after doxorubicin treatment might be beneficial in the treatment of ER positive tumors. Also, that a delayed administration of Akt inhibitor might be beneficial in the treatment of ER negative tumors after prior exposure to doxorubicin. Based on these assumptions we tested the ATP-competitive Akt inhibitor A-443654. A-443654 binds to the ATP-binding site, which causes a transient increase in S473 phosphorylation in Akt and disrupts its ability to phosphorylate downstream targets (Luo, Shoemaker et al. 2005). The doxorubicin-resistant ER positive MCF7 cell line was more sensitive to A-443654 treatment than its doxorubicin-naïve parental cell line, while this was not observed for the doxorubicin-resistant ER negative MDA-MB-231 cell line.

After 2 hours of A-443654 treatment pAkt increased in the doxorubicin-naïve MDA-MB-231 and MCF7 cell lines in a dose-dependent manner. The doxorubicin resistant cell lines also showed increased pAkt, although not as profound as the doxorubicin-naïve parental cell lines. After 24 hours of A-443654 treatment, pAkt remained upregulated while total Akt decreased in both the doxorubicin naïve and resistant MDA-MB-231 and MCF7 cell lines.

In a preclinical trial, we orthotopically implanted NOD SCID mice with either MDA-MB-231 or MCF7 breast cancer cells. When measureable tumors had developed, the mice was grouped into five treatment arms and received either sham treatment, doxorubicin, Akt inhibitor, combination treatment of Akt inhibitor and doxorubicin, or a delayed combination treatment where the Akt inhibitor treatment commenced after the second dose of doxorubicin. The Akt inhibitor had little effect in the MDA-MB-231 tumors as monotherapy, and two doses of doxorubicin halted tumor growth more than two weeks of continuous A-443654 treatment. In contrast, in MCF7 tumors A-443654 monotherapy caused tumor regression, similar to that of

doxorubicin alone. The combination of doxorubicin with delayed A-443654-treatment significantly inhibited tumor growth of MDA-MB-231 tumors compared to sham treatment, but no more than doxorubicin monotherapy. In MCF7 tumors there was a superior response to the combination treatment when A-443654 and doxorubicin treatment commenced simultaneously compared to sham treatment or A-443654 monotherapy.

The clinical relevance of *AKT1* expression with respect to anthracycline resistance was examined in two patient cohorts after short-term and long-term exposure to anthracyclines. Tumor biopsies were extracted before and 24 hours after treatment in the recently completed dose-dense trial (NCT00496795), and while *AKT1* mRNA levels increased in the ER positive tumors that responded to treatment, no change was observed in the non-responder group. Paradoxically, the mRNA levels of *PTEN* as well as *AKT1* increased concomitantly in the ER positive responder group. After 16 weeks of anthracycline treatment, no significant change was seen in *AKT1* mRNA levels in neither group, but *PTEN* mRNA increased in the non-responder group, and most prominently in the ER negative tumors.

In short, an initial activation of Akt characterizes ER positive breast cancers exposed to anthracyclines that respond to treatment. Also, when resistance to anthracyclines has developed, estrogen receptor positive cancer cells exhibit a particular sensitivity to Akt inhibition.

4.3 Paper 3

The *PTEN pseudogene (PTENP1)* has been shown previously to adsorb microRNAs targeting PTEN for degradation in prostate cancer cell lines (Poliseno, Salmena et al. 2010). Here we examined how *PTENP1* influences tumor progression in ER positive and negative breast cancer through ceRNA interactions. *PTENP1* overexpression increased PTEN protein in the ER negative breast cancer cell lines C3HBA and MDA-MB-231, and in the non-tumorigenic ER negative MCF10a cell line. However,

in the ER positive MCF7 and T47D cell lines, there was no influence on PTEN protein expression when *PTENP1* was overexpressed.

When examining the effect of *PTENP1* overexpression on cell proliferation we found that *PTENP1* decreased proliferation in C3HBA, whereas no change was observed in MDA-MB-231. In contrast, *PTENP1* increased cell proliferation in the ER positive MCF7 and T47D. By implanting the *PTENP1*-overexpressing cell lines C3HBA, MDA-MB-231 and MCF7 in mice, we found the same growth pattern as indicated by the cell proliferation results. While *PTENP1* overexpression decreased tumor growth in the C3HBA cell line, *PTENP1* overexpression had no effect on tumor growth in MDA-MB-231. However, a decrease in metastatic propensity was observed in mice with MDA-MB-231 tumors, transduced with *PTENP1*. Finally, *PTENP1* transduction increased tumor growth in the ER positive MCF7 tumors.

In contrast to the *in vitro* findings, *PTENP1* overexpression did not affect PTEN protein expression in any of the cell lines *in vivo*. Also, there was no effect of *PTENP1* overexpression with respect to downstream PI3K-Akt signaling measured by western blotting in the ER negative C3HBA or MDA-MB-231 cell lines. In the ER positive MCF7 cell line we observed increased Akt phosphorylation in *PTENP1* overexpressing tumors, but there was no effect on downstream S6K activation. This points to alternate pathways responsible for the change in tumor growth.

Due to the lack of effect on PI3K-Akt signaling by *PTENP1* transduction in tumor tissue, we expanded our search with a global gene expression microarray to identify other genes that might be influenced by *PTENP1* overexpression *in vivo*. Since mice lack the *PTENP1* gene, we chose the C3HBA murine cell line. By examining a *PTENP1*-naïve system we expected to observe stronger gene expression alterations induced specifically by *PTENP1* overexpression, as compared to a biological system wherein *PTENP1* is present and expressed at baseline. Using a mouse-specific microarray, we found that the gene most profoundly upregulated by *PTENP1 in vivo* was *TCFAP2C*, which encodes the protein AP2. This is a protein that can inhibit breast cancer growth, and is important for maintenance of a luminal phenotype (Cyr,

Kulak et al. 2014). Based on this we examined the *PTENP1* overexpressing tumors for AP2. We found AP2to be upregulated in C3HBA and MDA-MB-231, and weakly downregulated in the *PTENP1* overexpressing MCF7 tumors.

Because of the known interaction between AP2and ER, we also examined whether *PTENP1* overexpression influenced ER levels. In the ER negative tumors C3HBA and MDA-MB-231, no change in ER expression was observed subsequent to *PTENP1* overexpression. Strikingly, both ER and *ESR1* gene expression was significantly decreased in the *PTENP1* overexpressing ER positive MCF7 tumors.

We then examined if *PTENP1* overexpression influenced the level of the five verified microRNAs shared between *PTEN* and *PTENP1*; hsa-miR-19b-3p, hsa-miR-20a-5p, hsa-miR-21-5p, hsa-miR-26a-5p and hsa-miR-214-3p (Poliseno, Salmena et al. 2010). The five microRNAs were expressed in all the three cell lines C3HBA, MDA-MB-231 and MCF7, but *PTENP1* overexpression did not influence the expression of any of the examined microRNAs, except hsa-miR-26a which increased subsequent to *PTENP1* overexpression in the MCF7 cell line.

We also examined whether *PTENP1* influenced sensitivity towards doxorubicin. Interestingly, MCF7 became more resistant to doxorubicin when *PTENP1* was silenced by siRNA, where knockdown of *PTENP1* did not influence the sensitivity to doxorubicin in MDA-MB-231. In contrast, *PTENP1* overexpression sensitized MDA-MB-231 to doxorubicin, but had no effect on the doxorubicin sensitivity in the ER positive MCF7 and T47D cell lines.

To search for similar associations in patient breast cancer biopsies, we downloaded the *Breast Invasive Carcinoma* (TCGA, Provisional) dataset from the TCGAs database cBioportal.org. This dataset was selected because it had the largest number of breast cancer samples reporting both protein levels by RPPA and mRNA levels by sequencing. Due to the sequence similarities between *PTEN* and *PTENP1* we wanted to avoid using data from microarrays, where recognition sites for these two genes may overlap. In the TCGA dataset we found that *PTENP1* upregulated tumors expressed higher levels of *PTEN* mRNA as well as PTEN protein compared to

tumors with low or no *PTENP1* expression. Furthermore, there was lower ER expression in *PTENP1* upregulated tumors, whereas no difference in *TFAP2C* was found. AP2protein was not present in the RPPA array from this dataset and could therefore not be assessed.

Overall, the main finding in this paper is that the *PTENP1* 3`UTR transcript exerts a biological function in breast cancer, with opposite effects on tumor growth in ER positive and negative breast cancer.

5. Discussion

The current work merges preclinical and clinical research data to examine the relevance of *PTEN*, *PTENP1* and PI3K-Akt-mTOR signaling in breast cancer. Functional experiments were conducted preclinically to examine further the clinical findings we observed, and the results presented demonstrate that *PTEN* and *PTENP1*, as well as PI3K signaling are actively involved in breast cancer progression, and response to breast cancer therapy.

Our finding that a high intratumoral *PTEN* expression predicts worse survival in patients with preserved *TP53* is surprising considering that loss of PTEN protein has been characterized as an important protumorigenic event, which is frequently observed in a variety of cancers (Steck, Pershouse et al. 1997, Leslie and Foti 2011, Millis, Ikeda et al. 2016). p53 is a transcriptional regulator of PTEN (Stambolic, MacPherson et al. 2001, Agrawal and Eng 2006, Pappas, Xu et al. 2017), and in line with this we found *PTEN* and *TP53* mRNA expression levels to correlate. We established a weak correlation between PTEN protein and *PTEN* mRNA expression in our patient cohorts, and there was no significant prognostic impact of lack of PTEN staining. The weak correlation between PTEN protein and mRNA indicates that the mechanisms behind the negative impact of high *PTEN* mRNA expression levels may occur at the mRNA or post-translational level.

PTEN mRNA share MREs with several mRNA transcripts involved in cancer progression. A ceRNA network has been characterized where both *PTEN* and *PTENP1* are involved as well as other key genes involved in human cancer progression, such as *ZEB2*, *CNOT6L*, *VAPA* and *VCAN* (Poliseno and Pandolfi 2015). In another study, *RUNX1*, *STAT3*, *VEGFA* and *RB1* were identified as *PTEN* ceRNAs (Sumazin, Yang et al. 2011). At high *PTEN* mRNA transcript levels, *PTEN* interacts with microRNAs and influences the expression level of the microRNA targets within this network. *PTEN* may also act as a ceRNA to p53-regulating transcripts such as *RNF38*, *TP53INP1* and *HIPK2*, which may influence p53 by ubiquitylation, phosphorylation and transcription respectively (Zarringhalam, Tay et al. 2017). This

is particularly interesting in view of our results where high *PTEN* expression was prognostic only for *TP53* wt tumors. This adds a layer of complexity to expression studies, and is important to keep in mind when evaluating candidates for therapy resistance or oncogenicity based on protein or RNA expression studies.

Simultaneously, in normal cells there exist no perfect correlation between mRNA expression and protein expression levels. We estimate that 30-80% of variation in protein expression is caused by variations in mRNA expression levels, and lack of correlation can be caused by post-transcriptional or post-translational regulation such as mRNA degradation, microRNA regulation, protein degradation and protein secretion or measurement errors (Vogel and Marcotte 2012, Kosti, Jain et al. 2016). On a global scale, mRNA levels correlate to protein levels, but due to a large variation in tissue specific post-transcriptional regulation, this correlation varies between tissues (Franks, Airoldi et al. 2017). Also, in cancer tissues the mRNA-protein correlation is lower than in normal tissues (Kosti, Jain et al. 2016), indicating that deregulated post-transcriptional processes are common in cancer as well.

A high expression of *PTEN* mRNA cannot be used to guide treatment options in breast cancer based on its lack of predictive power herein, but these results points to biological mechanisms involving *PTEN* to be involved in worse survival for patients with locally advanced breast cancer. These alternate mechanisms may be ceRNA interactions, and more specifically interactions acting on p53 stability and activation.

In Paper 2 we focused on mechanisms of chemoresistance to explore candidate therapeutic targets in the PI3K-Akt-mTOR pathway. While PTEN protein levels were not affected by 24 hours doxorubicin treatment, we found that Akt was activated after 24 hours exposure to an IC30 dose of doxorubicin. Akt was also activated without doxorubicin treatment after long-term treatment where chemoresistance had developed, indicating a role for Akt activation in resistance to anthracyclins. Upregulation of *AKT1* was a common response 24 hours after anthracycline treatment in ER positive human breast cancers that responded to treatment, whereas no increase in *AKT1* was seen after 16 weeks of treatment in the responder group. Oncogene-

induced senescence is a protective mechanism against tumor progression in normal cells in the event of an oncogenic mutation; accordingly, most oncogenic mutations never give rise to tumors. Both PTEN loss and Akt overexpression has been shown to cause oncogene-induced senescence through Rb and p53 (Courtois-Cox, Jones et al. 2008). Chronic Akt activation in breast cancer could cause oncogene-induced senescence and apoptosis by downregulating MDM2 and increasing p53 (Nogueira, Park et al. 2008, Astle, Hannan et al. 2012). Interestingly, 24h after the first anthracycline treatment *AKT1* increased in 6 of the tumors that responded to treatment, and only 2 out of the 6 harbored a *TP53* mutation. Among the ER positive tumors, after 16 weeks there was no difference in *AKT1* level between the responder groups.

In light of the above findings, and since doxorubicin-resistant ER positive cell lines exhibited increased sensitivity to Akt inhibition, Akt inhibitors may become useful as a second line treatment when resistance has developed. This was observed in another preclinical trial using the Akt inhibitor MK-2206, where a synergistic response to Akt inhibition was achieved when Akt inhibition was combined with multiple chemotherapeutics, including doxorubicin (Hirai, Sootome et al. 2010). The response was sequence dependent, where Akt inhibitor + docetaxel treatment was less effective if given simultaneously, and more effective if the Akt inhibitor was given after docetaxel. However, the clinical benefit of Akt inhibitors in this setting remains to be defined in ongoing clinical trials (Avan, Narayan et al. 2016). Akt is important for insulin signaling and glucose homeostasis, and a known side effect of Akt inhibition is hyperglycaemia and hyperinsulinaemia (Brown and Banerji 2017). In our preclinical trials, the mice treated with Akt inhibitor lost 7% of weight during 14 days of treatment and developed sore skin at the injection site. This precluded long-term treatment with this particular drug. However, other ATP-competitive Akt inhibitors have been developed recently which are orally available and better tolerated (Avan, Narayan et al. 2016).

When suppressing Akt constitutively, the cell initiates feedback mechanisms, and prolonged Akt inhibition increases receptor tyrosine kinase (RTK) expression

upstream of Akt. Thus, combining Akt inhibitors with HER2 inhibitors have yielded promising results *in vivo* (Chandarlapaty, Sawai et al. 2011). Intermittent dosing has also been shown to be beneficial, by relieving feedback mechanisms, and increasing the patients tolerance for the drug (Davies, Greenwood et al. 2012).

PIK3CA mutations, PTEN loss/mutations or *HER2* amplification correlate to response to Akt inhibition, whereas *RAS* mutations are associated with resistance (Davies, Greenwood et al. 2012). This is in line with our results where MCF7 tumors was more sensitive to Akt inhibitor treatment than MDA-MB-231 tumors, given that MCF7 harbors a *PIK3CA* mutation and MDA-MB-231 harbors a *RAS* mutation (www.broadinstitute.org/ccle). The RAS-ERK pathway is an alternative/escape pathway when Akt inhibition fails to cause apoptosis. The RAS-ERK pathway is located downstream of oncogenic *PIK3CA*, and inhibition of Akt also activates ERK signaling through feedback mechanisms. Marie Will *et.al* observed that apoptosis was induced only if both Akt and ERK were inhibited, and that a pulsatile dosage of Akt and ERK inhibitors increased the efficacy and decreased the toxicity of such drugs (Marie Will 2014). It is clear that choosing inhibitors and combinations of inhibitors is a difficult exercise. To achieve a substantial therapeutic gain one should monitor the response using different inhibitors and dose schedules in order to adapt treatment to counteract feedback mechanisms causing resistance.

The relevance of the pseudogene *PTENP1* in breast cancer has not been examined functionally before. The role of *PTENP1* as a protector of *PTEN* was established in prostate cancer (Poliseno, Salmena et al. 2010), and the role of *PTENP1* has been examined in several cancer types. *PTENP1* is expressed in the majority of cancer subtypes, and *PTEN* and *PTENP1* expression is highly correlated. *PTENP1* was associated with decreased proliferation and tumor growth in squamous cell carcinomas of the head & neck (Liu, Xing et al. 2017). In endometrial cancer, no correlation was observed between *PTENP1* expression and response to treatment or survival, but the authors reported a trend towards a lower frequency of disease progression in patients with *PTENP1*-expressing tumors (Ioffe, Chiappinelli et al. 2012). In gastric cancer, *PTENP1* expression was lower than in adjacent normal

tissue, and interestingly high *PTENP1* expression was associated with lower tumor size, lower invasion depth and fewer lymph node metastasis (Guo, Deng et al. 2016, Zhang, Guo et al. 2017). In clear-cell renal carcinoma, lack of *PTENP1* expression correlated to worse survival outcome (Gan Yu 2014).

In contrast to previous work in other types of cancer, in Paper 3 we found a diverging role of *PTENP1* in breast cancer, depending on breast cancer subtype. Overexpression of *PTENP1* did not affect PTEN protein levels in the ER positive cell lines, but ER protein expression was reduced in the *PTENP1* overexpressing MCF7 tumors. *PTENP1* reduced growth in the ER negative cell lines and increased proliferation in the two ER positive cell lines tested. Hsa-mir-26a is known to target and downregulate *ESR1* in addition to *PTEN* and *PTENP1* (microRNA.org), and this microRNA was upregulated by *PTENP1* overexpression in all the three cell lines tested. Importantly, while expression levels are low, the tissue specificity of noncoding RNAs is high. Actually, noncoding RNA expression is more tissue-specific than mRNA and protein expression (Gloss and Dinger 2016). Thus, it is plausible that *PTENP1* can have different downstream effects in different subtypes of breast cancer. Among noncoding RNAs the context is highly important, and the microRNA-mRNA titration ratio is important for possible outcomes of deregulation of one spesific ceRNA (Johnsson, Ackley et al. 2013, Bosson, Zamudio et al. 2014).

Although we did not find high *PTENP1* expression to be of any prognostic value in Paper 1, the subgroup of TNBC with high *PTENP1* expression deviated from all the other subgroups with longer survival, although not statistically significant in these patient cohorts (Online resource 3). However, combining these clinical observations in Paper 1 with the functional assessments in Paper 3, where *PTENP1* expression sensitized MDA-MB-231 to doxorubicin, indicates that *PTENP1* sensitizes cancer cells to anthracyclin, that there is a protective role of the pseudogene *PTENP1* in ER negative breast cancer. The protective role in ER negative breast cancer is probably mediated via increased PTEN, while other mechanisms are invoked in ER positive breast cancer that influences ER signaling and increases proliferation.

6. Conclusion

Paper 1

PTEN loss is common in cancer (Millis, Ikeda et al. 2016) and is associated with metastatic events (Juric, Castel et al. 2015) and endocrine resistance (Shoman, Klassen et al. 2005). Despite this, all tumors we evaluated expressed *PTEN* mRNA and few had *PTEN* mutations. There was also a weak correlation between *PTEN* mRNA expression and PTEN protein staining measured by IHC. This indicates that posttranscriptional modifications could be responsible for the lack of PTEN protein staining. Having a high intratumoral *PTEN* mRNA expression was shown to be a negative prognostic marker for long term survival in metastatic breast cancer, but only for patients with *TP53* wt tumors. *PTEN* mRNA levels however did not predict response to treatment, regardless of *TP53* status. Accordingly, a high intratumoral *PTEN* mRNA levels is associated with worse survival, but is not a predictive factor to guide breast cancer treatment.

Paper 2

We found that activation of Akt is a common response to anthracycline treatment, and that inhibition of Akt yields a therapeutic benefit. However, the benefit is time- and context-dependent. Also, there are different mechanisms involved in short-term and long-term response to chemotherapy. We observed that 24 hours after the first chemotherapy dose, in patients with ER positive tumors, increased *AKT1* mRNA levels was associated with subsequent response to therapy. Also, the *in vitro* study demonstrated that persistent exposure to doxorubicin activated Akt extensively, and increased the sensitivity to Akt inhibition in doxorubicin-resistant MCF7.

The patients that could benefit from adding an Akt inhibitor to treatment are the patients who already have a high constitutive Akt signaling, or that have previously been exposed to anthracyclines. Accordingly, using Akt inhibitors to enhance the effect of chemotherapy should be explored in anthracycline-resistant breast cancers, and in particularly ER positive breast cancer.

Paper 3

During the recent years, the biological function of non-coding RNAs in cancer progression has been a rapidly expanding area of research. Non-coding RNAs were mostly considered inactive evolutionary leftovers that were accidentally transcribed. In Paper 3 we observed that in breast cancer the pseudogene *PTENP1* has a "Janus face"; i.e. *PTENP1* overexpression suppressed tumor growth in the ER negative C3HBA murine tumor model and the MDA-MB-231 human tumor model, while it increased tumor growth in the ER positive MCF7 human tumor model. Non-coding RNA transcription is highly cell-specific in normal cells, and we have shown a diverging response in different breast cancer subtypes. In ER negative breast cancers *PTENP1* increased the level of AP2γ, a transcription factor involved in maintaining a luminal phenotype. In addition, upregulating *PTENP1* dramatically reduced ERα expression in MCF7 tumors. The notion that *PTENP1* expression may decrease ERα expression in breast cancer was strengthened based on data extraction from the TCGA database. There we found that breast cancers having upregulated *PTENP1* expression had a lower ERα expression than tumors without upregulated *PTENP1* expression.

In short, the non-coding pseudogene *PTENP1* has a biological effect that affects breast cancer progression through ceRNA interactions, which is governed by the hormone receptor status of the tumor.

7. Future perspectives

The work presented in this thesis show that high intratumoral expression of *PTEN* mRNA is a negative prognostic marker in patients with preserved p53 function. Based on PTEN´s role as a tumor suppressor, this is rather counterintuitive and the mechanisms responsible remain to be elucidated. Although *PTEN* mRNA and PTEN protein levels were positively correlated, PTEN protein expression was not prognostic in our cohorts of patients with breast cancer. In most studies, lack of PTEN protein staining is associated with treatment resistance and aggressive malignancies (Wang, Hao et al. 2017), and it is generally accepted that PTEN loss is important in tumor development and progression (Wikman, Lamszus et al. 2012, Juric, Castel et al. 2015). However, PTEN is also involved in DNA repair and protects cells from genotoxic stress, and in that context, PTEN upregulation could promote chemoresistance by halting cell proliferation (Bassi, Ho et al. 2013).

In Paper 1 we found PTEN protein and *PTEN* mRNA levels to be weakly correlated, but several biopsies with high *PTEN* mRNA had no PTEN staining. Only 25% of cancer biopsies show a correlation between loss of the PTEN protein and *PTEN* mRNA (Zhang, Zhang et al. 2013), indicating that deregulated post-translational mechanisms mediate PTEN loss despite ongoing *PTEN* gene expression. This may in part be caused by ceRNA interactions, but also by deubiquitylating enzymes that interact with PTEN and destabilize or translocate the PTEN protein between the cytoplasm and nucleus. It would be of interest to examine the mechanisms causing these tumors to express high *PTEN* mRNA but little or no PTEN protein, as this may represent novel "druggable" targets.

Intriguingly, it has been reported that a high *PTEN* mRNA expression in lung adenocarcinoma is an adverse prognostic factor, but only in female patients (Inamura, Togashi et al. 2007). This raises several questions, such as the mechanisms and growth factors governing the adverse response in women, and whether hormone levels are imposing the negative impact of high *PTEN* expression. Based on our findings in breast cancer and that in lung cancer above, it seems that the hormonal

environment wherein these cancers develop may dictate the biological effect of *PTEN*. Investigating this further could have therapeutic implications in both breast cancer treatment and lung cancer.

Another possible reason for low correlation between *PTEN* mRNA expression and PTEN protein expression is the formation of alternative splice variants. Splice variants of *PTEN* is differentially expressed in cancer vs. normal tissues, they are also increasingly expressed in aged blood and EBV-transformed lymphoblastoid cell lines, and may be a cellular response to stress (Liu, Malaviarachchi et al. 2010). Furthermore, p53 promotes altered *PTEN* splice variants when overexpressed (Agrawal and Eng 2006), and p53 is thereby not only functioning as a transcriptional regulator of PTEN, but is also involved in the post-transcriptional regulation of PTEN through splicing. Agrawal *et.al* demonstrated that ectopically overexpressing p53 induced *PTEN* splice variants SV-5b and 5c that increased cyclin D1 promoter activity. Accordingly, it would be of interest to examine the expression of *PTEN* splice variants in the patient cohorts where we analyzed *PTEN* expression to evaluate whether the expression of SV-5b or 5c is associated with worse survival in patients harboring tumors with preserved p53 function.

Turning to PTEN, the PI3K-Akt-mTOR pathway and chemoresistance, we established that cell lines made resistant to anthracyclines expressed a higher degree of phosphorylated Akt than its parental chemotherapy-naïve cell line when exposed to an acute dose of anthracyclines. This makes Akt a promising druggable candidate using small molecule inhibitors to directly target drug resistance. The Akt inhibitor A-443654 tested in this work is not a drug that should be pursued further in its current form. This is due to the severe side effects of the compound. Severe weight loss, probably due to induced hyperglycaemia, prevented prolonged use of the drug regardless of its anti-cancer effect. However, other Akt inhibitors have been developed which can be taken orally and with tolerable side effects. There are several phase 2 clinical trials testing Akt inhibitors, particularly as a mode to resensitize platinum resistant ovarian cancer to treatment (Brown and Banerji 2017). It would be of interest to assess whether these newer and less toxic Akt inhibitors exhibit the

same increased cytotoxicity to anthracyclin-resistant breast cancer cell lines, compared to chemotherapy-naïve parental cell lines, and whether they could be used in combination with anthracyclines to counteract acquired chemoresistance.

During the recent years, massive parallel sequencing has increased our knowledge not only about the genomic landscape of cancer, but also the transcriptome. The number of non-coding RNAs transcribed, transcends the number of protein coding RNAs. Their tissue, cell and developmental expression specificity tell us that they are critically important in regulation, but their specific function is yet to be revealed (Gloss and Dinger 2016). *PTENP1* is but one of many noncoding RNAs. When we add proteomics, epigenomics and metabolomics, including all the possible interactions between RNAs and proteins, the complexity of "big data" becomes far too great to manage individually. To be able to construct models, navigate them and understand signal transduction in cancer and resistance to therapy, we will become more dependent on utilizing collective databases and systems biology as a tool (Nam 2017). This will change translational research, moving from hypothesis-testing to hypothesis-generating research where we can make clinical predictions for individual phenotypes (Biesecker 2013). These predictions however, must be tested by bench science for validity but provides exciting opportunities for elucidating biological mechanisms, and the personalization of cancer therapy.

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BRIEF REPORT

High *PTEN* gene expression is a negative prognostic marker in human primary breast cancers with preserved p53 function

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Abstract

Purpose PTEN is an important tumor suppressor in breast cancer. Here, we examined the prognostic and predictive value of *PTEN* and *PTEN pseudogene (PTENP1)* gene expression in patients with locally advanced breast cancer given neoadjuvant chemotherapy.

Methods The association between pretreatment *PTEN* and *PTENP1* gene expression, response to neoadjuvant chemotherapy, and recurrence-free and disease-specific survival was assessed in 364 patients with locally advanced breast cancer given doxorubicin, 5-fluorouracil/mitomycin, or epirubicin versus paclitaxel in three phase II prospective studies. Further, protein expression of PTEN or phosphorylated Akt, S6 kinase, and 4EBP1 was assessed in a subgroup of 187 tumors.

Results Neither *PTEN* nor *PTENP1* gene expression level predicted response to any of the chemotherapy regimens tested ($n = 317$). Among patients without distant metastases ($n = 282$), a high pretreatment *PTEN* mRNA level was associated with inferior relapse-free (RFS; $p = 0.001$) and disease-specific survival (DSS; $p = 0.003$). Notably, this association was limited to patients harboring *TP53* wild-type tumors (RFS; $p = 0.003$, DSS; $p = 0.009$). *PTEN* mRNA correlated significantly with *PTENP1* mRNA levels ($r_s = 0.456$, $p < 0.0001$) and PTEN protein staining ($r_s = 0.163$, $p = 0.036$). However, no correlation between PTEN, phosphorylated Akt, S6 kinase or 4EBP1 protein staining, and survival was recorded. Similarly, no correlation between *PTENP1* gene expression and survival outcome was observed.

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Availability of data and materials

Apart from patient data presented in the article, the full data set is not made publicly available due to ongoing scientific work.

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Conclusion High intratumoral *PTEN* gene expression was associated with poor prognosis in patients with locally advanced breast cancers harboring wild-type *TP53*.

Keywords Locally advanced breast cancer · PTEN · p53 · Prognosis · Predictive factors

Introduction

Mutations in the *TP53* tumor suppressor gene, encoding the p53 protein, are associated with lack of response to anthracycline- and mitomycin-containing chemotherapy as well as poor prognosis in breast cancer [1–7]. However, some patients experience lack of response to these chemotherapeutic compounds despite a preserved tumor p53 function, pointing to additional resistance mechanisms [8]. Apart from p53, PTEN is an important tumor suppressor which is frequently inactivated in breast cancer, thus enabling increased signaling of the crucial growth-promoting PI3K-Akt-mTOR pathway [9, 10]. PI3K-Akt-mTOR signaling is involved in resistance to endocrine- and HER2-directed therapy clinically [9, 11], as well as resistance to chemotherapy in preclinical trials [12, 13]. This suggests that PTEN expression may influence response to cancer treatment.

While *PTEN* somatic mutations are rare, PTEN protein expression is frequently lost in breast carcinomas, pointing to transcriptional and post-transcriptional regulation as possible mechanisms [14, 15]. Of notice, PTEN and p53 reciprocally interact to preserve each other's protein levels [16]. Further, in vitro data from prostate cancer cell lines suggest that *PTEN pseudogene (PTENP1)* mRNA transcripts may regulate the *PTEN* expression level by competing for *PTEN*-degrading micro RNAs (miRNAs) [17].

The aim of the present study was to assess the prognostic role of pretreatment *PTEN* and *PTENP1* gene expression levels in patients with locally advanced breast cancer, stratified by *TP53* mutations status, and the predictive role of *PTEN* and *PTENP1* gene expression levels toward chemotherapy response. In addition, we examined protein expression levels of PTEN as well as key signaling molecules in the PI3K-Akt-mTOR pathway [9]. For this purpose, we used tumor material collected from patients with locally advanced breast cancer treated with different chemotherapy regimens in phase II trials conducted between 1991 and 2007 [1–5].

Methods

Patient material

Pretreatment tumor samples were available from patients with locally advanced breast cancer (T3/T4 and/or N2/N3) included in three neoadjuvant phase II trials described in detail previously [1, 3–5, 18] and outlined in Fig. 1. Dates of enrollment of the first participants to the trials were 18/1-91 (Study 1), 1/6-93 (Study 2), and 24/11-97 (Study 3). In Study 1, patients were given neoadjuvant doxorubicin, 14 mg/m^2 qW for 16 weeks. In Study 2, each patient received 5-fluorouracil 1000 mg/m^2 and mitomycin 6 mg/m^2 (FUMI) q3w for 12 weeks. In Study 3, patients were randomized to either epirubicin 90 mg/m^2 (Arm A) or paclitaxel 200 mg/m^2 q3w (Arm B), administered in 4–6 courses. Further, in Study 3, patients with suboptimal tumor response to either drug switched to the opposite chemotherapy regimen [5, 18].

Response rates (according to the The Union for International Cancer Control criteria), TNM status, estrogen receptor (ER), and *TP53* mutation data have been reported previously [1, 5, 18], and are summarized in Table 1, along with the current assessment of *PIK3CA* and HER2 status. Follow-up data were available for >10 years or up to time of death for all patients in the trials. A total of 317 patients were assessed for chemotherapy response with respect to gene and protein expression. Among these, 282 patients with stage 3 disease at diagnosis were used for survival analysis.

Tumor samples

In each protocol, tumor samples were collected by incisional biopsies prior to commencing cancer therapy. Samples were snap frozen and stored in liquid nitrogen until DNA/RNA analysis. In the present investigation, tumor RNA was available from 325 patients; 81 patients from Study 1, 32 patients from Study 2, and 212 patients from Study 3. Among patients with tumor RNA available, seven lacked response data and 43 had primary metastatic disease, leaving 318 patients for response evaluation and 282 patients for survival analysis with respect to gene expression results (Fig. 1).

Pretreatment formalin-fixed paraffin-embedded (FFPE) tumor tissue was available from 193 patients in Study 3 as tissue microarrays (TMAs), but due to the lack of tumor tissue in some core biopsies or staining artifacts, incl. missing cores, only 187 patients could be evaluated for any particular protein. Among patients with TMA tumor tissue available, seven lacked response data, 18 had primary metastatic disease, whereas one patient did not undergo breast surgery and was unfit for calculation of recurrence-free survival, leaving 179 patients for response evaluation and 169 patients for survival analysis with respect to protein staining results (Fig. 1).

Basic genomic procedures

Procedures, primers, and antibodies used for RNA and DNA analysis are described in detail in Online Resource 1.

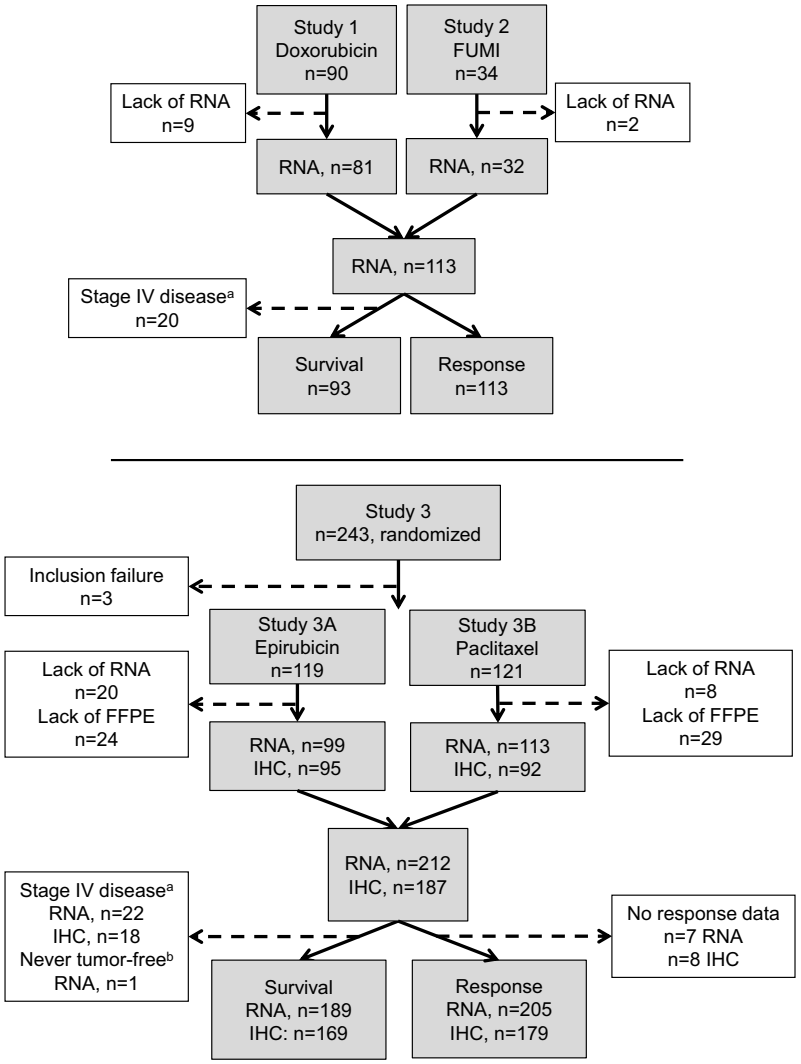


Fig. 1 Flow chart depicting the number of patients with locally advanced breast cancer recruited in Studies 1–3, and the number of samples available from each trial for RNA and immunohistochemistry (IHC) analysis. In Study 3, patients randomized to either epirubicin or paclitaxel were switched to the opposite regimen if tumor regression on the first regimen was insufficient; survival analysis was performed for all patients randomized to each regimen (intention-to-treat) and separately for those patients without crossover (w/o cross) to the opposite regimen. [a]Patients with stage IV disease were excluded from survival analysis. [b]One patient with progressive disease (PD) never became tumor-free, and recurrence-free or disease-free survival could therefore not be assessed. *FFPE* formalin-fixed paraffin-embedded tissue, *IHC* immunohistochemistry

Immunohistochemistry (IHC) and in situ hybridization (ISH)

Procedures used for IHC and ISH analysis are described in detail in Online Resource 1. The antibodies used for protein analysis were monoclonal anti-Akt (phosphorylated Ser 473), monoclonal anti-HER2 (4B5, Dako), polyclonal anti-PTEN, polyclonal anti-S6 kinase (S6K, phosphorylated Ser 371, Abcam), mouse monoclonal anti-S6K (phosphorylated Thr 389), and polyclonal anti-4EBP1 (phosphorylated Thr 70). All antibodies were developed in rabbit, and purchased from Cell Signaling unless specified

Table 1 Baseline patient and tumor characteristics

Treatment	Study 1[a] *Doxorubicin*	Study 2[a] *FUMI*	Study 3A[b] *Epirubicin*	Study 3B[b] *Paclitaxel*
Patients	90	34	119	121
Accrual	1991–1997	1993–2001	1997–2003	1997–2003
Age (years)				
Range	32–88	37–82	28–70	25–70
Median	64	67	49	48
T stage				
T2[c]	3	2	1	1
T3	54	15	99	90
T4	33	17	18	30
N stage				
N0[d]	30	9	52	45
N1	34	14	48	59
N2	26	11	17	17
N3	0	0	1	0
M stage				
M0	78	24	109	106
M1	12	10	10	15
ER				
Negative	13[e]	11[e]	52	49
Positive	77	23	66	69
Unknown	0	0	1	3
HER2				
Negative[f]	24	27	63	66
Positive	6	6	30	28
Unknown	60	1	26	27
TP53				
TP53 wt[g]	64	16	84	89
TP53 mut.	26	18	23	25
Unknown	0	0	12	7
Response[h]				
PD	5	9	10	14
SD	45	13	49	47
PR	31	10	56	47
CR	0	0	4	5
Unknown	0	0	0	8
TMA[i]				
Stage 3	0	0	88	81
Stage 4	0	0	7	11
RNA/DNA[j]				
Stage 3	71	22	90	99
Stage 4	10	10	9	14
PTEN[k]				
PTEN wt	0	0	80	99
PTEN mut.	0	0	2	2
Unknown	0	0	27	4
PIK3CA[l]				
PIK3CA wt	26	20	82	92
PIK3CA mut.	4	12	25	22

Table 1 continued

Treatment	Study 1[a] *Doxorubicin*	Study 2[a] *FUMI*	Study 3A[b] *Epirubicin*	Study 3B[b] *Paclitaxel*
Unknown	51	0	12	7

[a] Data from Studies 1–2 were pooled for statistical analysis due to a low number of patients in Study 2

[b] Data from Study 3 were split into Study 3a (epirubicin) and 3b (paclitaxel), based on the primary chemotherapy given

[c] T2 tumors only included if axilla stage N2. T stage and all subsequent tumor characteristics given for stage 3 and 4 combined

[d] N stage by clinical assessment alone

[e] ER negative if tumor ER concentration <10 fmol/mg in Study 1–2. ER assessed by standard IHC in Study 3

[f] For Studies 1–2; HER2 assessment available from a subset of the tumors by in situ hybridization only. For Study 3: HercepTest IHC was performed on all tumors, and HER2 in situ hybridization for tumors with staining score 2 by IHC

[g] *TP53* mutation status, whole exome assessed by Sanger sequencing. *wt* wild-type, *mut* mutation

[h] Progressive disease (PD), stable disease (SD), partial response (PR), complete response (CR)

[i] Subset of patients from whom formalin-fixed paraffin-embedded (FFPE) tumor tissue was available for protein analysis to correlate against gene expression results (*PTEN*), response rates (stage 3 and 4 disease), or survival (stage 3 only)

[j] Subset of patients from whom tumor RNA was available for gene expression analysis to correlate against response rates (stage 3 and 4 disease) or survival (stage 3 only)

[k] Subset of patients from whom tumor DNA was available for *PTEN* mutation analysis

[l] Subset of patients from whom tumor DNA was available for *PIK3CA* mutation analysis to correlate against response rates (stage 3 and 4 disease) or survival (stage 3 only)

otherwise. Immunostaining was evaluated by two independent researchers, and given a semi-quantitative score of 0 (no staining) to 3 (strong staining). Whereas both nuclear and cytoplasmic staining were assessed for PTEN, cytoplasmic staining was scored for 4EBP1, and nuclear staining for Akt and S6K. In a combined PI3K pathway analysis, absent PTEN protein staining, phosphorylated Akt staining, phosphorylated S6K staining, and *PIK3CA* mutation were each given a score of one each, and "PI3K pathway activation" was defined as a score of two or higher.

Statistics

Correlation analysis between *PTEN* mRNA expression level and PTEN staining was performed using Spearman's rho. Mann–Whitney test was used for comparison of mRNA or protein staining levels between tumor subgroups. The Chi-square test was used to assess the correlations between *PIK3CA* mutation status and phosphorylation status of Akt, S6 K, 4EBP1 proteins or between *PIK3CA* mutations and response to chemotherapy. Chi-square test was also used to assess the correlation between IHC staining and chemotherapy response. Survival data were assessed by Cox regression analysis calculating hazard ratios for each parameter. For Kaplan–Meier plots, patient subgroups were compared by the log-rank test. Due to a smaller number of patients, the survival data from Studies 1 to 2 were analyzed in concert, as described previously [1]. Recurrence-free (RFS) and disease-specific survival (DSS) were defined as time from inclusion in the trial until breast cancer recurrence or death due to breast cancer, respectively. Deaths for reasons other than breast cancer, or patients still alive at the time of analysis, were treated as censored observations. *PTEN* and *PTENP1* gene expression values were sorted for each of the three trials separately and divided by the median value into two groups defined as *PTEN* or *PTENP1* "low" (i.e., below the median) and "high" (i.e., above the median). Multivariate analysis was performed using Cox regression to evaluate the independent prognostic impact of *PTEN, PTENP1, TP53, PIK3CA*, HER2, and ER status in this cohort of locally advanced breast cancers. Statistical analyses were performed using the SPSS 22/PASW 17.0 and Graph Pad Prism v6 software packages. All p-values reported are two-tailed, and $p < 0.05$ was considered statistically significant.

Results

PTEN, PTENP1, and TP53 gene expression

Baseline patient and breast cancer characteristics from Studies 1-3 are summarized in Table 1. *PTEN* gene expression by quantitative/real-time PCR (qPCR) was detectable in all 318 tumors with a defined treatment

response (Fig. 2a). In contrast, *PTENP1* expression was undetectable in 96 tumors (30%; Fig. 2b). There was a significant, albeit not uniform correlation between *PTEN* and *PTENP1* mRNA expression levels ($r_s = 0.456$, $p < 0.0001$; Fig. 2c). Whereas *PTEN* mutations were identified in four out of 183 breast cancers (2.2%), *PIK3CA* mutations were found in 63 out of 220 (29%), and *TP53* mutations in 92 out of 253 (36%) tumors analyzed (Table 1). Among the four tumors with *PTEN* mutations, two had *PTEN* gene expression above and two below the median (data not shown). No significant differences in *PTEN* or *PTENP1* gene expression were observed in subgroups stratified by ER, HER2, *PIK3CA*, or *TP53* mutation status or by comparison of triple-negative breast cancer (ER/PGR/HER2 negative; TNBC) vs. non-TNBC (data not shown). *TP53* gene expression was undetectable in seven out of 273 tumors (2.5%), and a significant correlation was observed between *TP53* and *PTEN* gene expression in these 273 tumors from Studies 1 to 3 where both transcripts were measured ($r_s = 0.227$, $p < 0.0002$). This correlation

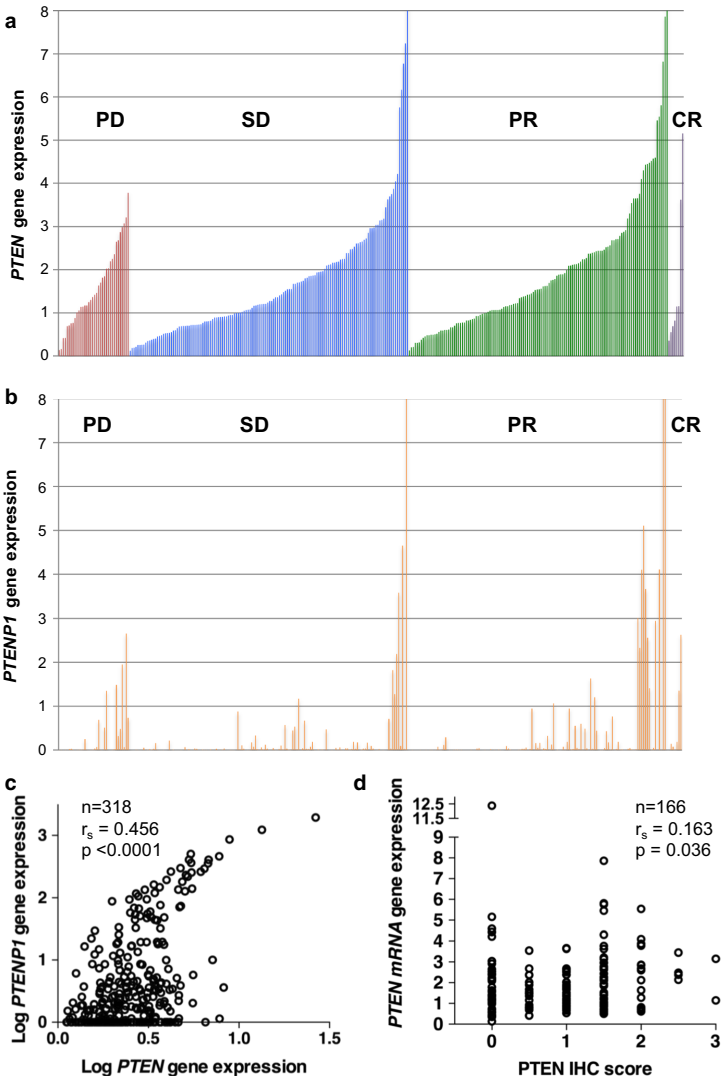


Fig. 2 **a** Gene expression of *PTEN* in locally advanced human breast cancers prior to starting neoadjuvant epirubicin, paclitaxel, doxorubicin, or 5-FU/mitomycin (FUMI), Studies 1–3 combined. Sorted by response group and increasing *PTEN* levels. **b** Gene expression of *PTEN pseudogene (PTENP1)* in locally advanced human breast cancers prior to starting neoadjuvant chemotherapy, sorted by response group and increasing *PTEN* levels (same as **a**). **c** Scatter plot depicting the correlation between *PTEN* and *PTENP1* gene expression in breast cancers from the epirubicin/paclitaxel, doxorubicin, FUMI trials combined. **d** Scatter plot depicting the correlation between *PTEN* gene expression and PTEN protein expression in breast cancers from the epirubicin/paclitaxel, doxorubicin, FUMI trials combined. *PTEN* and *PTENP1* mRNA levels in **a**–**d** are depicted as the mean gene expression of three separate real-time RT-PCR runs, as a fraction of *RPLP2* expression, and corrected for cDNA pool. Gene expression in **a**–**b** is not depicted beyond eight times the *RPLP2* expression to visualize better differences between the tumor samples. *PD* progressive disease, *SD* stable disease, *PR* partial response, *CR* complete response

between *TP53* and *PTEN* mRNA levels remained significant ($r_s = 0.150$, $p < 0.05$), if 47 out 212 tumors with known *TP53* or *PTEN* mutations (Study 3) were excluded from the analysis.

PTEN and PI3K pathway protein expression

IHC staining results for PTEN, and phosphorylated Akt (Ser 473), S6K (Ser 371 or Thr 389), and 4EBP1 (Thr 70) are summarized in Online Resource 2. High-quality immunostaining was observed for all antibodies used, apart from phosphorylated S6K (Thr 389) which yielded poor staining of the tissue microarrays. At the same time, it has been established previously that phosphorylation at the S6K Ser371 phosphorylation site is essential for Thr389 phosphorylation [19], indicating that the staining results for Ser371 should correlate to Thr389 staining. A weak correlation ($r_s = 0.163$, $p = 0.036$) was established between *PTEN* gene expression and the corresponding PTEN protein staining level in 166 tumors from which both RNA and TMA tissue blocks were available (Fig. 2d). However, there was no correlation between a low *PTEN* gene expression level and increased Akt (Ser 473) or S6K (Ser 371 or Thr 389) phosphorylation in breast cancers from which both RNA and IHC tissue samples were available for such comparisons ($n = 163$). Also, there was no correlation between the absence of PTEN protein staining and increased Akt (Ser 473) or S6K (Ser 371 or Thr 389) phosphorylation by comparison of IHC tissue samples (data not shown). "PI3K pathway activation," defined as two or more of the following: absent PTEN staining, phosphorylated Akt, phosphorylated S6K, and/or *PIK3CA* mutations, was observed in 117 out of 159 breast cancers in Study 3. *PTEN* gene expression was significantly higher ($p = 0.028$) in tumors with pathway activation, compared to tumors without pathway activation (data not shown). However, if split into ER-positive or ER-negative tumors, *PTEN* gene expression was not significantly higher in neither group in tumors with pathway activation. Akt phosphorylation was significantly more prevalent in tumors harboring *PIK3CA* mutations (27 out of 38 tumors), as compared to *PIK3CA* wild-type tumors (55 out of 132 tumors; $p = 0.002$, data not shown). However, there was no correlation between *PIK3CA* mutation status and the proportion of tumors with phosphorylation of S6K (Ser371), S6K (Thr389), or 4EBP1 further downstream in the PI3K pathway. In TNBC, a high frequency of absent PTEN staining, and low level of Akt-S6K-4EBP1 phosphorylation was observed, as expected for this breast cancer subtype (Online Resource 2). However, there was no significant difference in PTEN staining between TNBC and non-TNBC tumors (data not shown).

Predictive variables toward chemotherapy response

No association was recorded between pretreatment *PTEN* or *PTENP1* gene expression and response to neither of the chemotherapies given ($n = 320$ patients with stage 3/4 disease), irrespective of *TP53* mutation, *PIK3CA* mutation, HER2 or ER status (data not shown). Furthermore, no association between *PIK3CA* mutation status and response to chemotherapies was detected across the three trials ($n = 267$). Finally, the protein staining intensity for PTEN ($n = 179$), phosphorylated Akt ($n = 178$), S6K (Ser 371, $n = 173$), S6K (Thr 389, $n = 183$), and 4EBP1 ($n = 175$), yielded no predictive information toward chemotherapy response among patients in Study 3.

Prognostic impact of *PTEN* gene expression

Excluding patients with stage 4 disease from the analysis, high *PTEN* gene expression, defined as a *PTEN* mRNA level above the median, was associated with significantly shorter RFS (hazard ratio (HR) for recurrence 1.78, 95% confidence interval (CI) 1.26–2.50, $p = 0.001$), and DSS (HR for breast cancer-specific death 1.72, 95% CI 1.20–2.47, $p = 0.003$) across the pooled cohort of patients with stage 3 disease ($n = 282$, Fig. 3a–d). Among tumors wild-type for *TP53*, a high *PTEN* level remained a negative prognostic marker, with inferior RFS as well as DSS (HR 1.82, 95% CI 1.22–2.72, $p = 0.003$ and HR 1.78, 95% CI 1.16–2.73, $p = 0.009$, respectively; Figs. 3c, d, 4a, b). In contrast, no significant association between outcome and *PTEN* gene expression level was observed in patients with tumors harboring *TP53* mutations (Fig. 3c, d, 4c, d). These findings were consistent across each individual trial (Online Resource 3).

If stratified by ER status, high intratumoral *PTEN* gene expression was associated with inferior RFS (HR 2.20, 95% CI 1.41–3.44, $p = 0.001$) and DSS (HR 2.18, 95% CI 1.34–3.54, $p = 0.002$) among patients with ER-positive tumors only; no effect was observed among patients harboring ER negative tumors (Fig. 3c, d). Moreover, the negative prognostic impact of a high *PTEN* level was evident only in ER-positive tumors harboring wild-type *TP53* (Fig. 3c, d), with inferior RFS (HR 2.37, 95% CI 1.41–3.97, $p = 0.001$) and DSS (HR 2.30, 95% CI 1.31–4.04, $p = 0.004$). No prognostic impact of *PTEN* mRNA level was recorded in patients with ER-negative tumors, irrespective of *TP53* status (Fig. 3c, d). In contrast, *PTEN* gene expression above the median was associated with inferior survival outcome among both HER2 negative (RFS; HR 1.69, 95% CI 1.07–2.69, $p = 0.026$, DSS; HR 1.63, 95% CI 0.99–2.65, $p = 0.053$) and HER2-positive tumors (RFS; HR 2.52, 95% CI 1.07–5.91, $p = 0.034$, DSS; HR 3.16, 95% CI 1.19–8.39, $p = 0.021$, Fig. 3c, d).

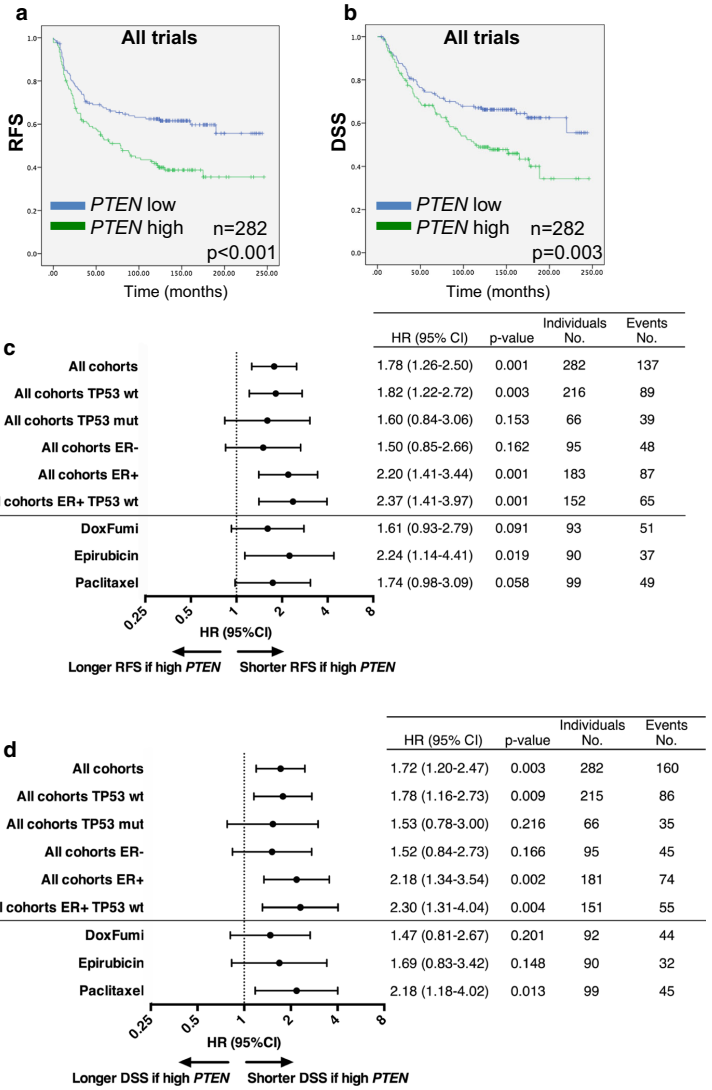


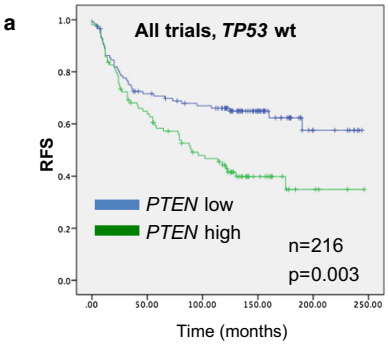
Fig. 3 **a**–**b** Recurrence-free (RFS) and disease-specific survival (DSS) after neoadjuvant chemotherapy in patients with locally advanced breast cancer after neoadjuvant epirubicin, paclitaxel, doxorubicin, or 5-FU/mitomycin (FUMI), Studies 1–3 combined. Groups are split by *PTEN* gene expression above or below the median. Censored values are marked with +. n indicates the number of patients used for the survival analysis. **c**–**d** Forest plot for the association between tumor *PTEN* gene expression level and recurrence-free (**c**) or disease-free survival (**d**) in patients with locally advanced breast cancer. Results are presented as individual hazard ratios (HRs) with corresponding 95% confidence intervals (CIs). HR > 1 indicates that the survival of patients with tumor *PTEN* gene expression above the median (*PTEN* high) is shorter than that of patients with *PTEN* low tumors, while HR < 1 indicates the opposite. *RFS* recurrence-free survival, *DSS* disease-specific survival, *wt* wild-type, *mut* mutated, *ER* estrogen receptor

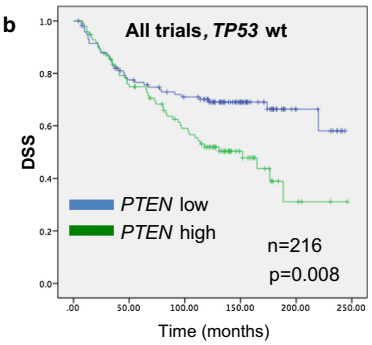
Finally, the negative prognostic impact of high *PTEN* mRNA levels was observed exclusively for *PIK3CA* wild-type tumors (RFS; HR 1.89, 95% CI 1.23–2.91, $p = 0.004$, DSS; HR 1.94, 95% CI 1.33–3.07, $p = 0.005$), with no impact of *PTEN* level in *PIK3CA* mutated tumors (Online Resource 3).

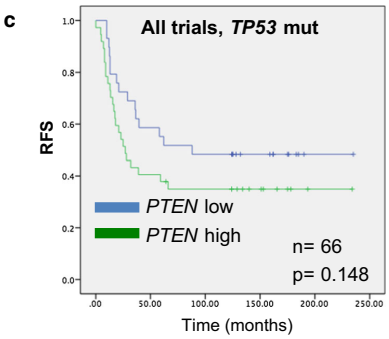
Patients with stage 4 disease ($n = 44$) were excluded from the above survival analysis. However, a high *PTEN* gene expression was associated with significantly shorter DSS (HR for breast cancer-specific death 2.06, 95% CI 1.08–3.01, $p = 0.027$) also for patients with primary metastatic disease (data not shown).

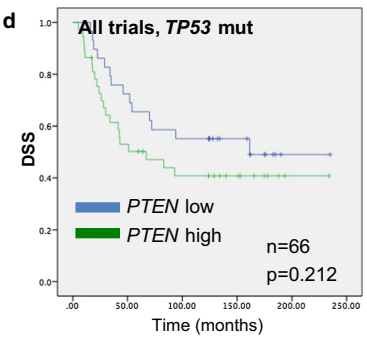
Validation using the cancer genome atlas (TCGA) public dataset

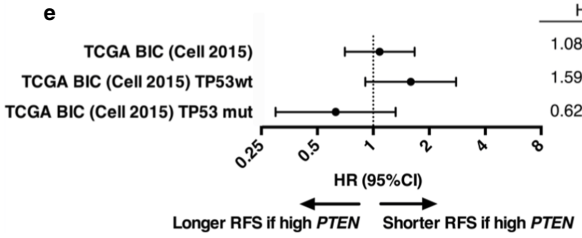
To validate our findings in another patient cohort, *PTEN* gene expression data were extracted from the cBioPortal database [20, 21], and normalized to *RPLP2* expression in the same dataset. These gene expression data are based on



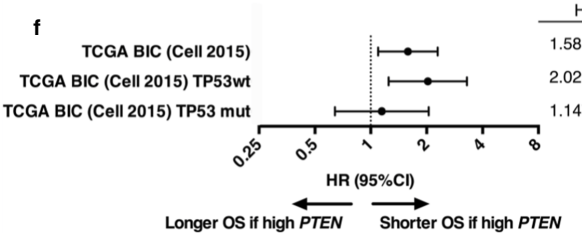








	HR (95% CI)	p-value	Individuals No.	Events No.
TCGA BIC (Cell 2015)	1.083 (0.703-1.668)	0.718	816	85
TCGA BIC (Cell 2015) TP53wt	1.591 (0.908-2.787)	0.105	536	52
TCGA BIC (Cell 2015) TP53 mut	0.627 (0.298-1.320)	0.219	280	33



	HR (95% CI)	p-value	Individuals No.	Events No.
TCGA BIC (Cell 2015)	1.585 (1.096-2.292)	0.014	816	118
TCGA BIC (Cell 2015) TP53wt	2.029 (1.249-3.297)	0.004	536	71
TCGA BIC (Cell 2015) TP53 mut	1.147 (0.641-2.053)	0.645	280	47

◀**Fig. 4 a–d** Recurrence-free (RFS) and disease-specific survival (DSS) after neoadjuvant chemotherapy in patients with locally advanced breast cancer after neoadjuvant epirubicin, paclitaxel, doxorubicin, or 5-FU/mitomycin (FUMI), Studies 1–3 combined. Groups are split by *PTEN* gene expression above or below the median, and stratified by *TP53* mutation status. Censored values are marked with +. *n* indicates the number of patients used for the survival analysis. **e–f** Forest plot for the association between tumor *PTEN* gene expression level and recurrence-free (**e**) or overall survival (**f**) in patients with early breast cancer with data extracted from the *The Cancer Genome Atlas (TCGA) Breast Invasive Carcinoma (Cell, 2015)* cohort. Results are presented as individual hazard ratios (HRs) with corresponding 95% confidence intervals (CIs). HR > 1 indicates that the survival of patients with tumor *PTEN* gene expression above the median (*PTEN* high) is shorter than that of patients with *PTEN* low tumors, while HR < 1 indicates the opposite. *RFS* recurrence-free survival, *OS* overall survival, *wt* wild-type, *mut* mutated

RNA sequencing in the *Breast Invasive Carcinoma (Cell 2015)* analysis [22], which are in whole based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/. Patient outcome for 816 patients with primary breast cancer was compared for tumors with *PTEN* mRNA levels above or below the median. A negative prognostic impact of high *PTEN* gene expression was observed for overall survival (OS) (HR 1.59, 95% CI 1.10–2.29, $p = 0.014$), but not for RFS (Fig. 4e, f). Among tumors wild-type for *TP53*, a high *PTEN* level remained a negative prognostic marker, with inferior OS (HR 2.03, 95% CI 1.25–3.30, $p = 0.004$; Fig. 4e, f). In contrast, no prognostic value was established for *PTEN* gene expression in tumors harboring *TP53* mutations. DNA sequencing data from the same cohort identified *PTEN* mutations in 42 tumors (5.1%), and 13 tumors thereof exhibited *PTEN* gene expression above and 29 tumors exhibited *PTEN* gene expression below the median. A weak negative correlation ($r_s = -0.090$, $p = 0.010$) was established between the presence of *PTEN* mutations and the corresponding *PTEN* gene expression level in the 816 tumors from the TCGA dataset.

Other prognostic variables

No survival difference was observed between patients with tumor *PTENP1* gene expression above or below the median within the pooled cohort of patients with stage 3 disease, nor within any of the subgroups (Online Resource 4). Also, there was no prognostic impact of *PTENP1* mRNA level in patients with stage 4 disease (data not shown). Similarly, no prognostic impact of either *PIK3CA* mutation status ($n = 238$), PTEN protein expression level ($n = 168$), phosphorylated Akt ($n = 167$), S6K ($n = 162$), or 4EBP1 ($n = 165$) assessed by immunohistochemistry was recorded with respect to RFS and DSS for patients with stage 3 disease (Online Resource 5). Further, in patients with stage 4 disease where tissue was available for IHC ($n = 18$), no correlation was observed between PTEN protein expression and DSS (data not shown).

Multivariate analysis

Multivariate analysis revealed *PTEN* expression level and *TP53* mutation status to be independent prognostic variables for RFS as well as DSS (Table 2). No significant interaction between *PTEN* mRNA level and *TP53* status with respect to outcome was recorded (Table 2).

Discussion

TP53 inactivating mutations are associated with resistance to anthracycline- and mitomycin-containing chemotherapy and poor prognosis in patients with locally advanced breast cancer [1–7]. Among *TP53* wild-type breast cancers revealing primary resistance to anthracyclines, mutations in the p53 upstream activator *CHEK2* [23] or low expression levels of *ATM* [24] have been observed. Yet, additional factors are known to influence p53 activation in response to genotoxic stress [25, 26]. One such factor is the PTEN protein encoded by the *PTEN* gene [10]. In the present

Table 2 Prognostic indicators of survival by multivariate analysis

Variable	Recurrence-free survival			Disease-specific survival		
	HR (95% CI)	*p* value	Events/patients	HR (95% CI)	*p* value	Events/patients
PTEN low	1.00	0.040	57/147	1.00	0.005	51/146[a]
PTEN high	1.48 (1.02–2.14)		80/135	1.69 (1.17–2.42)		70/135
TP53 wt	1.00	0.001	98/216	1.00	0.040	86/215[a]
TP53 mut	1.75 (1.24–2.46)		39/66	1.51 (1.02–2.24)		35/66
*Interaction PTEN*TP53*		0.927			0.776	

The parameters included in the multivariate analysis were *PTEN* gene expression (high vs. low) and *TP53* mutation status (wild-type vs. mutated)

wt wild-type, *mut* mutated, *HR* hazard ratio, *CI* confidence interval

[a] One case censored before the earliest event in a stratum for disease-free survival

work, we provide data demonstrating the negative prognostic role of high *PTEN* gene expression levels in tumor tissue from patients with locally advanced breast cancer. Notably, the prognostic role of *PTEN* was observed exclusively in patients whose tumors contain preserved *TP53* wild-type status, in accordance with the known functional crosstalk between PTEN and p53 [16, 25, 27–29]. Moreover, our data suggest that the biological impact of *PTEN* in human breast cancer is mediated via mRNA interactions, given a lack of prognostic impact of PTEN protein staining, and a lack of correlation between PTEN and PI3K-Akt-mTOR signaling activity.

To the best of our knowledge, the prognostic role of *PTEN* gene expression by qPCR has not been assessed in patients with breast cancer previously. In a study of 70 patients with stage 2 breast cancer, a gene expression profile of "PTEN loss," including reduced *PTEN* gene expression, was predictive of poor survival, whereas PTEN protein staining had no prognostic value [30]. However, *PTEN* gene expression was categorized only as up- or downregulated in this microarray analysis, with no further quantification [30]. Another study found *PTEN* gene expression to be significantly higher in 93 human breast cancer samples as compared to healthy breast tissue; however, the potential impact on survival was not assessed [31].

While our clinical data are provocative to suggest a negative prognostic role of high intratumoral *PTEN* gene expression in patients with stage 3 breast cancer, our findings were confirmed by mining the TCGA dataset, to extract RNA sequencing data from 816 patients with stage 1–3 breast cancer [22]. Again, inferior overall survival was observed among patients with high intratumoral *PTEN* mRNA levels, and in particular, for patients with *TP53* wild-type tumors. In this validation cohort, recurrence-free survival did not differ for patients with high versus low *PTEN* levels, as opposed to our findings. This could be attributed to a high proportion of stage 1–2 breast cancer in the TCGA cohort (74%), with a better prognosis, regardless of *PTEN* gene expression, compared to patients with high-risk stage 3 disease in our trials.

The biological reason why high *PTEN* gene expression was associated with an inferior prognosis in our clinical material remains to be elucidated. While a weak correlation between *PTEN* gene expression and PTEN protein staining was observed, PTEN protein levels had no prognostic impact, pointing to biological interactions at the mRNA level as a probable reason.

Firstly, PTEN and p53 influence each other at the transcriptional level as well as through protein interaction [25]. Apart from binding to and stabilizing the p53 protein [16], PTEN inhibits *MDM2* transcription, thus reducing MDM2-mediated p53 degradation [27]. Furthermore, p53 binds to the genomic *PTEN* locus and increases *PTEN* transcription [28, 29]. Notably, while we found *PTEN* and *TP53* to correlate at the mRNA expression level, this was observed among tumors harboring wild-type *TP53* only. Similar, *PTEN* expression correlated to outcome only among *TP53* wild-type tumors. Interestingly, in vitro data indicate that nuclear PTEN modulates the response to genotoxic stress by control of DNA repair in cancer cells with preserved p53 function [32]. While the role of PTEN as a regulator of PI3K cytoplasmic signaling has been extensively studied, the role of nuclear PTEN to influence cell cycle arrest and DNA repair remains less defined [33, 34]. However, the prognostic impact of PTEN protein staining did not differ if nuclear staining was assessed separately, as opposed to combined nuclear and cytoplasmic staining in the current patient cohort.

Secondly, *PTEN* mRNA share miRNA binding sites with multiple gene transcripts implicated in cancer progression [35], and high *PTEN* gene expression could skew the balance between these transcripts in a pro-tumorigenic manner by adsorbing miRNAs which would otherwise target and degrade important oncogenes [36]. Moreover, *PTEN* and the protein non-coding *PTEN pseudogene (PTENP1)* share multiple miRNA binding sites [17], and altering the *PTEN* mRNA level could influence *PTENP1* degradation by competing for the same miRNAs [17, 35]. *PTEN* and *PTENP1* could even interact via *PTENP1* antisense transcripts which bind to the *PTEN* promoter and reduce *PTEN* mRNA expression [37]. While being protein non-coding, *PTENP1* transcripts are biologically active and tumor suppressive in various solid cancers [17, 38–40]. Loss of *PTENP1* on chromosome 9p was identified in 11 out of 118 human breast cancers in data extracted from array-based comparative genomic hybridization databases by Poliseno et al. [17].

To the best of our knowledge, we present the first analysis of *PTENP1* gene expression in human breast cancer. We found *PTENP1* to be expressed in 222 out of 318 human breast cancer samples analyzed. However, the positive correlation between *PTEN* and *PTENP1* transcript levels established in the current report, and the known tumor inhibitory role of *PTENP1*, do not indicate that the negative prognostic impact of high *PTEN* levels is mediated via its pseudogene. Accordingly, no prognostic impact of *PTENP1* was observed in univariate analysis in our patient cohort.

Thirdly, methodological issues associated with immunohistochemistry, such as formalin fixation, antigen retrieval, antibody specificity, and inter-observer variability could explain the lack of strong correlation between *PTEN* mRNA and PTEN protein levels. In comparison, *PTEN* mRNA analysis was performed using a standardized qPCR assay with specific primers and validated PCR

products which were quantified independently of the observers.

PTEN is a known inhibitor of the growth-promoting PI3 K-Akt-mTOR pathway [9, 41], and lack of PTEN protein expression is generally associated with increased PI3K-Akt-mTOR signaling [9, 42]. While a significant association between PTEN and phosphorylated Akt by IHC was established previously in 655 breast cancers [43], such an association was not observed in another patient cohort [44], and there was no correlation between the loss of PTEN staining and increased Akt phosphorylation in neither of these two trials [43, 44]. In our current TMA analysis, negative PTEN staining was not associated with increased Akt or S6K phosphorylation levels in 163 locally advanced breast cancers, clearly indicating a lack of biological interaction between PTEN and the PI3K-Akt-mTOR pathway in this setting.

The lack of prognostic impact of PTEN protein expression among 168 patients in the current study is in accordance with several large clinical trials in early breast cancer [30, 43–46]. In the recent CLEOPATRA trial in HER2-positive metastatic breast cancer, a low PTEN protein expression was associated with worse OS, but at the same time an improved progression-free survival, whereas the presence of *PIK3CA* mutations was a definite negative prognostic marker [47]. In the BOLERO-2 trial, patients with ER-positive metastatic breast cancer experienced the same survival benefit from adding the mTOR inhibitor everolimus to exemestane, regardless of "PI3K activation", defined as low PTEN staining, or *AKT1*, *PIK3CA*, *PIK3R1* or *PTEN* mutations [48]. Finally, the prognostic impact of *PIK3CA* in breast cancer is not well established [49], and our data are consistent with the findings in a recent study, reporting no influence of *PIK3CA* mutation status on survival outcome among 1008 patients with breast cancer at high risk of relapse [50].

Conclusions

We establish that high *PTEN* gene expression in locally advanced human breast cancers is a marker of poor prognosis, across three neoadjuvant trials with 282 patients. Furthermore, the prognostic impact of *PTEN* gene expression is evident only among patients with *TP53* wild-type breast cancers. This should be examined further to assess whether the outcome of patients with these breast cancer characteristics could be improved by alternative therapeutic measures in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval The study protocols were approved by the Regional Committee for Medical and Health Research Ethics West, reference numbers 192/91-69.91, 39/92-69.91, 06/597, and 082.96. All patients provided informed consent before inclusion in the protocols, which also included future studies on prognostic and predictive factors.

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High *PTEN* gene expression is a negative prognostic marker in human primary breast cancers with preserved p53 function

Breast Cancer Research and Treatment

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Online Resource 1.

Basic genomic procedures

RNA was extracted from breast cancer samples using either Trizol (Invitrogen) (Studies 1-2) or the Qiagen RNeasy protocol (Study 3). RNA concentrations were measured by a NanoDrop spectrophotometer (Thermo Scientific), and cDNA was made from 500 ng of RNA using Transcriptor Reverse Transcriptase kit w/random hexamers (Roche). DNA for *PTEN* and *PIK3CA* analysis was extracted using the Qiagen DNeasy kit.

Real-time qPCR was performed using dual labeled hydrolysis probes (TIB MOLBIOL). The Light Cycler 480 (Roche) was used to detect the PCR products. Lack of gene expression was defined as lack of gene amplification after 37 cycles. Gene of interest was given as a ratio to the housekeeping gene *RPLP2*, and as a mean of three independent runs, normalized to a cDNA pool of 6 breast cancer cell lines. *PTEN* was amplified much earlier than *PTENP1* during the qPCR procedure, demonstrating higher absolute levels of *PTEN* than *PTENP1* transcripts. The relative *PTENP1/RPLP2* gene expression was therefore divided by 98 ($2^{\triangle Cp}$) since *PTENP1* appeared on average 6.62 cycles after *PTEN* (ΔCp) on real-time RT-PCR.

For each of the three trials patients were sorted based on increasing *PTEN* or *PTENP1* gene expression in the tumors, and the groups were split by gene expression above or below the median.

Gene expression of *PTEN* and the known transcribed, processed *PTENP1* [1] was analyzed using cDNA produced from DNAse-treated RNA. Due to the 98% sequence homology between *PTEN* and *PTENP1* [1], the specificity of all PCR reactions was verified by Sanger sequencing.

PTEN 3´UTR primers from [2]:

PTEN_F2: CTTCTCCATCTCCTGTGTAATCAA

PTEN_R2: GTTGACTGATGTAGGTACTAACAGCAT

PTEN_FAM: FAM6-CCAGTGCTAAAATTCA-BBQ

PTENP1 3´UTR:

PTENP1_F18: TGCAGTTAGCTAAGAGAAGTTTCTG

PTENP1_R20: CCATTCCCCTAACCCAAATAC

PTENP1_FAM: FAM6-AGGGTTTTGCTGCATTCTTGCAT-BBQ

RPLP2:

RPLP2_F	GACCGGCTCAACAAGGTTAT
RPLP2_R	CCCCACCAGCAGGTACAC
RPLP2_Cy5	Cy5-AGCTGAATGGAAAAAACATTGAAGACGTC-BBQ

TP53:

TP53_F	CGAGCACTGCCCAACAA
TP53_A	GCCTCATTCAGCTCTCGGAA
TP53_TM	FAM6-CACGGATCTGAAGGGTGAAATATTCTCCA—BBQ

Hot-spot *PIK3CA* mutations in exons 10 and 21 (previously exon 9 and 20) [3] were assessed in 275 pre-treatment breast cancer samples from Studies 1-3; tumor DNA was used as PCR template with the primers listed below. *PTEN* mutation status was assessed in 183 pre-treatment breast cancer samples from Study 3, using cDNA as the template for nested PCR with the primers listed below. PCR products were analyzed at Center for Medical Genetics and Molecular Medicine, Haukeland University Hospital, by Sanger sequencing using the BigDye v1.1 reaction mix (Applied Biosystems).

PIK3CA sequencing primers:

PI3KCA_ex10_F	TGAAAATGTATTTGCTTTTTCTG
PI3KCA_ex10_R	ACATGCTGAGATCAGCCAAA
PI3KCA_ex10_F2	GGGAAAAATATGACAAAGAAAGC
PI3KCA_ex10_R2	CTGAGATCAGCCAAATTCAGTT
PI3KCA_ex10_F3	GGAAAAATATGACAAAGAAAGCTATATAAG
PI3KCA_ex10_R3	ACAGAGAATCTCCATTTTAGCAC
PI3KCA_ex21_F	CATTTGCTCCAAACTGACCA
PI3KCA_ex21_R	CCTGCTGAGAGTTATTAACAGTGC
PI3KCA_ex21_F2	GCTCCAAACTGACCAAACTGTTC
PI3KCA_ex21_R2	TGGAATCCAGAGTGAGCTTTC

PI3KCA_ex21_seqF	CTCAATGATGCTTGGCTCTG
PI3KCA_ex21_seqR	AGAAAATGAAAGCTCACTCTG
PI3KCA_ex21_seqF0	GGAGATGTGTTACAAGGCTTATCTA
PI3KCA_ex21_seqR2	GCATTGAACTGAAAAGATAACTGAGAAA

PTEN sequencing primers (nested PCR):

PTEN outerF	TCCAGAGCCATTTCCATC
PTEN outerR	TGTCAAAACCCTGTGGATG

PTEN inner primerF	CTCCTCCTTTTTCTTCAGC
PTEN inner primerR	CAAGAGGGATAAAACACCAT

PTEN sequencingF	GAGTAACTATTCCCAGTCAGAGG
PTEN sequencingR	AACTGAGGATTGCAAGTTCC

Immunohistochemistry (IHC) and *in situ* hybridization (ISH)

Tissue microarrays (TMAs) were created from Study 3 with quadruplicate 1 mm cores from formalin-fixed paraffin embedded (FFPE) tumor material collected at the time of diagnosis. Briefly, tumor sections (4 μm) from the TMAs were de-paraffinized and rehydrated, before antigen retrieval at 98°C for 1 hour in DAKO Target Retrieval Buffer (pH 6.0 or 9.0). After blocking with diluted goat serum for 30 min, the slides were incubated overnight (+4°C) with the primary antibody. After blocking endogenous peroxidase activity for 15 min with DAKO peroxidase block, a biotinylated goat anti-rabbit secondary antibody was applied for 30 min (Vector Laboratories). The antigen-antibody complex was revealed with avidin-biotin-peroxidase (ABC) for 30 min according to the manufacturer's instructions (Vectastain® ABC Kit, Vector). The immune reaction was visualized by incubation with diamino-benzidine tetrahydrochloride (Vector). The sections were then counterstained with haematoxylin (Fisher), dehydrated and mounted with Entellan (Electron Microscopy Services). Parallel sections were run for all the experiments without primary antibody, to assure the specificity of the immune reactions.

HER2 assessment was performed according to international guidelines [4], using the Ventana-Roche HercepTest and the INFORM HER2 Dual ISH DNA Probe Cocktail (Roche) assays. Briefly, HER2 immunostaining (HercepTest) scores 0 and 1 were classified as HER2 negative, score 2 as equivocal and score 3 as HER2 positive. Biopsies with equivocal results were analyzed by dual-color dual-hapten brightfield *in situ* hybridization (DDISH, Ventana-Roche) using the INFORMER HER2 Dual ISH DNA Probe Cocktail, the UltraView Red ISH DIG Detection and ultraView SISH DNP Detection Kits. HER2 and chromosome 17 centromere (CEP17) were counted in 20 tumor cell nuclei and specimens with equivocal ratio HER2/CEP17 (1,8-2,2) an additional 20 nuclei were counted. A ratio below 1.8 was considered negative for Her2, and above 1.8 was considered HER2 amplified and therefore HER2 positive.

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Online Resource 2.

		All samples		ER+		ER-		TNBC		TP53 wt		TP53 mut		PIK3CA wt		PIK3CA mut	
	Score	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
PTEN (c+n)*	0	55	29	23	22	32	40	13	36	31	23	18	49	43	32	6	17
	0.5	20	11	9	9	11	14	7	19	15	11	4	11	15	11	4	11
	1	38	20	19	18	18	22	7	19	30	22	6	16	30	22	6	17
	1.5	48	26	33	32	14	17	6	17	40	30	5	14	31	23	14	39
	2	17	9	13	13	4	5	1	3	13	10	3	8	11	8	5	14
	2.5	5	3	4	4	1	1	1	3	4	3	0	0	4	3	0	0
	3	4	2	3	3	1	1	1	3	1	1	1	3	1	1	1	3
	Total	187	100	104	100	81	100	36	100	134	100	37	100	135	100	36	100
PTEN (c)	0	64	34	29	28	35	43	14	39	40	30	18	49	50	37	8	22
	1	88	47	52	50	34	42	17	47	72	54	10	27	63	47	19	53
	2	30	16	20	19	10	12	4	11	21	16	7	19	21	16	7	19
	3	5	3	3	3	2	2	1	3	1	1	2	5	1	1	2	6
	Total	187	100	104	100	81	100	36	100	134	100	37	100	135	100	36	100
PTEN (n)	0	76	41	30	29	46	57	21	58	44	33	25	68	58	43	11	31
	1	40	21	22	21	17	21	6	17	31	23	7	19	30	22	8	22
	2	59	32	43	41	15	19	7	19	52	39	3	8	41	30	14	39
	3	12	6	9	9	3	4	2	6	7	5	2	5	6	4	3	8
	Total	187	100	104	100	81	100	36	100	134	100	37	100	135	100	36	100
pAkt (Ser473) (n)	0	96	52	49	47	46	58	21	60	67	50	21	57	77	58	11	29
	1	50	27	30	29	19	24	10	29	36	27	10	27	34	26	12	32
	2	36	19	23	22	13	16	4	11	28	21	5	14	20	15	13	34
	3	4	2	2	2	2	3	0	0	2	2	1	3	1	1	2	5
	Total	186	100	104	100	80	100	35	100	133	100	37	100	132	100	38	100
pS6K (Ser371) (n)	0	49	27	12	12	36	44	18	50	30	23	16	43	35	27	11	32
	1	53	29	25	26	27	33	12	33	40	31	9	24	41	31	8	24
	2	76	42	58	59	18	22	6	17	56	44	12	32	54	41	14	41
	3	3	2	3	3	0	0	0	0	2	2	0	0	1	1	1	3
	Total	181	100	98	100	81	100	36	100	128	100	37	100	131	100	34	100
pS6K (Thr389) (n)	0	177	93	94	88	81	99	35	97	128	93	34	92	125	93	37	95
	1	9	5	9	8	0	0	0	0	5	4	3	8	6	4	2	5
	2	5	3	4	4	1	1	1	3	4	3	0	0	4	3	0	0
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Total	191	100	107	100	82	100	36	100	137	100	37	100	135	100	39	100
p4EBP1 (Thr70) (c)	0	14	8	6	6	8	10	5	14	11	8	2	5	10	8	3	8
	1	70	38	44	44	24	30	8	22	52	40	10	27	44	34	18	49
	2	83	45	41	41	42	52	21	58	56	43	22	59	63	48	15	41
	3	16	9	9	9	7	9	2	6	11	8	3	8	13	10	1	3
	Total	183	100	100	100	81	100	36	100	130	100	37	100	130	100	37	100

Immunohistochemistry results for PTEN and phosphorylated Akt (pAkt, Ser473), S6K (pS6K, Ser371 or Thr389) and 4EBP1 (p4EBP1, Thr70) proteins. Staining was performed on tissue microarray sections of pretreatment breast cancer samples from Study 3, and results are summarized for all samples, or subgrouped based on estrogen receptor (ER) status, triple negative breast cancer status (TNBC;

ER/PGR/HER2 negative breast cancer), *TP53* or *PIK3CA* mutation status. (c) = cytoplasmic staining. (n) = nuclear staining. *PTEN (c+n) score is given as (c+n)/2.

High *PTEN* gene expression is a negative prognostic marker in human primary breast cancers with preserved p53 function

Breast Cancer Research and Treatment

Authors:

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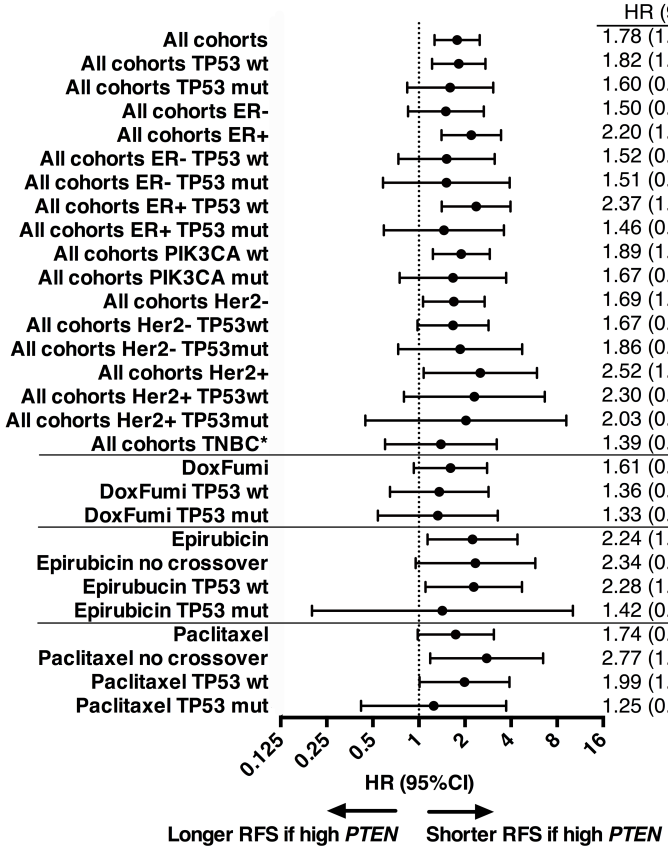
Online Resource 3

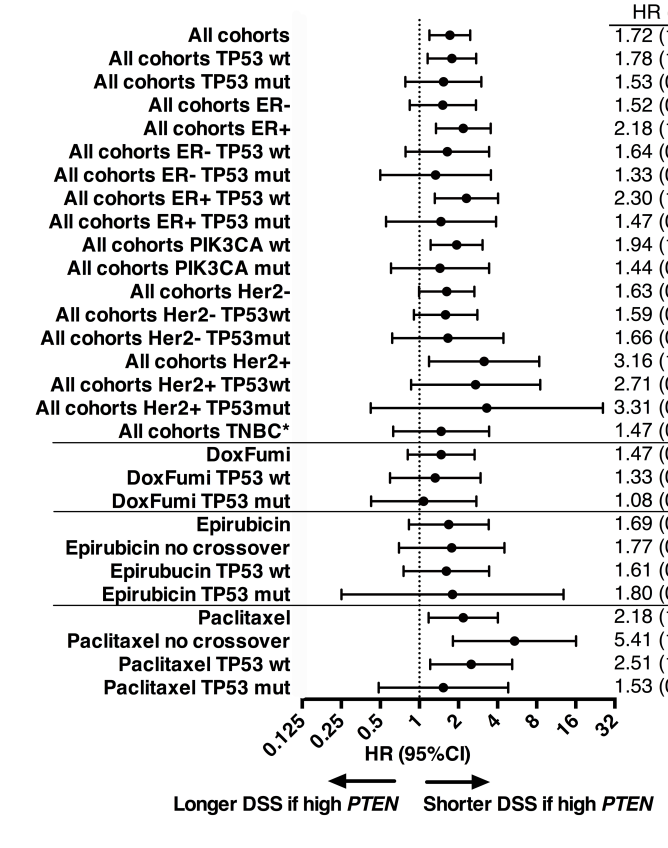
a-b Forest plot for the association between tumor *PTEN* gene expression level and recurrence-free (**a**) or disease-free survival (**b**) in patients with locally advanced breast cancer. Results are presented as individual hazard ratios (HRs) with corresponding 95% confidence intervals (CIs) for Study 1 (doxorubicin trial), Study 2 (FUMI trial) and Study 3 (epirubicin/paclitaxel trial) combined (i.e. all cohorts) or split by subgroups. HR>1 indicates that the survival of patients with tumor *PTEN* gene expression above the median (*PTEN* high) is shorter than that of patients with *PTEN* low tumors, while HR<1 indicates the opposite. RFS: recurrence-free survival, DSS: disease-specific survival, wt: wildtype, mut: mutated, ER: estrogen receptor, PGR: progesterone receptor, TNBC: triple negative breast cancer (ER/PGR/HER2 negative breast cancer), *for patients in Study 1 and 2 PGR status was not available, and TNBC was defined as ER/HER2 negative tumors

a



	HR (95% CI)	p-value	Individuals No.	Events No.
All cohorts	1.78 (1.26-2.50)	0.001	282	137
All cohorts TP53 wt	1.82 (1.22-2.72)	0.003	216	89
All cohorts TP53 mut	1.60 (0.84-3.06)	0.153	66	39
All cohorts ER-	1.50 (0.85-2.66)	0.162	95	48
All cohorts ER+	2.20 (1.41-3.44)	0.001	183	87
All cohorts ER- TP53 wt	1.52 (0.74-3.13)	0.260	60	31
All cohorts ER- TP53 mut	1.51 (0.58-3.92)	0.395	35	17
All cohorts ER+ TP53 wt	2.37 (1.41-3.97)	0.001	152	65
All cohorts ER+ TP53 mut	1.46 (0.59-3.60)	0.413	31	22
All cohorts PIK3CA wt	1.89 (1.23-2.91)	0.004	181	91
All cohorts PIK3CA mut	1.67 (0.75-3.72)	0.210	57	27
All cohorts Her2-	1.69 (1.07-2.69)	0.026	138	76
All cohorts Her2- TP53wt	1.67 (0.98-2.85)	0.060	104	58
All cohorts Her2- TP53mut	1.86 (0.73-4.73)	0.192	34	18
All cohorts Her2+	2.52 (1.07-5.91)	0.034	58	29
All cohorts Her2+ TP53wt	2.30 (0.80-6.64)	0.122	39	16
All cohorts Her2+ TP53mut	2.03 (0.45-9.20)	0.359	19	13
All cohorts TNBC*	1.39 (0.60-3.22)	0.439	48	23
DoxFumi	1.61 (0.93-2.79)	0.091	93	51
DoxFumi TP53 wt	1.36 (0.65-2.85)	0.417	65	29
DoxFumi TP53 mut	1.33 (0.54-3.28)	0.536	28	22
Epirubicin	2.24 (1.14-4.41)	0.019	90	37
Epirubicin no crossover	2.34 (0.95-5.76)	0.064	61	22
Epirubucin TP53 wt	2.28 (1.11-4.72)	0,026	74	33
Epirubicin TP53 mut	1.42 (0.20-10.13)	0,724	16	4
Paclitaxel	1.74 (0.98-3.09)	0.058	99	49
Paclitaxel no crossover	2.77 (1.19-6.47)	0.019	57	25
Paclitaxel TP53 wt	1.99 (1.01-3.91)	0.047	77	36
Paclitaxel TP53 mut	1.25 (0.42-3.72)	0.689	22	13

b



	HR (95% CI)	p-value	Individuals No.	Events No.
All cohorts	1.72 (1.20-2.47)	0.003	282	160
All cohorts TP53 wt	1.78 (1.16-2.73)	0.009	215	86
All cohorts TP53 mut	1.53 (0.78-3.00)	0.216	66	35
All cohorts ER-	1.52 (0.84-2.73)	0.166	95	45
All cohorts ER+	2.18 (1.34-3.54)	0.002	181	74
All cohorts ER- TP53 wt	1.64 (0.78-3.45)	0.189	60	29
All cohorts ER- TP53 mut	1.33 (0.50-3.56)	0.565	35	16
All cohorts ER+ TP53 wt	2.30 (1.31-4.04)	0.004	151	55
All cohorts ER+ TP53 mut	1.47 (0.55-3.89)	0.442	31	19
All cohorts PIK3CA wt	1.94 (1.33-3.07)	0.005	181	80
All cohorts PIK3CA mut	1.44 (0.60-3.44)	0.410	56	22
All cohorts Her2-	1.63 (0.99-2.65)	0.053	137	67
All cohorts Her2- TP53wt	1.59 (0.91-2.80)	0.105	104	52
All cohorts Her2- TP53mut	1.66 (0.62-4.45)	0.316	34	16
All cohorts Her2+	3.16 (1.19-8.39)	0.021	58	26
All cohorts Her2+ TP53wt	2.71 (0.86-8.53)	0.088	39	15
All cohorts Her2+ TP53mut	3.31 (0.42-25.93)	0.255	19	11
All cohorts TNBC*	1.47 (0.63-3.45)	0.628	48	22
DoxFumi	1.47 (0.81-2.67)	0.201	92	44
DoxFumi TP53 wt	1.33 (0.59-2.96)	0.493	64	25
DoxFumi TP53 mut	1.08 (0.42-2.75)	0.875	28	19
Epirubicin	1.69 (0.83-3.42)	0.148	90	32
Epirubicin no crossover	1.77 (0.70-4.52)	0.229	61	19
Epirubucin TP53 wt	1.61 (0.76-3.45)	0.216	74	28
Epirubicin TP53 mut	1.80 (0.25-12.90)	0.560	16	4
Paclitaxel	2.18 (1.18-4.02)	0.013	99	45
Paclitaxel no crossover	5.41 (1.81-16.13)	0.002	56	21
Paclitaxel TP53 wt	2.51 (1.21-5.20)	0.013	77	33
Paclitaxel TP53 mut	1.53 (0.49-4.8)	0.486	22	12

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Breast Cancer Research and Treatment

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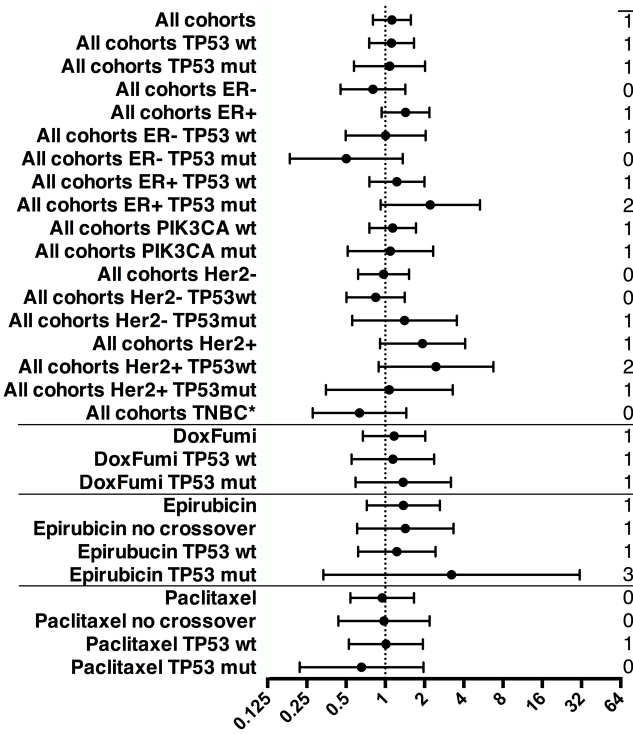
Online Resource 4

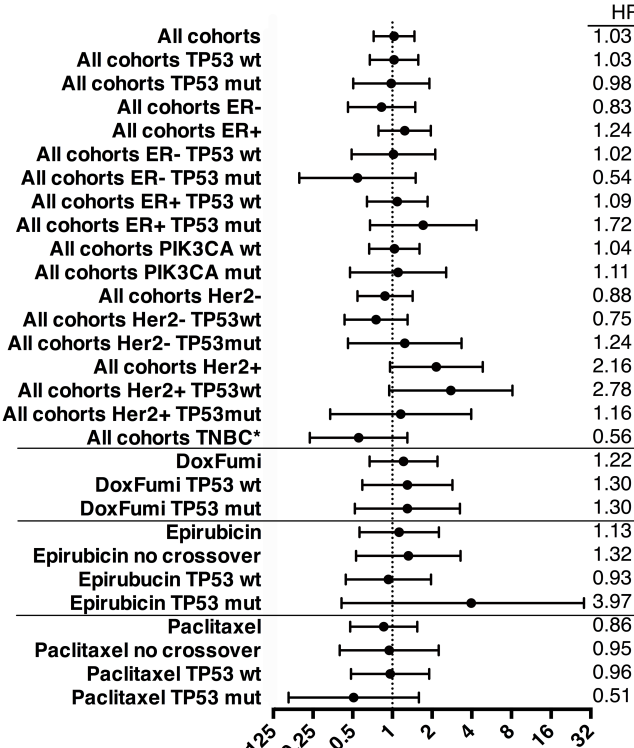
a-b Forest plot for the association between tumor *PTEN pseudogene* (*PTENP1)* gene expression level and recurrence-free (**a**) or disease-free survival (**b**) in patients with locally advanced breast cancer. Results are presented as individual hazard ratios (HRs) with corresponding 95% confidence intervals (CIs) for Study 1 (doxorubicin trial), Study 2 (FUMI trial) and Study 3 (epirubicin/paclitaxel trial) combined (i.e. all cohorts) or split by subgroups. HR>1 indicates that the survival of patients with tumor *PTENP1* gene expression above the median (*PTENP1* high) is shorter than that of patients with *PTENP1* low tumors, while HR<1 indicates the opposite. RFS: recurrence-free survival, DSS: disease-specific survival, wt: wildtype, mut: mutated, ER: estrogen receptor, PGR: progesterone receptor, TNBC: triple negative breast cancer (ER/PGR/HER2 negative breast cancer), *for patients in Study 1 and 2 PGR status was not available, and TNBC was defined as ER/HER2 negative tumors. **one case was censored before the earliest event in a stratum

a



	HR (95% CI)	p-value	Individuals No.	Events No.
All cohorts	1.12 (0.80-1.57)	0.496	282	137
All cohorts TP53 wt	1.12 (0.75-1.66)	0.583	216	98
All cohorts TP53 mut	1.08 (0.58-2.02)	0.811	66	39
All cohorts ER-	0.80 (0.45-1.42)	0.455	95	48
All cohorts ER+	1.43 (0.94-2.18)	0.098	183	87
All cohorts ER- TP53 wt	1.01 (0.50-2.04)	0.986	60	31
All cohorts ER- TP53 mut	0.50 (0.19-1.36)	0.176	35	17
All cohorts ER+ TP53 wt	1.23 (0.75-2.00)	0.411	152	65
All cohorts ER+ TP53 mut	2.22 (0.92-5.33)	0.076	31	22
All cohorts PIK3CA wt	1.14 (0.75-1.72)	0.539	181	91
All cohorts PIK3CA mut	1.09 (0.51-2.33)	0.816	57	27
All cohorts Her2-	0.97 (0.62-1.52)	0.898	138	76
All cohorts Her2- TP53wt	0.84 (0.50-1.41)	0.516	104	58
All cohorts Her2- TP53mut	1.40 (0.56-3.54)	0.472	34	18
All cohorts Her2+	1.93 (0.91-4.10)	0.086	58	29
All cohorts Her2+ TP53wt	2.45 (0.89-6.75)	0.083	39	16
All cohorts Her2+ TP53mut	1.08 (0.35-3.29)	0.899	19	13
All cohorts TNBC*	0.63 (0.28-1.45)	0.280	48	23
DoxFumi	1.17 (0.67-2.03)	0.583	93	51
DoxFumi TP53 wt	1.14 (0.55-2.37)	0.721	65	29
DoxFumi TP53 mut	1.37 (0.59-3.19)	0.462	28	22
Epirubicin	1.38 (0.72-2.63)	0.333	90	37
Epirubicin no crossover	1.43 (0.61-3.34)	0.413	61	22
Epirubucin TP53 wt	1.22 (0.62-2.43)	0.558	74	33
Epirubicin TP53 mut	3.23 (0.34-31.01)	0.310	16	4
Paclitaxel	0.95 (0.54-1.66)	0.848	99	49
Paclitaxel no crossover	0.98 (0.44-2.19)	0.958	57	25
Paclitaxel TP53 wt	1.01 (0.53-1.95)	0.972	77	36
Paclitaxel TP53 mut	0.66 (0.22-1.97)	0.452	22	12

b



	HR (95% CI)	p-value	Individuals No.	Events No.
All cohorts	1.03 (0.72-1.47)	0.87	281**	121
All cohorts TP53 wt	1.03 (0.67-1.57)	0.895	215	86
All cohorts TP53 mut	0.98 (0.01-1.91)	0.982	66	35
All cohorts ER-	0.83 (0.46-1.49)	0.533	95	45
All cohorts ER+	1.24 (0.79-1.96)	0.355	182	74
All cohorts ER- TP53 wt	1.02 (0.49-2.12)	0.96	60	29
All cohorts ER- TP53 mut	0.54 (0.20-1.50)	0.24	35	16
All cohorts ER+ TP53 wt	1.09 (0.64-1.85)	0.748	151	55
All cohorts ER+ TP53 mut	1.72 (0.68-4.35)	0.256	31	19
All cohorts PIK3CA wt	1.04 (0.67-1.61)	0.875	181	80
All cohorts PIK3CA mut	1.11 (0.48-2.56)	0.816	56	22
All cohorts Her2-	0.88 (0.54-1.42)	0.602	137	67
All cohorts Her2- TP53wt	0.75 (0.43-1.30)	0.310	104	52
All cohorts Her2- TP53mut	1.24 (0.46-3.34)	0.669	34	16
All cohorts Her2+	2.16 (0.96-4.85)	0.63	58	26
All cohorts Her2+ TP53wt	2.78 (0.95-8.15)	0.062	39	15
All cohorts Her2+ TP53mut	1.16 (0.34-3.98)	0.815	19	11
All cohorts TNBC*	0.56 (0.24-1.30)	0.175	48	22
DoxFumi	1.22 (0.67-2.20)	0.519	92	44
DoxFumi TP53 wt	1.30 (0.59-2.85)	0.514	65	25
DoxFumi TP53 mut	1.30 (0.52-3.23)	0.574	28	19
Epirubicin	1.13 (0.56-2.25)	0.737	90	32
Epirubicin no crossover	1.32 (0.53-3.29)	0.549	61	19
Epirubucin TP53 wt	0.93 (0.44-1.96)	0.853	74	28
Epirubicin TP53 mut	3.97 (0.41-28.42)	0.233	16	4
Paclitaxel	0.86 (0.48-1.54)	0.614	99	45
Paclitaxel no crossover	0.95 (0.40-2.25)	0.904	57	21
Paclitaxel TP53 wt	0.96 (0.49-1.91)	0.913	77	33
Paclitaxel TP53 mut	0.51 (0.16-1.59)	0.245	22	12

High *PTEN* gene expression is a negative prognostic marker in human primary breast cancers with preserved p53 function

Breast Cancer Research and Treatment

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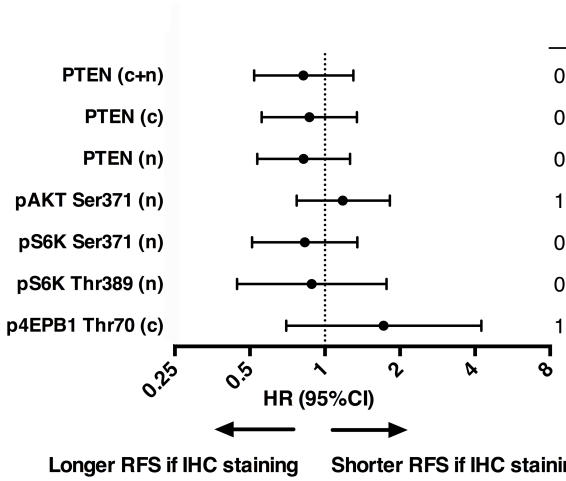
Online Resource 5

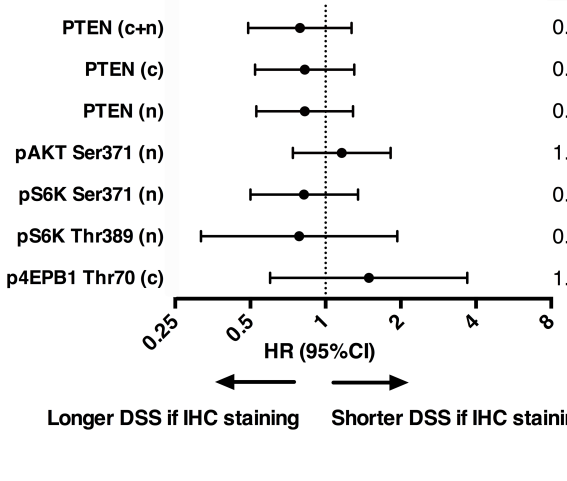
a-b Forest plot for the association between tumor protein staining by immunohistochemistry (IHC) and recurrence-free (**a**) or disease-free survival (**b**) in patients with locally advanced breast cancer. Results are presented as individual hazard ratios (HRs) with corresponding 95% confidence intervals (CIs), based on tissue microarray staining and survival data from Study 3 (epirubicin/paclitaxel trial). HR>1 indicates that the survival of patients with protein staining is shorter than that of patients without protein staining in the tumors, while HR<1 indicates the opposite. Number of samples analyzed per protein vary due to technical issues during tissue microarray staining. *One case censored before the earliest event in a stratum. RFS: recurrence-free survival, DSS: disease-specific survival, wt: wildtype, mut: mutated, ER: estrogen receptor, pAkt: phosphorylated Akt (Ser 473), pS6K (Ser 371): phosphorylated S6 kinase (Ser 371), pS6K (Thr 389): phosphorylated S6 kinase (Thr 389), p4EBP1: phosphorylated 4EBP1 (Thr 70), c: cytoplasmic staining, n: nuclear staining

a



	HR (95% CI)	p-value	Individuals No.	Events No.	No staining Reference	Staining Indicator
PTEN (c+n)	0.82 (0.52-1.30)	0.407	168*	86	49	120
PTEN (c)	0.87 (0.56-1.35)	0.521	168*	86	58	111
PTEN (n)	0.82 (0.54-1.26)	0.367	168*	86	68	101
pAKT Ser371 (n)	1.18 (0.77-1.82)	0.452	167*	83	89	79
pS6K Ser371 (n)	0.83 (0.51-1.35)	0.456	162*	82	42	121
pS6K Thr389 (n)	0.89 (0.44-1.77)	0.885	172*	88	155	18
p4EPB1 Thr70 (c)	1.72 (0.70-4.24)	0.242	165*	83	14	152

b



	HR (95% CI)	p-value	Individuals No.	Events No.	No staining Reference	Staining Indicator
PTEN (c+n)	0.79 (0.49-1.27)	0.333	168*	80	49	120
PTEN (c)	0.83 (0.52-1.30)	0.408	168*	80	58	111
PTEN (n)	0.83 (0.53-1.29)	0.396	168*	80	68	101
pAKT Ser371 (n)	1.16 (0.74-1.82)	0.510	167*	77	89	79
pS6K Ser371 (n)	0.82 (0.50-1.35)	0.430	162*	77	42	121
pS6K Thr389 (n)	0.78 (0.32-1.94)	0.598	172*	81	161	12
p4EPB1 Thr70 (c)	1.49 (0.60-3.69)	0.388	165*	78	14	152

II

Activation of Akt characterizes estrogen receptor positive human breast cancers which respond to anthracyclines

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ABSTRACT

Anthracyclines are key components of human breast cancer chemotherapy. Here, we explored the role of Akt signaling in anthracycline resistance.

The antitumor activity of doxorubicin and Akt inhibitor A-443654 alone or combined was examined in estrogen receptor (ER) positive and negative human breast cancer cell lines. Further, we examined mRNA changes induced by anthracyclines in locally advanced breast cancers biopsied before and after treatment in two clinical trials.

Doxorubicin increased Akt phosphorylation in ER positive MCF7 and T47D cell lines, with no effect in ER negative MDA-MB231 breast cancer cells. A-443654 was significantly more cytotoxic in doxorubicin-resistant compared to doxorubicin-naïve MCF7. This difference was not observed in MDA-MB231. Among 24 patients, *AKT1* gene expression increased 24 hrs after the initial epirubicin exposure in ER positive tumors responding to therapy (n=6), as compared to ER positive non-responders (n=7) or ER negative tumors (n=11). In contrast, *AKT1* mRNA changes after 16 weeks of doxorubicin were unrelated to clinical response and ER status (n=30).

In conclusion, rapid Akt activation was observed in ER positive breast cancers which responded to anthracyclines. Increased cytotoxicity of A-443654 in doxorubicin-resistant MCF7 cells indicates a possible role for Akt inhibitors in ER positive breast cancers where chemoresistance evolves.

INTRODUCTION

Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR)-S6 kinase (S6K) signaling (in short: PI3K signaling) is upregulated in 25% of human breast cancers and has been associated with resistance to endocrine as well as HER2 directed therapy [1–3]. *PIK3CA*, encoding the p110α subunit of PI3K, harbors activating mutations in up to 45% of luminal A breast cancers [4], which are typically estrogen receptor (ER) positive tumors. Thus, therapeutic inhibition of the PI3K signaling pathway with the mTOR inhibitor everolimus can be used to counteract acquired resistance to aromatase inhibitors and prolong survival among patients with ER positive breast cancer [3]. Moreover, activating *PIK3CA* mutations are observed in ER negative breast cancer as well [4], and mTOR inhibition, combined with trastuzumab and paclitaxel, prolonged progression-free survival significantly among patients with hormone receptor negative, HER2 positive breast cancer [5]. However, the potential to treat chemoresistant breast cancer by inhibiting PI3K signaling has not been thoroughly addressed thus far.

Phosphatase and tensin homolog (PTEN) is the main endogenous inhibitor of PI3K activation [6]. While experimental studies revealed loss of PTEN function to be associated with reduced sensitivity to doxorubicin in breast and prostate cancer models, chemosensitivity was restored by concomitant mTOR inhibition [7, 8]. Furthermore, increased Akt phosphorylation is observed in doxorubicin-resistant ER positive, but not in ER negative breast cancer cell lines [9–11]. In line with this, inhibitors of the PI3K-Akt-mTOR pathway can be employed to enhance anthracycline sensitivity in ER positive breast

cancers [10, 11] Whereas the introduction of Akt inhibitors in clinical trials has been slower than PI3K and mTOR inhibitors [12], the key position of Akt as a signal hub for important pro-tumorigenic pathways [6] makes such trials highly relevant.

In the present work we assessed the influence of doxorubicin treatment on PTEN and Akt-mTOR-S6K signaling, and the interaction between doxorubicin and the Akt inhibitor A-443654 in ER positive and negative human breast cancer cell lines *in vitro* and *in vivo*. In particular, cell lines made resistant to doxorubicin by continous drug exposure were compared with doxorubicin-naïve cells to decipher the role of Akt-mTOR-S6K signaling in breast cancer chemoresistance. Furthermore, the short-term and long-term changes in *PTEN* and *AKT1* gene expression subsequent to anthracycline exposure were assessed in patients with locally advanced breast cancers.

RESULTS

Influence of doxorubicin treatment on Akt activity and PI3K signaling in doxorubicin-naïve breast cancer cell lines

A sublethal concentration of doxorubicin (24 hrs exposure) was established by the WST-1 assay, to facilitate subsequent assessment of increased cytotoxicity when the A-443654 Akt inhibitor was introduced. The IC30 was approximately 1.5-2.0 μM for MB231 and MCF7 and 0.5-1.0 μM for T47D (Supplementary Figure 1A). Based on this, doxorubicin was used at a concentration of 1.5 μM for MB231, 2 μM for MCF7 and 0.7 μM for T47D for the *in vitro* experiments. Each experimental setup was conducted in three parallel cell cultures.

Doxorubicin increased phosphorylated Akt (p-Akt) in the ER positive MCF7 and T47D human breast cancer cell lines (Figure 1B, Supplementary Figure 1B). In contrast, p-Akt was not influenced by doxorubicin in the ER negative MB231 cell line (Figure 1A). Whereas doxorubicin had no impact on PTEN protein levels in neither cell line, mTOR phosphorylation levels increased in MB231 and decreased in MCF7 cells (Figure 1A-1B), although not significant by densitometry (Figure 1E-1F). Phosphorylated S6K was weakly expressed in both cell lines, and a non-significant decrease in S6K phosphorylation levels was observed in the MCF7 cell line after doxorubicin exposure (Figure 1A-1B).

AKT1 mRNA levels as determined by qPCR analysis remained unaltered 24 hours after doxorubicin exposure in all three cell lines (Supplementary Figure 2A). While *PTEN* mRNA levels decreased in MB231 cells, no change was observed in MCF7 and T47D cells subsequent to doxorubicin treatment (Supplementary Figure 2A). The reason why decreased *PTEN* mRNA levels did not translate into decreased PTEN protein levels in MB231 cells exposed to doxorubicin remains to be elucidated, but the rapid changes in gene expression induced by the chemotherapy could take longer to translate into a change in protein levels, due to a half-life of more than 8 hrs for PTEN [13]. Furthermore, there is no strong correlation between *PTEN* mRNA and PTEN protein levels in human breast cancer, which could be explained by post-transcriptional and post-translational mechanisms modifying protein expression and stability [14].

Influence of doxorubicin treatment on Akt activity and PI3K signaling in doxorubicin-resistant cell lines

We performed the same experiments as outlined above in MB231 and MCF7 cells made resistant to doxorubicin through long-term doxorubicin exposure (see Methods & materials).

While doxorubicin exposure for 24 hours increased *AKT1* gene expression in doxorubicin-resistant MCF7 cells, no significant change in *AKT1* expression was observed subsequent to doxorubicin expression in MB231 cells (Supplementary Figure 2B). Notably, *PTEN* gene expression was profoundly reduced 24 hrs after doxorubicin exposure in doxorubicin-resistant MB231, whereas a minor *PTEN* increase was observed in doxorubicin-resistant MCF7 cells (Supplementary Figure 2B). While p-Akt increased at the protein level in doxorubicin-resistant compared to doxorubicin-naïve MB231 cells, no change in downstream signaling was observed. The level of p-Akt was increased in doxorubicin-resistant MB231 cells, compared to doxorubicin-naïve cells, but without any changes in downstream signaling (Figure 1A, 1C). In doxorubicin-resistant MCF7 cells, the protein levels of PTEN, p-Akt, mTOR and p-mTOR were higher compared to doxorubicin-naïve cells (Figure 1B, 1D). However, an additional pulse of doxorubicin treatment did not change PTEN or Akt-mTOR-S6K protein levels further in doxorubicin-resistant MB231 or MCF7 cells, compared to sham treatment (Figure 1C-D).

Akt inhibition in doxorubicin-naïve and resistant MB231 and MCF7 cell lines

Next, we examined the cytotoxicity of the Akt inhibitor A-443654, alone or combined with doxorubicin, in the ER negative MB231 and ER positive MCF7 cell lines. Moreover, based on the increased Akt phosphorylation levels observed in the doxorubicin-resistant cell lines, we compared the doxorubicin-naïve and resistant cell lines with respect to Akt inhibitor cytotoxicity.

First, it was established that the IC30 concentration of A-443654 was 1.0 μM in MB231 and 0.5 μM in the MCF7 cell line (Supplementary Figure 2C). A-443654 is a known ATP competitive inhibitor of Akt, which causes a transient increase in Akt phosphorylation at S473 [15].

In line with this, 2 hrs exposure to A-443654 increased Akt phosphorylation in a dose-dependent manner in MCF7 as well as MB231 cells (Figure 2A-2B). The induction of Akt by A-443654 in ER negative MB231 cells was not influenced by doxorubicin resistance (Figure 2A). However, in ER positive MCF7 cells, the induction of Akt phosphorylation by A-443654 was significantly less prominent in doxorubicin-resistant compared to doxorubicin-naïve cells (Figure 2B), suggesting that long-term doxorubicin exposure exhausts the ability to activate Akt and could influence the response to A-443654. Of notice, the baseline phosphorylation level of Akt in doxorubicin-naïve MB231 differed in Figure 1A and 2A, possibly due to the use of different dissolvents given to control cells in the two experiments; HPMC was used as dissolvent

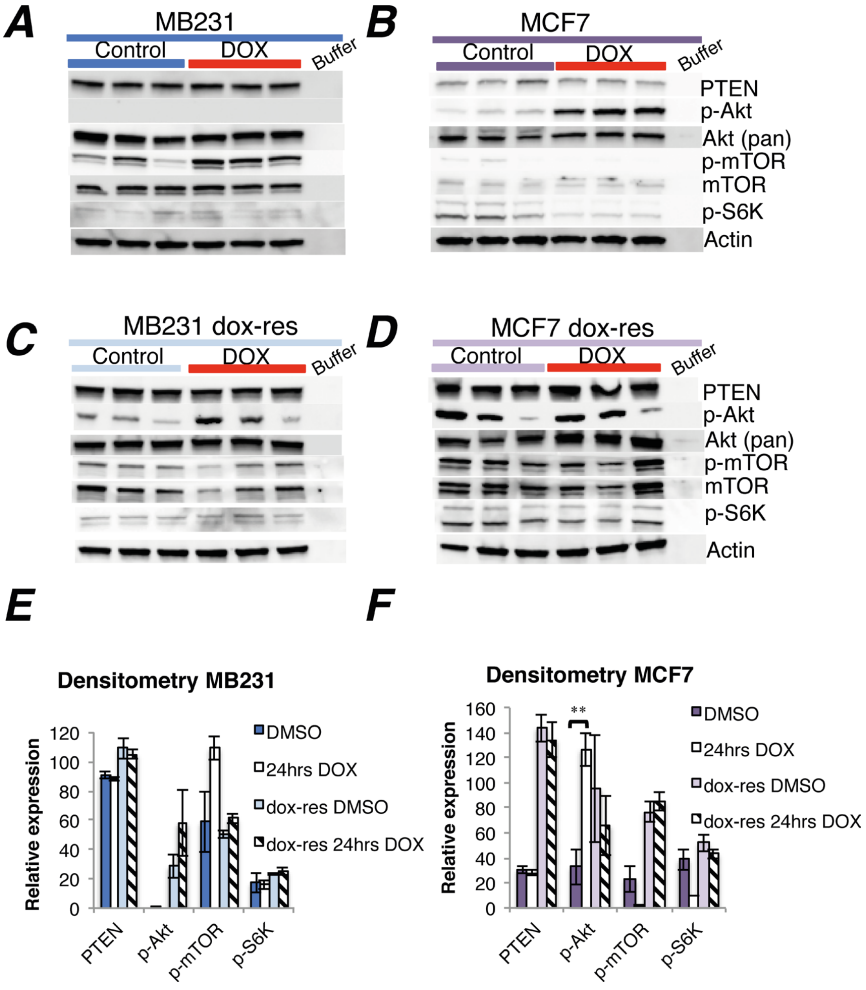

Figure 1: Doxorubicin treatment of doxorubicin-naïve and doxorubicin-resistant human breast cancer cell lines. (A-B) Western blots of PTEN and Akt-mTOR-S6K signaling in MB231 and MCF7 breast cancer cells *in vitro*, either doxorubicin-naïve **(A-B)** or doxorubicin-resistant (**C-D**, dox-res). Drug exposure lasted 24 hrs, at either 1.5 μM for MB231 and 2 μM for MCF7 or an equivalent volume of DMSO (stock solvent for doxorubicin) for control wells, three independent experiments per group. Whole cell lysate, 30 μg protein loaded per lane. **(E-F)** Densitometries for western blots **(A-D)** depict the relative protein expression, normalized to actin. Phosphorylated Akt (p-Akt) and mTOR (p-mTOR) were normalized to actin and thereafter to total Akt and mTOR, respectively. Bars represent the mean protein expression for experiments performed in triplicate ± SEM. **p<0.01

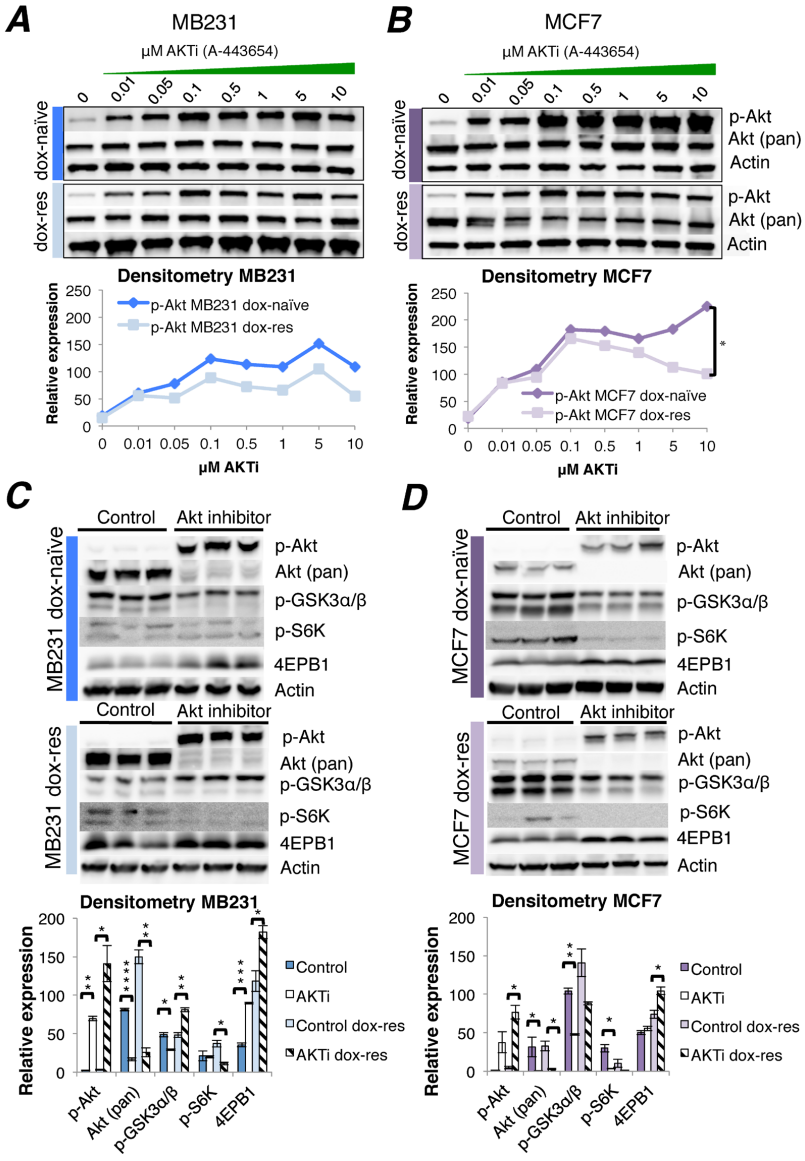

Figure 2: Akt inhibitor treatment of doxorubicin-naïve and doxorubicin-resistant human breast cancer cell lines. (A-B) Western blots of Akt phosphorylation induced by increasing doses of the Akt inhibitor A-443654, 0-10 μM, 2 hrs exposure in doxorubicin-naïve or doxorubicin-resistant MB231 **(A)** and MCF7 **(B)** human breast cancer cells *in vitro*. Whole cell lysate, 30 μg protein loaded per lane. Densitometries for western blots **(A-B)** depict the relative protein expression, normalized to actin and total Akt. Phosphorylated Akt increased significantly in doxorubicin-naïve (dox-naïve), compared to doxorubicin-resistant MCF7 cells (dox-res), at AKTi concentrations above 0.5 μM. *p<0.05. **(C-D)** Western blot analysis of Akt and downstream signaling in doxorubicin-naïve or doxorubicin-resistant MB231 **(C)** and MCF7 **(D)** human breast cancer cells, after 24 hrs exposure to A-443654 (MB231: 1 μM, MCF7 0.5 μM) *in vitro*. Densitometries for western blots **(C-D)** depict the relative protein expression, normalized to actin. Bars represent the mean protein expression in experiments performed in triplicate ± SEM.

for A-443654 (Figure 2A), whereas DMSO was the dissolvent for doxorubicin (Figure 1A).

To decipher the consequence of Akt inhibition in a wider time frame, Akt phosphorylation and downstream signaling was assessed after 24 hrs of A-443654 (IC30) exposure (Figure 2C-2D). As compared to 2 hrs, total Akt was profoundly reduced after 24 hrs, suggesting the Akt inhibitor may induce protein degradation. In parallel, phosphorylated Akt remained upregulated after 24 hrs of doxorubicin exposure in the doxorubicin-naïve as well as the doxorubicin-resistant MCF7 and MB231 cell lines, which is in accordance with the reported activity of A-443654 [16]. With respect to downstream signaling, it was clearly reduced by A-443654 in the doxorubicin-naïve MCF7 cell line, with decreased GSK3 and S6K phosphorylation and increased 4EBP1 protein levels (Figure 2D), and the same signaling inhibition was observed in the doxorubicin-resistant MCF7 cell line. The influence of A-443654 on Akt downstream signaling was less pronounced in the MB231 cell line (Figure 2C). Whereas reduced GSK3 and increased 4EBP1 phosphorylation was observed in doxorubicin-naïve MB231, Akt inhibition had no influence on phosphorylated S6K. In doxorubicin-resistant MB231, A-443654 reduced S6K and increased 4EBP1 phosphorylation, in accordance with protein synthesis inhibition, but at the same time phosphorylated GSK3 protein levels increased, indicating glycogen synthase and cell cycle activation. All in all, these results point to a stronger dependence on Akt downstream signaling for cell proliferation in MCF7 than in MB231 breast cancer cells when doxorubicin resistance evolves.

Indeed, the Akt inhibitor exhibited significantly increased cytotoxicity in doxorubicin-resistant compared to doxorubicin-naïve MCF7 cells (Figure 3B). In contrast, the cytotoxicity of A-443654 was significantly reduced in doxorubicin-resistant compared to doxorubicin-naïve MB231 cells (Figure 3A). The cytotoxicity of A-443654 was not augmented by doxorubicin in neither cell line (Figure 3A-3B).

Efficacy of doxorubicin and Akt inhibition in doxorubicin-naïve MB231 and MCF7 xenografts *in vivo*

The efficacy of sham treatment, A-443654 or doxorubicin, alone or in combination, was assessed in NOD/SCID mice implanted orthotopically with doxorubicin-naïve MB231 or MCF7 human breast cancer (n=5-6 mice/group).

The Akt inhibitor A-443654 was ineffective as monotherapy in MB231 tumors in mice, but inhibited tumor growth significantly in MCF7 tumors (Figure 4A-4B). Doxorubicin treatment yielded significant tumor inhibition in both cancer subtypes. In MB231, the combination of doxorubicin and A-443654 inhibited tumor growth significantly compared to A-443654 or sham treatment, but only if A-443654 was postponed for a week after commencing doxorubicin administration (treatment group B). In contrast, co-administration of A-443654 and chemotherapy (treatment group A) diminished the tumor growth inhibition induced by doxorubicin alone. In MCF7, the combination of doxorubicin and A-443654 yielded significant tumor growth inhibition as compared to A-443654 or sham treatment, but only if the Akt inhibitor and doxorubicin were administered concomitantly (treatment group A). In both breast cancer models there was no significant difference in tumor response between doxorubicin alone and doxorubicin combined with A-443654.

Subcutaneous Akt inhibitor injections caused a 7% weight loss after 14 days of treatment, which was comparable to combined treatment with doxorubicin and A-443654. However, the observed weight loss precluded further extension of the A-443654 treatment period, to assess whether long-term Akt inhibiton could augment the efficacy of doxorubicin. Unfortunately, two mice in the doxorubicin and one mouse in the doxorubicin and A-443654 group (A) had to be euthanized and taken out of the MCF7 trial due to accidental injection of doxorubicin into the gut wall and subsequent gut necrosis.

In a separate experiment, mice exposed to the same treatment regimens as above were euthanized after 14 days and tumor tissue extracted for molecular analysis (doxorubicin-naïve MB231; n=3 mice/group and MCF7; n=2 mice/group). As monotherapy, A-443654 or doxorubicin yielded a heterogenous increase in Akt phosphorylation in MCF7 and to a lesser extent in MB231 xenografts, although not significant by densitometry (Figure 4C-4D). Combined treatment with A-443654 and doxorubicin increased Akt phosphorylation in MCF7 xenografts significantly, whereas the phosphorylation level of Akt in MB231 was unaffected by the combination regimen (Figure 4C-4D). While Akt phosphorylation increased substantially subsequent to 24 hours of A-443654 treatment *in vitro* (Figure 2A-2D), this increase was less pronounced in MB231 and MCF7 xenografts after two weeks of A-443654 treatment (Figure 4C-4D).

Potential effects of Akt inhibition was further monitored by analyzing downstream target effects (S6K phosphorylation status). In doxorubicin-naïve MB231 tumors, protein analysis demonstrated significantly reduced S6K phosphorylation after treatment with doxorubicin alone or combined with A-443654 (Figure 4C). In doxorubicin-naïve MCF7 tumors, A-443654 or doxorubicin, either alone or in combination, reduced mTOR phosphorylation (Figure 4D). Gene expression analysis of *PTEN, AKT1* and *S6K* in tumors extracted 14 days after commencing therapy (Supplementary Figure 3) demonstrated a significant decrease in *AKT1* in MCF7 tumors subsequent to doxorubicin exposure, but apart from this no definite

differences between the treatment groups were observed in neither MCF7 nor MB231.

Gene expression changes induced by anthracyclines in human breast cancers

Next, to compare with the preclinical results, we examined how anthracyclines affected acute and chronic tumor gene expression by analyzing breast cancer samples obtained before and 24 hours after the first epirubicin (60 mg/m^2 i.v.) course, or before and after 16 weeks of weekly doxorubicin (14 mg/m^2 i.v.). All 24 tumors collected in the dose dense epirubicin trial (ClinicalTrials.gov NCT00496795)expressed *PTEN, AKT1* and *S6K*, before and/or after treatment (Supplementary Figure 4). Among the patients treated with epirubicin, *AKT1* gene expression increased significantly (p=0.016) in tumors that subsequently regressed on treatment (PR; n=9), whereas no change was observed in tumors that did not respond (SD, PD; n=15, Figure 5A). The mRNA levels of *PTEN* and *S6K* did not change significantly, neither among responders nor non-responders (Figure 5A). Stratifying patients according to ER status, neither *AKT1*, *PTEN* nor *S6K* mRNA levels were influenced by epirubicin exposure among ER negative tumors (n=11), independent of clinical response to therapy (Figure 5B). Interestingly, among the ER positive tumors (n=13), *AKT1* (p=0.040) as well as *PTEN* levels (p=0.039) increased significantly in patients who responded to therapy (n=6); whereas no change in neither *AKT1* nor *PTEN* levels was observed among the non-responders (n=7, Figure 5B). Furthermore, *S6K* decreased significantly (p=0.027) in ER positive tumors that did not respond to therapy (n=7, Figure 5B).

After a median follow-up of 69 months, six out of 24 patients from the dose dense trial had developed breast cancer recurrence; no difference in gene expression changes between patients relapsing and those not relapsing was observed (Supplementary Figure 4).

To assess potential long-term effects of anthracycline treatment, tumor samples collected from 30 patients with locally advanced breast cancers, before and after 16 weeks of doxorubicin [30, 31], were examined for long-term gene expression changes of *PTEN* and *AKT1* (Supplementary Figure 5). Analysing all patients together, no change in neither *AKT1* nor *PTEN* expression was observed. However, stratifying patients based on response to therapy, *PTEN* expression increased significantly (p=0.033) among non-responders (patients having a PD or SD on therapy; n=17), in particular among ER negative non-responders (n=4; p=0.026; Figure 6A-6B). In contrast,

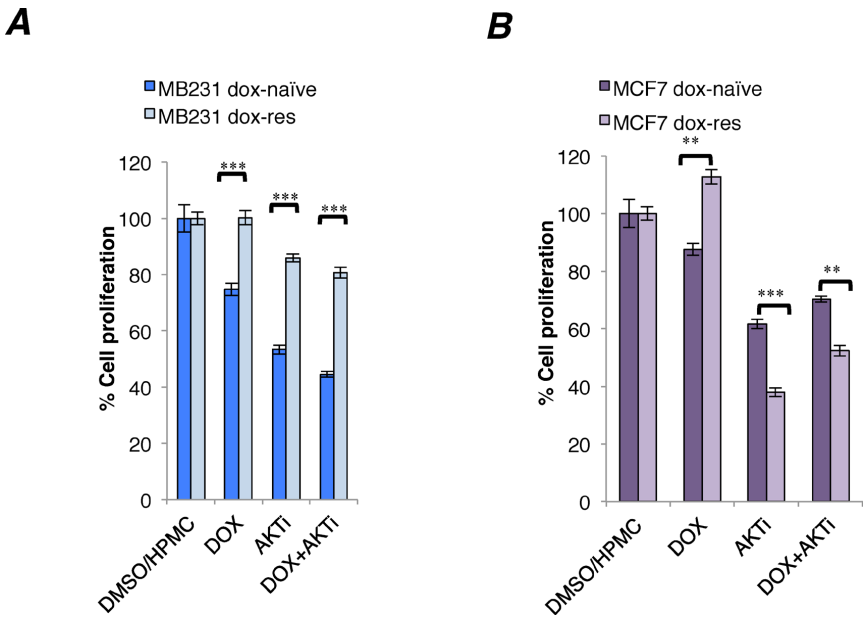

Figure 3: Cytotoxicity of A-443654 and doxorubicin in doxorubicin-naïve and doxorubicin-resistant human breast cancer cell lines. *In vitro* cytotoxicity of doxorubicin (1 μM), Akt inhibitor A-443654 at IC30 concentration, or the combination, in doxorubicin-naïve or resistant MB231 **(A)** and MCF7 **(B)** cells, after 24 hrs drug exposure. WST-1 cell proliferation assay, absorbance read at optical density (OD) 450 nm, normalized to readings in control wells exposed to equivalent volumes of DMSO (doxorubicin stock solvent) and HPMC (dissolvent for A-443654). Bars depict the mean ± SEM.**p<0.01, ***p<0.001

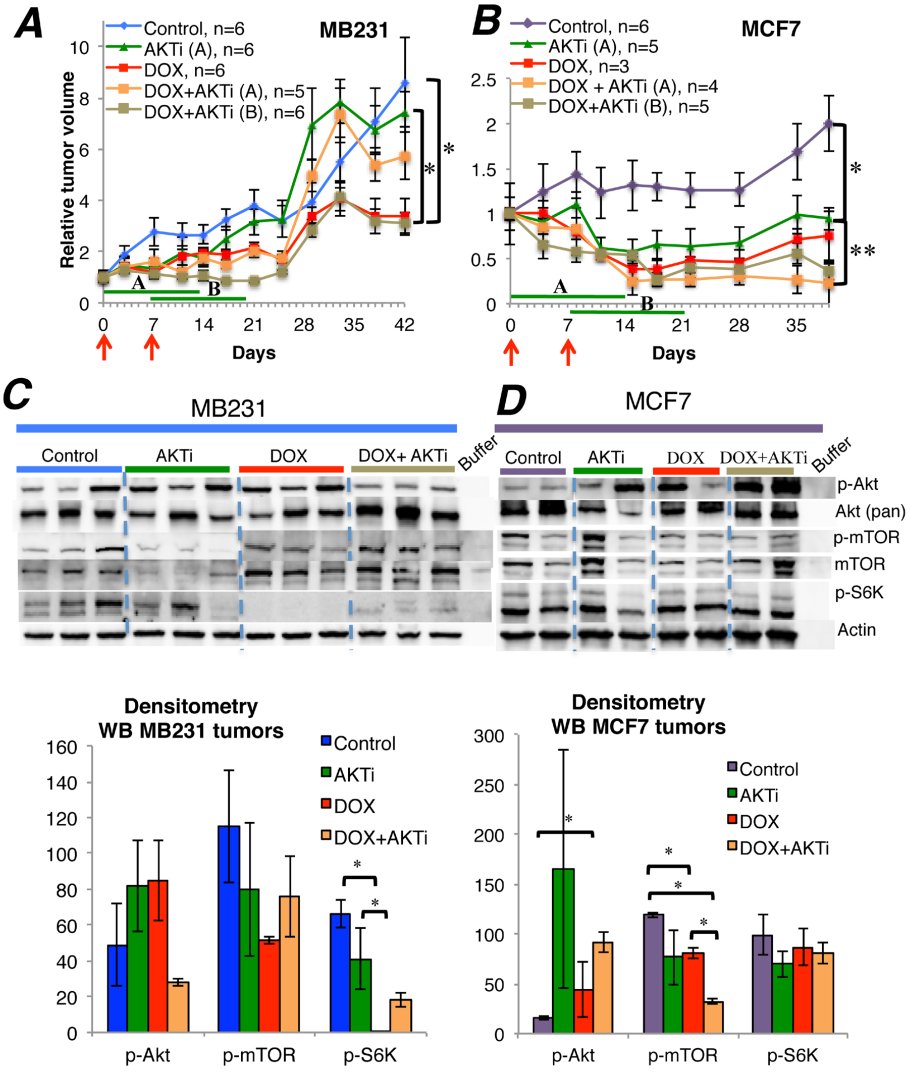

Figure 4: The influence of A-443654 and doxorubicin on tumor growth *in vivo*. **(A-B)** Tumor growth of MB231 and MCF7 breast cancer in NOD/SCID mice, given doxorubicin (DOX) 1.25 mg/kg i.p. qW twice (red arrows), Akt inhibitor A-443654 (AKTi) 3.75 mg mg/kg BID 14 days (green lines) or the combination. AKTi treatment commenced either at the first **(A)** or at the second **(B)** doxorubicin injection. Tumor volume is displayed as the mean ± SEM for each group, relative to tumor volume on the day treatment started. *p<0.05, **p<0.01. **(C-D)** Western blots for PTEN and Akt-mTOR-S6K signaling in MB231 **(C)** and MCF7 **(D)** tumors, harvested the last day of A-443654 treatment. Whole cell lysate, 30 µg protein loaded per lane. The sample order on the blot pictures has been rearranged to enhance readability. Densitometries for western blots **(C-D)** depict the relative protein expression, normalized to actin. Phosphorylated Akt (p-Akt) and mTOR (p-mTOR) were normalized to actin and thereafter to total Akt and mTOR, respectively. Bars represent the mean protein expression for experiments performed in duplicate **(D)** or triplicate **(C)** ± SEM. *p<0.05

while 22 out of 30 patients had relapsed during a median follow-up of 235 months, gene expression changes did not correlate to long-term outcome (Supplementary Figure 5).

The mutation status of *PIK3CA* and *TP53* was assessed in all the above tumor samples (Supplementary Figure 4 and Supplementary Figure 5). The low number of tumors harboring *PIK3CA* mutations precluded any correlation analysis between *PIK3CA* mutation status and the gene expression changes observed. Among tumors assessed before and 24 hours after the initial epirubicin course, no difference in *PTEN* or *AKT1* gene expression was observed regardless of response to

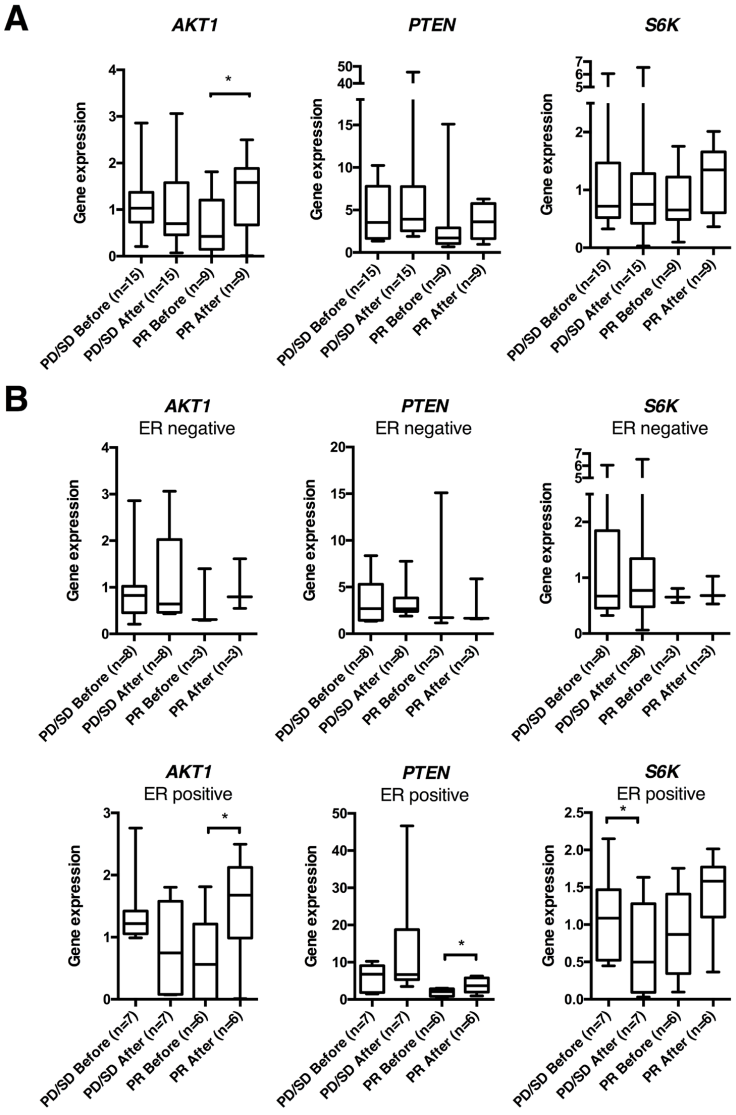

Figure 5: *AKT1, PTEN* and *S6K* gene expression in human breast cancers before and 24 hrs after epirubicin exposure. **(A)** Box plots of gene expression of *PTEN*, *AKT1* and *S6K* normalized to *RPLP2* in human breast cancer samples, before and 24 hrs after the first epirubicin dose, from patients in the dose dense trial. **(B)** Box plots from the same patient cohort as in **(A)**, but depicted separately for estrogen receptor (ER) positive and ER negative breast cancers. *p<0.05

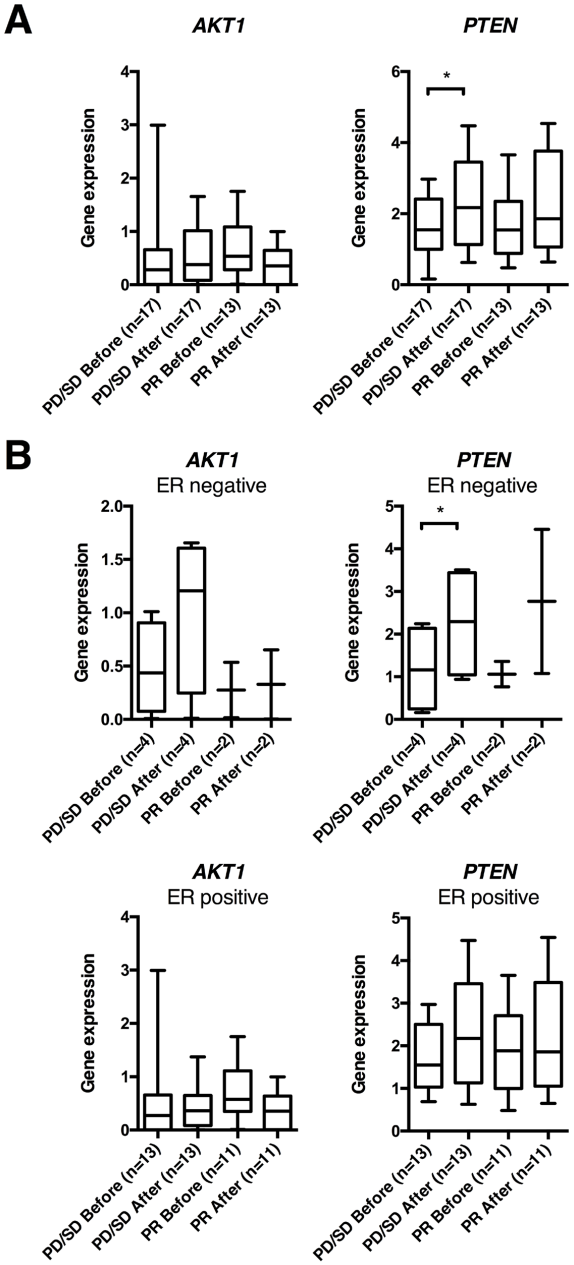

Figure 6: *AKT1* and *PTEN* gene expression in human breast cancers before and after 16 weeks of doxorubicin treatment. (A) Box plots of gene expression of *PTEN* and *AKT1* normalized to *RPLP2* in human breast cancer samples, before and after 16 weeks of doxorubicin treatment, from patients in the doxorubicin trial. **(B)** Box plots from the same patient cohort as in **(A)**, but depicted separately for estrogen receptor (ER) positive and negative breast cancers. *p<0.05

chemotherapy, if subdivided into *TP53* wt (n=16) and *TP53* mutated tumors (n=8). Upregulated *AKT1* 24 hrs after the first epirubicin exposure was observed in six out of 13 ER positive breast cancers which subsequently regressed on this treatment (Figure 5); and four out of these six tumors where *AKT1* increased harbored wt *TP53* status (Supplementary Figure 4).

In tumors examined before and after 16 wks of doxorubicin, if split by *TP53* mutation status (*TP53* wt; n=15, *TP53* mutated; n=15), *PTEN* increased significantly after treatment (p=0.02) in tumors harboring *TP53* mutations that did not respond to doxorubicin treatment (n=8), whereas no change was observed among responders (n=7) or among *TP53* wt tumors (data not shown). Also, there was no significant change in *AKT1*, among responders or non-responders to doxorubicin, if the subgroups were split by *TP53* mutation status.

DISCUSSION

Patients with ER positive as well as ER negative breast cancer obtain improved survival from adjuvant polychemotherapy [17], but the benefit of chemotherapy is less in typical luminal A tumors with strong ER expression compared to other subtypes [18]. The mutational landscape of breast cancer subtypes differ substantially, with a high prevalence of activating *PIK3CA* mutations in ER positive, luminal or HER2-enriched tumors, whereas inactivating *TP53* mutations are commonly observed in ER negative subtypes [4]. Moreover, while activating *AKT1* mutations are rare in human breast cancers, they occur more frequently among luminal or HER2-enriched (2-4%) than basal-like tumors (0%) [4], suggesting a selection pressure towards increased Akt signaling in these neoplasms. Accordingly, the PI3K-Akt-mTOR pathway has been targeted therapeutically to counteract acquired resistance to endocrine therapy and combined trastuzumab-chemotherapy in clinical trials [1, 3, 5, 19]. However, the importance of PI3K-Akt-mTOR signaling to chemoresistance has not been fully elucidated.

Here, we systematically explored alterations in this pathway in response to anthracycline and/or Akt inhibition in ER positive and negative breast cancer cell lines and their concomitant xenografts and to anthracycline treatment in human breast cancers.

We established that the Akt inhibitor A-443654 reduces cell proliferation both in the ER positive MCF7 and the ER negative MB231 cell line *in vitro*. However, Akt inhibition yielded tumor regression in MCF7 and not MB231 *in vivo*, and doxorubicin significantly augmented this tumor response only in MCF7 xenografts. Of notice, A-443654 caused significant weight loss which was intolerable beyond 14 days, and we therefore could not explore its full potential alone or combined with anthracyclines. Toxicity was a similar problem in the first clinical trials testing Akt inhibitors, although next generation compounds seem better tolerated [20] and should be tested in long-term combination schedules with anthracyclines. Importantly, the combined efficacy of A-443654 and doxorubicin was dependent on timing, where concomitant administration of the Akt inhibitor and chemotherapy was required for optimal tumor regression in MCF7 xenografts. In contrast, co-administration of A-443654 with doxorubicin reduced the efficacy of doxorubicin in MB231 xenografts. Doxorubicin yielded rapid upregulation of phosphorylated Akt in MCF7 cells *in vitro*, whereas long-term exposure and induction of doxorubicin resistance was required to upregulate phosphorylated Akt in MB231. This may explain the different efficacy with respect to timing between doxorubicin and the Akt inhibitor in the two cell lines.

Previous studies have revealed A-443654 to act as an ATP competitive inhibitor of Akt; as such, it increases Akt phosphorylation while at the same time inhibiting Akt downstream signaling [15, 16]. Accordingly, A-443654 rapidly increased Akt phosphorylation, which was more pronounced in the MCF7 as compared to the MB231 cell line, and suggesting a particular responsiveness of the PI3K-Akt-mTOR pathway in ER positive breast cancer cells. However, the cytotoxicity of A-443654 was comparable between MCF7 and MB231, and similar inhibition of Akt downstream signaling was observed in the ER positive and ER negative cell lines. In contrast, the activity of A-443654 was profoundly reduced in doxorubicin-resistant MB231 cells, where the inhibition of Akt signaling by A-443654 was abrogated. Interestingly, doxorubicin resistance enhanced the cytotoxicity of A-443654 significantly in MCF7, with maintained inhibition of Akt downstream signaling. The mechanisms behind the enhanced activity of A-443654 in doxorubicin-resistant MCF7 cells remain to be established. However, the baseline Akt phosphorylation level is higher in the doxorubicin-resistant MCF7 cell line, as compared to doxorubicin-naïve MCF7 cells, potentially explaining the increased cytotoxicity towards A-443654.

In line with previous observations [10, 21], we established that doxorubicin exposure increases Akt phosphorylation in the ER positive MCF7 and T47D human breast cancer cell lines, but not the ER negative MB231 cell line. Herein we expand upon these data to show that MCF7 cells made resistant to doxorubicin exhibit a higher constitutive Akt phosphorylation levels which is not affected by further doxorubicin exposure. The increased cytotoxicity of Akt inhibition in doxorubicin-resistant MCF7 breast cancer cells points to a potential use of such a drug class in ER positive breast cancer, in particular if resistance to anthracycline has developed and Akt phosphorylation levels are elevated. Importantly, while the Akt inhibitor exhibited increased cytotoxicity in doxorubicin-resistant compared to doxorubicin-naïve MCF7 breast cancer, we did not examine whether Akt inhibitors can be used to reverse doxorubicin resistance, but this issue should be addressed in future trials. However, upregulated PI3K-Akt-mTOR signaling is

clearly associated with chemoresistance, which has been shown in various preclinical cancer models, and chemotherapy response can be augmented in this setting by simultaneous PI3K or Akt inhibition [10, 11, 22–24], in particular in ER positive breast cancer [10, 22].

Finally, we provide clinical data demonstrating that increased *AKT1* gene expression 24 hours after epirubicin exposure characterizes ER positive, but not ER negative, primary breast cancers that subsequently regress on anthracycline treatment. Interestingly, no change in tumor *AKT1* expression was observed in patients after 16 weeks of doxorubicin, suggesting an intermittent Akt response where the efficacy of Akt inhibitors could depend on timing. Furthermore, an increased *AKT1* mRNA level 24 hrs after the first chemotherapy course could potentially be used as a biomarker identifying ER positive tumors likely to respond to chemotherapy. The reason why elevated *AKT1* mRNA is associated with good response remains to be elucidated. However, if high *AKT1* translates into increased Akt activation throughout the duration of chemotherapy, chronic Akt activation may promote senescence and apoptosis by downregulating MDM2 and increasing p53 in breast cancers with preserved p53 function [25, 26]. Of notice, among the ER positive breast cancers which responded to epirubicin, four out of six tumors with upregulated *AKT1* after chemotherapy harbored wt *TP53* status.

In contrast to the patient data, *AKT1* mRNA levels were not affected in the MB231 and MCF7 human breast cancer cell lines after 24 hrs chemotherapy exposure. The reason for this discrepancy remains to be elucidated, but could be due to the admixture of tumor cells and stroma in patient tumor samples in contrast to the pure tumor cell content in the *in vitro* cultures. Unfortunately, we did not have patient samples available for proteinanalysis of Akt and Akt signaling to compare with the gene expression data. Furthermore, the induction of Akt phosphorylation by A-443654 which was observed in MB231 and MCF7 *in vitro*, was not detected in the corresponding xenografts. Again, the admixture of tumor cells and stroma cells *in vivo*, as well as the heterogeneity between the xenografts may explain the lack of correlation between the *in vivo* and *in vitro* findings. Moreover, the xenografts used for the proteinanalysis were extracted after 14 days of treatment compared to the 2 hrs and 24 hrs of treatment in the *in vitro* experiments. The reason why decreased *PTEN* mRNA levels did not decrease PTEN protein levels in MB231 cells after 24 hrs exposure to doxorubicin also remains to be established. Whereas rapid changes in gene expression are induced by the chemotherapy, protein changes may take longer to develop due to the relatively long half-life of PTEN (>8 hrs) [13]. Furthermore, there is no strong correlation between *PTEN* mRNA and PTEN protein levels in human breast cancer [14], which could be explained by post-transcriptional and post-translational mechanisms modifying protein expression and stability.

In conclusion, our data point to upregulated Akt expression as a recurrent initial response to anthracyclines in ER positive human breast cancers, and in particular, among patients who respond to chemotherapy. Furthermore, we observed increased sensitivity to Akt inhibition in doxorubicin-resistant, compared to doxorubicin-naïve, ER positive MCF7 breast cancer cells. Accordingly, the benefit of Akt inhibition is clearly context-dependent, with respect to ER status and previous anthracycline exposure. Thus far, the role of Akt inhibitors to augment the efficacy of chemotherapy in solid tumors has not been dealt with to a large extent, despite promising preclinical and clinical data [23, 24, 27–29] and should be explored further, in particular in ER positive breast cancers.

MATERIALS AND METHODS

Ethical declaration

The authors declare that the experiments within this paper comply with the ethical standards and current laws in Norway.

Cell lines

The ER positive MCF7 and T47D and the ER negative MDA-MB-231 (MB231) human breast cancer cell lines were used for all the preclinical experiments. For cell growth conditions and cell line identity, see *Supplementary methods*. Doxorubicin (Adriamycin, Pfizer) was diluted to 2 mg/ml in DMSO 99% for cell culture experiments, stored as frozen aliquots (-20°C), and prepared fresh by dilution in complete cell culture medium for each experiment to preserve drug stability. Control cells were always incubated with an equivalent volume of DMSO 99% as cells exposed to medium containing doxorubicin. A-443654 (AbbVie) was dissolved in 0.2% hydroxypropyl methylcellulose (HPMC, Sigma) prior to use. For comparison, control cells were given an equivalent volume of HPMC as those cells exposed to A-443654.

Generation of doxorubicin-resistant cell lines

Doxorubicin-naïve MB231 and MCF7 cells were grown in gradually increasing doxorubicin concentrations over several months. When the cells were subconfluent they were exposed to growth medium containing twice the previous concentration of doxorubicin, and this was repeated until a dose was reached, where the cells would not expand any further. At this point, the cells had acquired resistance to doxorubicin 1.5 μM (MB231 dox-res) and doxorubicin 0.65 μM (MCF7 dox-res), each by exposure for 48 hrs. The cells were maintained in doxorubicin-free medium, but exposed to doxorubicin at their resistance

dose every two weeks to maintain resistance. Control cells were propagated in medium with an equivalent volume of DMSO. To determine the acute response to doxorubicin in doxorubicin-resistant cells, the cells were seeded at $3x10^5$ in 6-well dishes (Nunc) and incubated for 24 hrs until 70% confluence. The medium was then replaced by medium containing either doxorubicin or DMSO, and cells were incubated for another 24 hrs before the cells was harvested and RNA and protein isolated.

In vitro activity and cytotoxicity of A-443654 and doxorubicin

Subconfluent MB231 and MCF7 cells (in T25 flasks), either doxorubicin-naïve or doxorubicin-resistant, were exposed to increasing concentrations of Akt inhibitor A-443654 or HPMC (control) to assess the influence on Akt phosphorylation, and protein was harvested after 2 hrs. To evaluate the influence of the Akt inhibitor on Akt phosphorylation and downstream signaling after 24 hrs, cells were exposed to A-443654 at the IC30 (1 μM MB231, 0,5 μM MCF7), before harvesting protein.

To assess the influence of doxorubicin +/- Akt inhibitor A-443654 on cell viability, 5000 MB231, 1500 MCF7 or 20000 T47D cells were seeded per well in 96-well plates (Falcon), and allowed to attach over night in complete medium. Thereafter the drug(s) was added, before culturing the cells for another 24 hours. Cell proliferation after drug exposure was assessed by the WST-1 assay (Roche), as described in the manufacturer´s manual.

Gene expression analysis and western blots

Therapy-induced changes in gene and protein expression were assessed using qPCR and western blot analyses. Detailed methods, primers and antibodies are described in *Supplementary methods*.

In vivo cytotoxicity of A-443654 and doxorubicin

NOD/SCID mice were bred and mouse crossings performed inhouse at the Animal Facility, University of Bergen. Adult mice of fertile age were anesthetized with isoflurane (Baxter) before $1x10^6$ MCF7 or MB231 cells were injected orthotopically in the fourth left inguinal mammary gland. The tumors were measured every 3-4 days using Vernier calipers, and tumor volumes were calculated using the formula $a^2b/2$, where a and b are the shorter and longer diameter of the tumors respectively. Treatment commenced when the tumors had reached 4-6 mm in diameter, and the animals were stratified into groups according to tumor size. The mice were euthanized if signs of serious distress occurred or when the first tumor in any treatment group had a tumor diameter exceeding 17 mm.

The maximum tolerable dose (MTD) of doxorubicin, given weekly for two following weeks, with or without the Akt inhibitor A-443654 was initially assessed in non-tumor bearing NOD/SCID mice before commencing the therapy trial in xenograft-implanted mice. Doxorubicin was dissolved in 0.9% NaCl (Baxter) and injected i.p. once weekly for two consecutive weeks to establish an MTD of 1.25 mg/kg qW. A-443654 was dissolved in HPMC and administered subcutaneously at 3.75 mg/kg BID for 14 consecutive days, based on dosage reported elsewhere [16]. In the combination treatment groups, administration of A-443654 commenced either upfront (A), 24 hours after the first doxorubicin injection (to treat primary resistance due to high Akt signaling), or started as a delayed regimen (B) at the same time as the second doxorubicin injection (to counteract doxorubicin-induced acquired resistance due to upregulated Akt signaling). Control mice were given 0.2% HPMC s.c. and 0.9% NaCl i.p.

In a separate experiment, three MB231 mice and two MCF7 mice per group were sacrificed at the end of 14 days of treatment (as given in the treatment trial above) for protein analysis. All animals were euthanized by cervical dislocation two hours after the last injection of A-443654 or sham treatment, and tissue samples from the tumor as well as all organs snap-frozen on liquid N_2 and stored at -80°C.

The animal experiments were performed with the approval of and in accordance with guidelines by the Norwegian State Commission for Laboratory Animals.

Patient breast cancer samples

The short-term effect of anthracyclines on *PTEN*, *AKT1* and *S6K* gene expression was assessed in a selected cohort of 14 ER positive and 11 ER negative breast cancers biopsied before and 24 hrs after receiving their first course of epirubicin as part of the "dose dense trial" (ClinicalTrials.gov NCT00496795) wherein treatment-naïve patients with locally advanced breast cancer were given dose dense neoadjuvant epirubicin 60 mg/m^2 q2w (four courses) followed by docetaxel 100 mg/m^2 q2w (four courses). The presence of pre-treatment *PIK3CA* and *TP53* mutations in all tumor samples were examined as described in *Supplementary methods*. One ER positive tumor pair was excluded due to poor RNA quality, leaving 24 tumor pairs for analysis.

Furthermore, to analyze for long-term effects of anthracyclines on *PTEN* and *AKT1* mRNA levels, gene expression was assessed in paired tumor samples from 30 patients with treatment-naïve locally advanced breast cancer in the "doxorubicin trial", biopsied before and after 16 wks of neoadjuvant doxorubicin 14 mg/m^2 qW treatment [30, 31]. Briefly, tumor pairs for mRNA analysis were available from 24 patients with ER positive and six patients with ER negative locally advanced breast cancer.

The dose dense and doxorubicin clinical trials were approved by the Regional Ethical Committee of the Western health region in Norway (reference numbers: 192/91-69.91 and 079.06). All patients gave their informed consent before inclusion. Accordingly, all procedures performed in these clinical trials were in accordance with the national ethical standards and with the 1964 Helsinki declaration and its later amendments.

Statistics

SPSS 22/PASW 17.0 and Graph Pad Prism v6 software packages were used for statistical analyses. Correlation analysis between *AKT1* and *PTEN* or *S6K* mRNA expression levels was performed using Spearman´s rho. Comparison of gene or protein expression levels was performed using the Student's *t*-test for paired samples or two independent samples, as appropriate. All p-values reported are two-tailed, and $p<0.05$ was considered statistically significant.

Availability of data and materials

All raw data generated from the experiments presented are available from the corresponding author upon request. The data subset used for mutational calling of *TP53* and *PIK3CA* in the "dose dense trial" was extracted from whole exome sequencing data, and the DNA sequences are available from the corresponding author upon request.

Abbreviations

4EBP1=4E-binding protein 1, **AKTi**=Akt inhibitor, **ATCC**= American Type Culture Collection, **ER**=Estrogen Receptor, **Dox-res**=Doxorubicin resistant, **HPMC**= Hydroxypropyl Methylcellulose, **MB231**=MDA-MB-231, **mTOR**=mammalian Target Of Rapamycin, **MTD**=Maximum Tolerable Dose, **p-Akt**=phosphorylated Akt at Ser473, **PBS**=Phosphate-Buffered Saline, **p-GSK3α/ß**=phosphorylated glycogen synthase kinase 3α/ß at Ser21/9, **PI3K**= Phosphatidylinositol-4,5-bisphosphate 3-kinase, **p-mTOR**=phosphorylated mammalian target of rapamycin at Ser2448, **PTEN**=phosphatase and tensin homolog, **p-S6K**=phosphorylated S6K at Ser371, **RPMI**=Roswell Park Memorial Institute Medium-1640, **RT-PCR**=Reverse Transcriptase Polymerase Chain Reaction, **S6K**= S6 Kinase, **STR**=Short Tandem Repeat, **TP53**=Tumor Protein p53, **qPCR**=Quantitative PCR

Authors' contributions

HPE and PEL designed the studies. SY, HPE, SK and PEL wrote the manuscript. SY, EA and HPE performed and supervised the laboratory experiments, the preclinical studies, and analyzed the results. SK helped with experimental design and analyzed the mutational data extracted from the whole exome sequencing dataset. PEL was principal investigator for the clinical trials. IRS, HPE and PEL conducted the clinical trials, performed response evaluations and collected follow-up data. SY and HPE performed statistical calculations. All authors have read and approved the final version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare they have no conflicts of interest.

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Activation of Akt characterizes estrogen receptor positive human breast cancers which respond to anthracyclines

SUPPLEMENTARY MATERIALS

SUPPLEMENTARY METHODS

Cell lines and growth conditions

The human breast cancer cell lines MDA-MB-231 (MB231), MCF7 and T47D were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were grown at 37°C in 5% CO_2 atmosphere in Roswell Park Memorial Institute medium-1640 (RPMI), supplemented with non-essential amino acids, 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml of streptomycin and 400 uM L-glutamine (all products: Lonza, Basel, Switzerland). Additionally, the RPMI medium for T47D was supplemented with human insulin 108 U/ml (Life/Thermo Fisher). The cell lines were tested negative for mycoplasma using the Mycoplasma Detection Kit Venor GeM (Minerva Biolabs) before starting the experiments listed. Also, the cell lines were fingerprinted based on short tandem repeat (STR) markers with the AmpFLSTR® Profiler Plus® and AmpFLSTR® COfiler® PCR Amplification Kit, followed by capillary electrophoresis on the ABI PRISM® 310 Genetic Analyzer and STR allele identification using the Peak Scanner Software v1.0 (Applied Biosystems).

Basic genomic procedures

Breast cancer cells were grown on 6-well plates (Nunc), under conditions as described above, until subconfluency. After exposure to doxorubicin, the cells were rinsed gently with warm (37°C) phosphate-buffered saline (PBS), and RNA was isolated using the Qiagen RNeasy kit, as described in the product manual, followed by DNAse (Ambion) treatment.

The human breast cancer biopsies were snap-frozen immediately after extraction and stored on liquid nitrogen until analysis, while collecting prospectively response rates and patient survival data. DNA for mutation analysis was extracted by the Qiagen DNeasy kit, and RNA was extracted using either Trizol (Invitrogen) (doxorubicin study) or mirVana™ miRNA Isolation Kit, with phenol (Ambion) (dose dense study), followed by DNAse treatment.

RNA concentrations were measured by a NanoDrop spectrophotometer (Thermo Scientific), and cDNA was made from 500 ng of RNA using qScript reverse transcriptase and the qScript cDNA SuperMix with a blend of random and oligo(dT) primers (QuantaBio).

RT-PCR was undertaken using AmpliTaq Gold polymerase (Invitrogen), with reagents as recommended by the company, and PCR reactions were run with 30-35 cycles and the appropriate temperature settings.

Real-time qPCR was performed on a Light Cycler 480 (Roche), with the gene of interest given as the ratio to housekeeping gene RPLP2, and as a mean of three individual runs. Taqman probes were used to detect the PCR products for *PTEN* and *RPLP2*, whereas PowerSYBR®Green (Applied Biosystems) was used for *AKT1* and *S6K*. Lack of gene expression was defined as lack of gene amplification after 35 cycles. Gene of interest was given as a ratio to the housekeeping gene RPLP2, and as a mean of three independent runs, normalized to a cDNA pool of 6 breast cancer cell lines.

All PCR products were checked for specificity by Sanger sequencing.

Primers for RT-PCR and real-time qPCR

RT-PCR:
AKT1: F: TTGGCTGCACAAACGAGGGGAGTAC,
R: TGCGTTCGATGACAGTGGTCCAC
β-actin: F: AGATGACCCAGATCATGTTTG,
R: AGGAGCAATGATCTTGATCTTCATTGTG
PTEN: F: TTTCCATCCTGCAGAAGAAGC,
R: TAAATATGCACATATCATTAC
S6K: F: TGGACCATATGAACTTGGCATG,
R: CTTTCCATAGCCCCCTTTACC
Real-time qPCR (PowerSYBR®Green):
AKT1: F: TTGGCTGCACAAACGAGGGGAGTAC,
R: TGCGTTCGATGACAGTGGTCCAC
S6K: F: TGGACCATATGAACTTGGCATG,
R: CTTTCCATAGCCCCCTTTACC
Real-time qPCR (Taqman):
PTEN:
F: CTTCTCCATCTCCTGTGTAATCAA
R: GTTGACTGATGTAGGTACTAACAGCAT
FAM: CCAGTGCTAAAATTCA
RPLP2:
F: GACCGGCTCAACAAGGTTAT
R: CCCCACCAGCAGGTACAC
FAM: AGCTGAATGGAAAAAACATTGAAGACGTC

PIK3CA and *TP53* mutation analysis

PIK3CA and *TP53* mutation analysis was performed on pre-treatment breast cancer samples. All tumor samples

in the doxorubicin trial and eight out of 25 tumors in the dose dense trial were analyzed by Sanger sequencing. Briefly, tumor DNA was used as PCR template to assess hot-spot *PIK3CA* mutations in exons 10 and 21 (previously exon 9 and 20) [1] and *TP53* mutations (all exons), using primers as described previously [2, 3]. PCR products were analyzed at Center for Medical Genetics and Molecular Medicine, Haukeland University Hospital, by Sanger sequencing using the BigDye v1.1 reaction mix (Applied Biosystems). The remaining 17 tumors in the dose dense trial were analyzed for *PIK3CA* and *TP53* mutations by massive parallel whole exome sequencing on an Illumina HiSeq 2500, to an average sequencing depth of >200x. Somatic mutations were called using the intersect between the MuTect and Strelka mutation calling algorithms.

Western blots

Cells and tissues were homogenized and proteins lysed in a custom made total protein lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% deoxycholate, 1% Triton X-100) containing a protease inhibitor cocktail (Roche). Protein concentrations were measured by a bicinchoninic acid (BCA) assay (Pierce), and 30 µg protein was loaded per lane for all the immunoblots.

The protein lysates were fractionated using reducing SDS-PAGE gel electrophoresis with Mini-PROTEAN TGX Precast Gels (Bio-Rad), and transblotted by semi-dry technique to nitrocellulose membranes using the Transblot Turbo system (Bio-Rad). Thereafter the membranes were blocked with 5% bovine serum albumine (Sigma) for 60 min, before immunoblotting with the primary antibody. The immobilized antibody was detected using the appropriate horseradish peroxidase-conjugated secondary antibody (Promega) and a 1:5 mix of SuperSignal West Femto and Pico chemiluminescent solution (Pierce). The immunoreaction was visualized on a Fujifilm LAS 4000. Immunoblots for actin were made for all samples to assure equal protein loading for total protein analysis.

Densitometry of western blots was performed using Gel Analyzer 2010a and the band intensity for each protein was normalized to actin. Thereafter phosphorylated Akt and mTOR were normalized to total Akt and mTOR, respectively.

Antibodies for proteinanalysis

Rabbit anti-actin (Sigma), anti-Akt (pan), anti-phosphorylated Akt (Ser473), anti-mTOR, anti-phosphorylated mTOR (Ser2448), anti-phosphorylated p70 S6K (Ser371), anti-phosphorylated GSK3α/β (Ser21/9) or anti-PTEN and goat anti-4EBP1 (R&D). Actin, 4EBP1 and phosphorylated p70 S6K are polyclonal antibodies, all other antibodies are monoclonal. All primary antibodies were purchased from Cell Signaling, unless noted otherwise.

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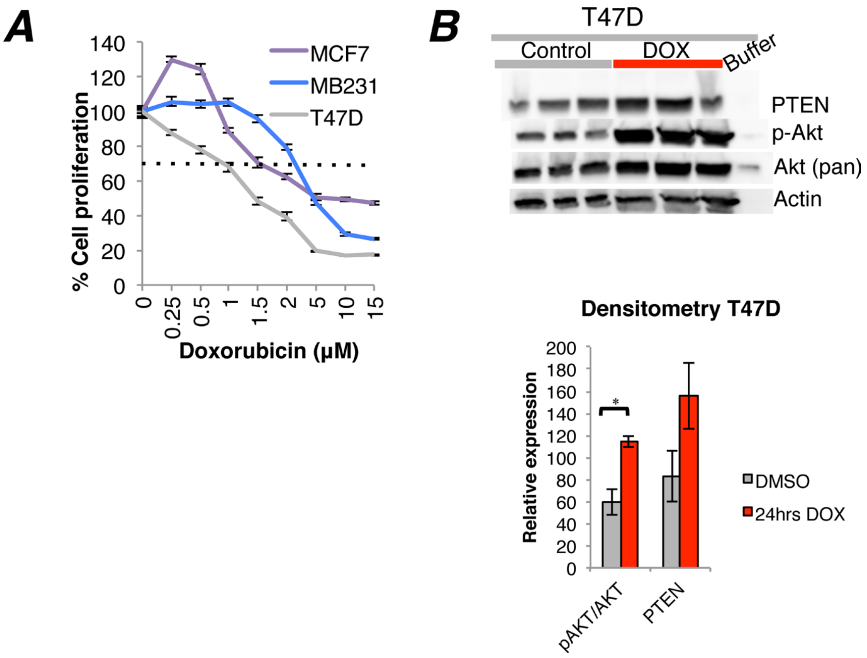
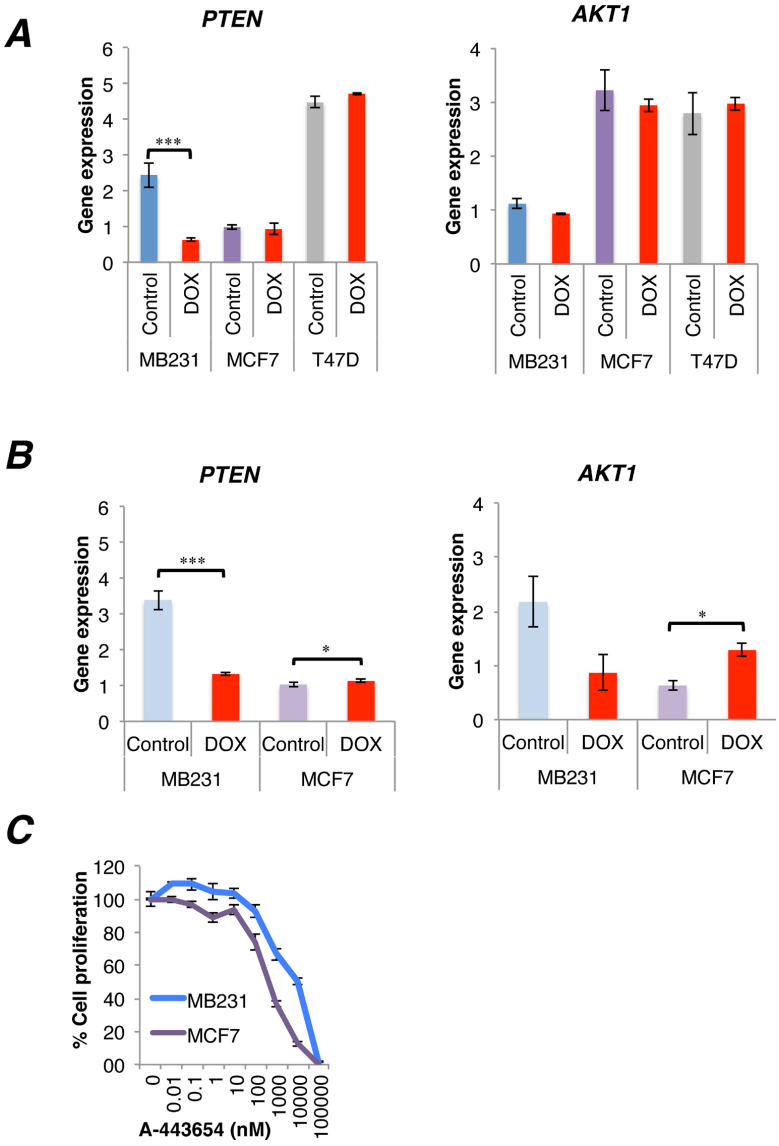
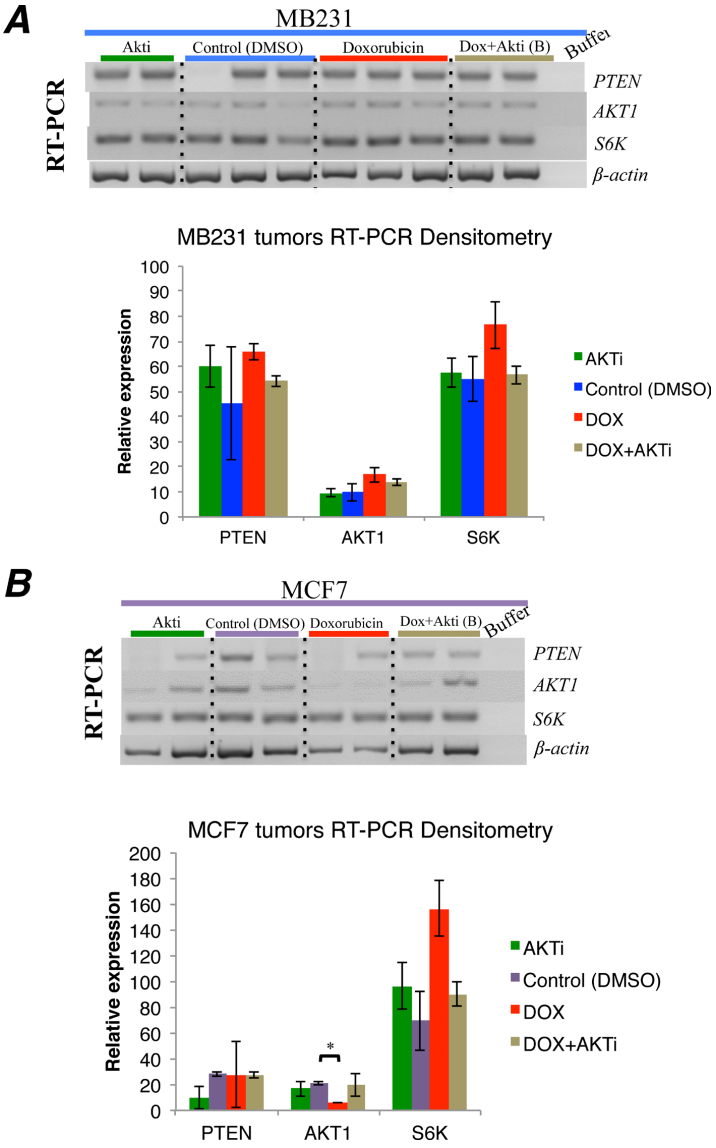
SUPPLEMENTARY FIGURES



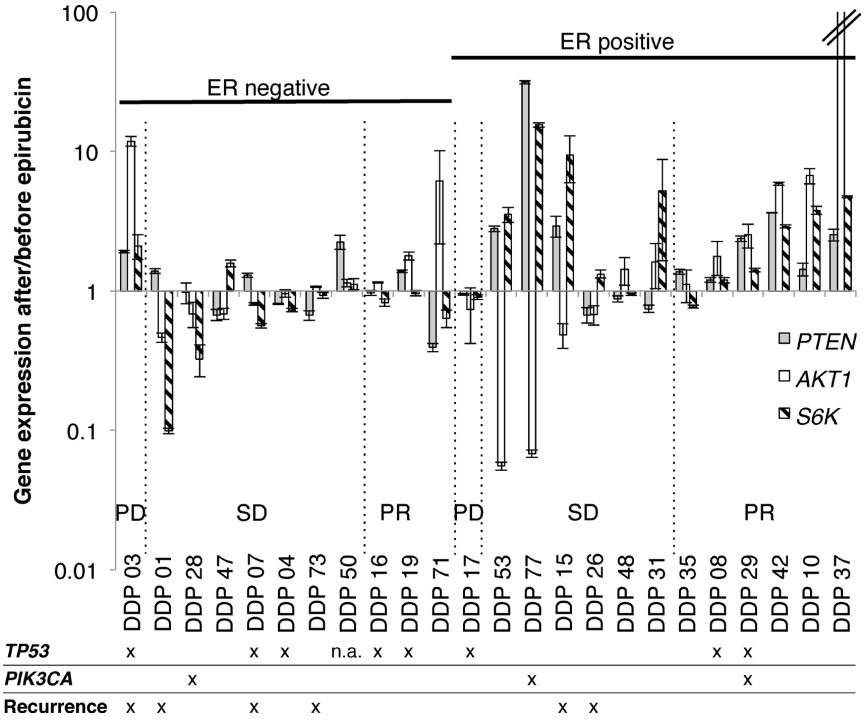
Supplementary Figure 1: (A) Doxorubicin cytotoxicity in MB231, MCF7 and T47D breast cancer cells, assessed by WST-1 cell proliferation assay, after 24 hrs drug exposure. Absorbance was read at optical density (OD) 450 nm, and readings normalized to control wells, incubated with an equivalent DMSO (doxorubicin stock solvent) dose as in the wells with the highest doxorubicin dose. Bars depict the mean ± SEM. The dotted line indicates the IC30 level. **(B)** Western blots of PTEN and Akt protein expression in T47D breast cancer cells *in vitro*, after exposure to doxorubicin or DMSO. Drug exposure lasted 24 hrs, at 0.7 μM doxorubicin or an equivalent volume of DMSO (stock solvent for doxorubicin) for control wells, three independent experiments per group. Whole cell lysate, 30 μg protein loaded per lane.



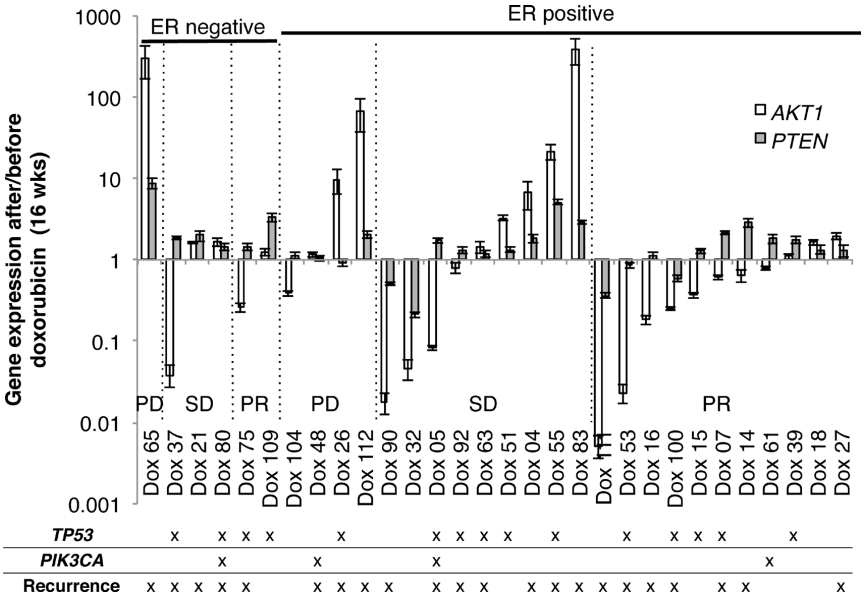
Supplementary Figure 2: (A) Gene expression in doxorubicin-naïve MB231, MCF7 and T47D breast cancer cells *in vitro* exposed to doxorubicin (MB231: 1.5 µM, MCF7: 2 µM, T47D: 0.7 µM), 24 hrs drug exposure. **(B)** Gene expression in doxorubicin-resistant MB231 and MCF7 breast cancer cells *in vitro* exposed to doxorubicin (MB231: 1.5 µM, MCF7: 2 µM), 24 hrs drug exposure. Bars depict the mean gene expression of three parallels ± SEM, normalized to *RPLP2,* and analyzed by real-time quantitative RT-PCR. *p<0.05. ***p<0.001. **(C)** Cytotoxicity of Akt inhibitor A-443654 in MB231 and MCF7 cells, assessed by WST-1 assay, 24 hrs drug exposure. Absorbance was read at optical density (OD) 450 nm, and readings normalized to control wells, incubated with an equivalent dose of HPMC (A-443654 dissolvent) as in the wells with the highest A-443654 dose. Data points depict the mean ± SEM.



Supplementary Figure 3: Gene expression in MB231 **(A)** or MCF7 tumors **(B)** in NOD/SCID mice, given sham treatment (DMSO and HPMC; Controls), doxorubicin (DOX) 1.25 mg/kg i.p. qW twice, Akt inhibitor A-443654 (AKTi) 3.75 mg mg/kg BID 14 days or the combination. Tumors were removed and analyzed 14 days after commencing the treatment. Only tumors from treatment group B, wherein AKTi treatment commenced at the second doxorubicin injection, were used in the current analysis. Densitometry depicts the relative gene expression, normalized to ß-actin. Bars depict the mean ± SEM. *p<0.05.



Supplementary Figure 4: Gene expression in human breast cancer samples before and 24 hrs after the first epirubicin dose (60 mg/m²), in 11 patients with estrogen receptor (ER) negative breast cancer and 13 patients with ER positive breast cancer included in the dose dense trial. Bars depict the ratio of gene expression after vs. before epirubicin, based on the mean of three separate real-time RT-PCR runs, normalized to *RPLP2* and corrected for cDNA pool. PD: Progressive disease after 4 courses epirubicin q2w, SD: Stable disease, PR: Partial response. Patients with breast cancers harboring *TP53* or *PIK3CA* mutations, and those with breast cancer recurrence are labelled beneath the diagram.



Supplementary Figure 5: Gene expression in human breast cancer samples before and after 16 weeks of doxorubicin qW 14 mg/m², in six patients with estrogen receptor (ER) negative breast cancer and 24 patients with ER positive breast cancer included in the doxorubicin trial. Bars depict the ratio of gene expression after vs. before 16 weeks of doxorubicin, based on the mean of three separate real-time RT-PCR runs, normalized to *RPLP2* and corrected for cDNA pool. PD: Progressive disease, SD: Stable disease, PR: Partial response. Patients with breast cancers harboring *TP53* or *PIK3CA* mutations, and those with breast cancer recurrence are labelled beneath the diagram.

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