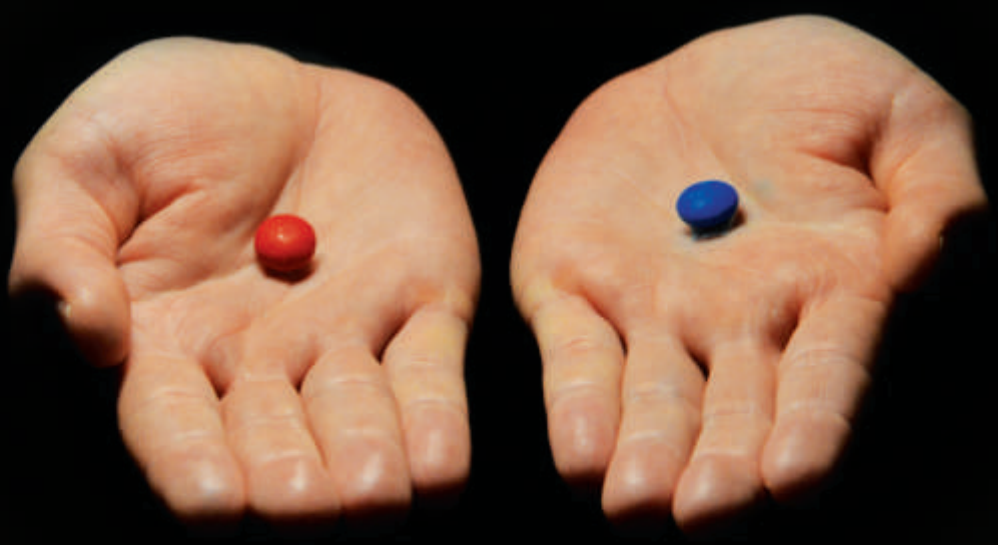
PIcKing the right treatment for the right patient

Anti-hormonal therapy resistance in breast cancer: *PIK3CA* related biomarkers and signaling pathways



Diana Esperanza Ramírez Ardila

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Het kiezen van de juiste behandeling voor de juiste patiënt:

Anti-hormonale therapie resistentie bij borstkanker: *PIK3CA* gerelateerde biomarkers en signaalpaden

PROEFSCHRIFT

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All is before us

This thesis is dedicated to my source of life

CONTENTS

CHAPTER I

General Introduction and outline of the thesis

1. Breast cancer

Breast cancer (BC) is a heterogeneous disease with different clinical, biological, molecular and phenotypical features resulting from accumulation of (epi)genetic alterations and/or altered expression of genes [1,2]. The diversity of these genotypes, as in all kind of cancers, has been generalized by Hanahan and Weinberg as physiologic changes including self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, tissue invasion, metastasis, reprogramming of energy metabolism and evading immune destruction that together dictate malignant growth [3]. Thus, its development is a multi-step process.

BC is also multi-factorial disease in which environmental factors, life-style and individual genetic background play important roles [4].

2. Epidemiology

According to the latest Press Release (No.223) on December 12 2013 by the International Association of Cancer Research (IARC, the intergovernmental agency forming part of the World Health Organisation of the United Nations): Breast cancer affects some 1.7 million women annually worldwide. It comprised 25.2% of cancers diagnosed in women, thus being the most common female cancer (World Cancer report 2014). It was estimated that more than 508.000 women died in 2011 (Global Health Estimates, WHO 2013).

Breast cancer incidence rates strongly vary worldwide. Incidence rates are higher in more developed countries such as Western Europe (90 new cases per 100.000 women annually) compared to developing countries like in eastern Africa (30 new cases per 100.000). Yet, their mortality rates are comparable (15 per 100.000). Although in the developed world the prevention strategies and resources to treat breast cancer may be easier available, it seems that industrialization process may have a higher impact in cancer incidence. Remarkably the incidence rates for breast cancer may increase with level of country income, however this also indicates better screening programs as well as aging of this population.

3. Risk and protective factors

The most important risk factors are female gender, older age, postmenopausal status [5], as well as family history (mutations in high penetrance genes, such as *BRCA1, BRCA2, CHECK2*, *TP53 and PTEN* explain approximately 25% of familial breast cancer) [6-9]. Factors related to increased or prolonged estrogen exposure have been identified as risk factors for BC development and progression i.e. lower age at menarche, late birth of first child, later menopause, lack or shorter periods (<12 months) of breast feeding. Long-term use of contemporary oral contraceptives (OCs) and current use for ≥ 5 years has been related to an increase in risk of getting premenopausal BC. Similarly, hormonal replacement therapy, specially combined estrogen-progestin menopausal therapy has been linked to enhance the risk of getting postmenopausal breast cancer [5].

As for every disease lifestyle is important and lack or little physical activity is a risk factor [10]. Other reported lifestyle risk factors include smoking, nightshifts or sleep disorders which affect circadian and neuroendocrine rhythms [5,11].

Environmental factors such endocrine disruptors have shown to cause and/or to be linked to strong causality in breast cancer [5]. Around 800 chemicals used in daily life are known or suspected to be endocrine disruptors (http://www.who.int/ceh/publications/endocrine/en/). DDT, PCB, DDE, PAH, PCB and BPA are the most important [12-19]. Maybe frequent mammographic examinations in some cases may increase the risk of getting BC. All this is still a controversial and therefore a debated topic [5].

Protective factors include amongst others the use of turmeric, garlic, as well as different vegetables and fruits which are considered chemopreventive, anti-proliferative, antioxidant and carcinogen-blocking agents which acts by diverse mechanisms which are investigated in clinical trials [20], and in cell line models [21-26]. Additional and more detailed information about risk as well as protective factors can be found for instance at the website breastcancer.org (see references for further details link[27]).

4. Estrogens and Estrogen Receptor (ER)

Estrogens, together with progesterone [28], prolactine [29] and growth hormone [30], play an important role in the regulation of proliferation and differentiation of the mammary glands. In mouse models, estrogens have been shown to exert a proliferative effect on mammary gland epithelial cells either directly or indirectly by neighboring stromal cells, through binding to estrogen receptors [30]. Thus, estrogens control important aspects of reproduction and homeostasis. Estrogens selectively bind to estrogen receptors (ER) of which 2 types have been identified: ER-alpha and ER-beta. Especially ER-alpha has been associated with carcinogenesis and serves as main target for endocrine treatments. Also, loss of ER-beta and TP53 results in breast tumorigenesis and cancer progression [31].

ER acts as ligand-activated nuclear transcription factors [32,33]. Once the estrogen binds to ER, the receptor dimerizes attracting co-activators and displacing co-repressors and then successively bind to specific estrogen response elements within promoter regions of estrogen-regulated genes. Some examples of ER co-activators such as AIB1 and PELP1/MNAR as well as the ER co-repressors RIP140, LCoR, MTA1, TR2, SAFB1/2, FKHR and NCoR have been reviewed by Dobrzycka et al. [34]. Due to these co-activators or repressors, expression of ER target genes can be either up- or down-regulated.

Additional genomic functions of ER as co-regulator for other transcription factors as well as ER non-genomic activities outside the nucleus, at the membrane, in the cytoplasm and for ER-beta in the mitochondria have been also described [35,36].

5. Prognosis

Breast cancer prognosis is strongly related to tumor characteristics such as tumor size, tumor grade and axillary lymph node involvement [37,38]. Mainly, two different and complementary classifications have been used for prognosis: TNM status and tumor grade by Bloom and Richardson. TNM classification is based on three clinico-pathological characteristics: diameter of the tumor (T), involvement of local lymph node metastases (N) and presence of distant metastases (M) at time of diagnosis [39]. Tumor grade classification determined by the Bloom and Richardson system categorizes breast tumors in three grades based on the degree of glandular differentiation, degree of nuclear atypia and mitotic index into: well differentiated (grade I), moderately differentiated (grade II) or poorly differentiated (grade III) [40]. Overall, women with larger breast tumors and/or increasing numbers of positive lymph-nodes based on the TNM staging have a worse breast cancer survival [41]. Likewise, poorly differentiated tumors, i.e. with high tumor grade, correlates with worse survival I [42].

Nowadays, most newly diagnosed breast tumors are predominantly found at an early disease stage. The majority of tumors (75%) are ER-positive and more often found in postmenopausal patients. Whereas ER-negative breast cancer is more frequently found (46%) in premenopausal patients and is associated with larger tumors. Despite the growth-stimulatory effect of estrogens, exerted by its receptor, breast tumors without detectable ER expression tend to grow more rapidly, are less differentiated and more aggressive.

6. Clinical significance of the intrinsic subtypes

Although breast cancer has been described as a heterogeneous disease, it is the introduction of high throughput molecular technologies and bioinformatics that enabled researchers to more accurately classify breast tumors, based on common gene expression patterns [43-45]. While general classification has been done on single ER protein expression, the multiple gene expression patterns described in the landmark study by Perou et al. can distinguish different intrinsic molecular subtypes. Initially, these subtypes were classified in four main groups: luminal, HER2-enriched, basal-like and normal-like [43]. Importantly, these subtypes were maintained among different microarray platforms, patients' series and races [46,47].

Later these subtypes were further refined based on larger sets and more detailed gene expression patterns. Luminal breast cancer tumors were ultimately subdivided in two groups: Luminal A and luminal B [48,49]. Additional characteristics of each of these subtypes have been added to better define them [1]. For instance, patients with basal-like tumors, nowadays better known as triple-negative tumors since they generally do not express ER, PR, nor HER-2, have the worst overall survival [48]. Moreover, luminal B compared to luminal A tumors have also a less favorable outcome [48,50]. Bone and (to a lesser extend) pleura are the most frequent sites of metastasis of luminal tumors; whereas HER-2 and basal-like tumors have more preference to metastasize to the brain [51].

7. Treatment and mechanisms of action

Treatment characteristics are defined in terms of response or time. For example, therapy given before the surgery is called neo-adjuvant therapy, but when followed after surgery it is called adjuvant treatment. When afterwards, the patient has a metastasis the given treatment is called first-line therapy and after every relapse will be followed by second-, third-, etc.-line therapy. Additionally, the effect of a therapy is evaluated based on response or survival analysis. Figure 1 illustrates general terms used in clinic for therapy and survival given in scientific publications.

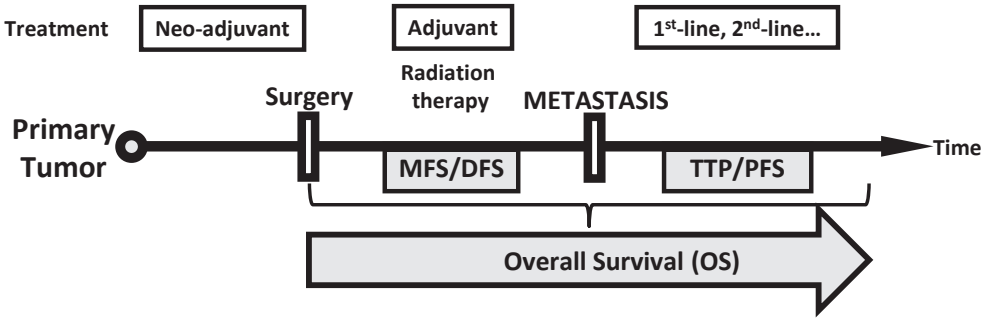

Figure 1 Terms used in clinic for therapy and survival. Terms mentioned refer to the moment in which a systemic treatment is given (White boxes, upper part); and to survival analysis (Grey boxes, bottom part). Survival is specified by their abbreviations: **MFS:** Metastasis Free Survival and **DFS**: Disease Free Survival are interchangeable; as well as **TTP**: Time to Progression and **PFS:** Progression Free Survival. Surgery and radiation therapy are local treatments.

Until 1970 treatment decisions were merely based on clinico-pathological characteristics, including tumor size, lymph node status and histological grade. Later on, and based on (targeted) treatment possibilities, evaluation of ER, PR and HER2 protein expression was added and combined in an algorithm. This algorithm is available online to assist decision making on adjuvant treatment in early breast cancer [52]. Generally, luminal tumors express estrogen receptors (ER) and count for approximately 75% of all breast cancer tumors. For these ER-positive tumors three types of hormonal therapy are currently given: SERMs like tamoxifen, aromatase inhibitors (AI), or SERDs like fulvestrant. Although each of these drugs have different mechanisms of action, they all aim at in preventing activation of ER and it signaling pathway.

While in premenopausal women, the main source of estrogens (estrone and estradiol) is the ovary, in post-menopausal women the estrogens are mainly derived from local conversion of circulating androgens (androstenedione and testosterone) by peripheral aromatase, predominantly in the adipose tissues. The adrenal glands are the main producers of these circulating androgens. However, approximately 10-25% are still being produced by the ovaries under the control of the Luteinizing Hormone (LH) [53-55].

Endoxifen and (4-OH)-tamoxifen, the active metabolites of tamoxifen, act as competitors of estrogen by irreversible binding to the ER which results in a

conformational change but not activation of ER. This then leads to blocking of the G0/G1 phase of the cell cycle, which results in the attenuation of cell proliferation [56,57], and possible induction of apoptosis [58].

Unlike tamoxifen, AI reduce local estrogen levels by inhibiting the aromatase enzyme responsible for conversion of androgens into estrogens. AI can inactivate aromatase enzymes by binding reversibly to heme moiety (like nonsteroidal AI such as letrozole or analstrozole) or by permanent binding to the active site of the enzyme (like steroidal AI such as exemestane) [59].

There are two types of aromatase inhibitors approved to treat breast cancer, i.e. steroidal and non-steroidal inhibitors. The irreversible steroidal inhibitors, such as exemestane, forms a permanent and deactivating bond with the aromatase enzyme. On the other hand, non-steroidal inhibitors such as anastrozole and letrozole, inhibit the synthesis of estrogen via reversible competition for the aromatate enzyme.

In postmenopausal women, AI have shown very limited improvement over tamoxifen in relation to overall survival (OS) [60]. In metastatic breast cancer, AI increase time to recurrence compared to tamoxifen [60,61] and have replaced tamoxifen as first-line treatment for advanced postmenopausal breast cancer [62,63]. Although less severe than tamoxifen, AI exhibit side-effects, including bone loss and fractures, rheumatoid arthralgia and even possibly effects on lipid metabolism and cognition [64].

Another therapy used for ER+ tumors, although less common, is Fulvestrant (Faslodex®), an estrogen receptor antagonist, inhibiting ER-alfa protein dimerization and additionally introduces a conformational change accelerating the proteosomal degradation of the estrogen receptor. Its efficacy is similar to other endocrine therapies [65,66]. Fulvestrant and other SERDs are nowadays considered of treatment for MBC patients who acquired resistance to AI-therapy due to specific mutations in the ligand binding domain of *ESR1* [67,68].

Drugs targeting HER2-positive tumors include: trastuzumab (Herceptin®), a monoclonal antibody against HER2; pertuzumab (Perjeta®), a HER2 and HER3 dimerisation inhibitor; ado-trastuzumab emtansine (Kadcyla®), Herceptin® linked to emtansine (a chemotherapeutic agent); and most recently lapatinib (Tykerb®), a dual tyrosine kinase inhibitor of both HER2 and EGF receptors, which has been developed to expand the options for treating HER2-positive breast cancer patients [69].

8. Tumor biomarkers: gene signatures, microRNAs and liquid biopsy

Besides single biomarkers, gene signatures, also called gene expression profiles, started to emerge ever since 2000 attempting to identify individual's prognosis and most optimal personalized treatments.

Gene signatures / gene expression profiles

A gene signature is a specified gene expression pattern that can be strongly correlated with clinical and/or tumor characteristics. This may result from a comparison between two defined groups based on one specific feature. For example, a gene signature defined based on clinical outcome, such as patients with good prognosis (no distant metastases >5 years) vs patients with poor prognosis (distant metastases <5 years).

Consequently, the expression pattern has a certain number of up and down-regulated genes that have a significant and strong correlation with the parameter of interest. These signatures are typically used to generate scores. Positive scores indicate the number of up-regulated genes in one group is higher compared to the number of up-regulated genes in the other group.

Overall, these expression profiles contribute on one side to reduce over-treatment and on the other side to consider alternative (targeted) therapies to treat predicted resistant tumors. Gene signatures may include different genes, while in some cases these genes enrich for related biological pathways [70]. They may contribute to the outcome prediction. Nowadays a large number of signatures with prognostic and/or predictive value has been proposed to provide additional information next to the currently used clinical-pathological features as tumor size and lymph-node status rather than to be a replacement [71].

Most of the signatures were in principle designed to predict recurrence, later on some of them were shown to have also predictive value for chemotherapy [72,73], for example:

MammaPrint®, a 70-Gene signature (which was one of the first FDA approved gene expression signatures), predicts high risk of developing metastasis in either ER-positive or ER-negative early breast cancer patients [74,75]. This validated (Raster study and Mindact trial) signature showed that patients classified as high risk had benefit from chemotherapy when added to endocrine treatment [76] whereas patients at low (genomic) risk, but classified at high clinical risk, showed no benefit from chemotherapy [77].

Oncotype DX®, a 21-Gene signature, predicts recurrence in lymph-node negative ER-positive breast cancer patients treated with adjuvant tamoxifen [78,79]. It also identifies patients who would benefit of additional chemotherapy [80].

EndoPredict®, a 12-Gene signature combined with tumor characteristics, scores the risk of distant metastasis in early-stage ER-positive, HER2-negative breast cancer patients treated with endocrine therapy alone. [81-84].

Other signatures, besides scoring the risk of distant recurrence 5 to 10 years after diagnosis, define patients who can benefit from hormonal treatment and thus may be of value in selecting patients for extended hormone therapy [85]:

Prosigna® 58-gene signature (Formerly called PAM50), is already approved by the FDA and has the CE mark. This signature, measures the risk of distant recurrence in

ER-positive postmenopausal breast cancer patients in early stage who will benefit from extended adjuvant hormonal therapy [43,48,86].

Breast Cancer Index™ 7-Gene signature, scores the risk of recurrence in early stage ER-positive HER2-negative lymph node-negative (NO) and N1 breast cancer patients. Additionally, it predicts the benefit from extended endocrine therapy [87-90].

Additional signatures have been described to predict response to hormonal therapies in breast tumors including for tamoxifen and AI [91,92], respectively. Furthermore, the prognostic 76-gene signature of Zhang Y. et al., demonstrated for its classified high-risk patients benefit from adjuvant tamoxifen therapy [93,94].

MicroRNAs

Next to mRNA expression signatures, also expression of microRNAs have been profiled and evaluated for a relation with disease and treatment outcome. MicroRNAs (miRNAs) are a class of small non-protein-coding RNAs, evolutionarily conserved, that control gene mRNA expression. This mRNA expression can be either inhibited, degraded [95,96] or enhanced [97-100] via sequence-specific interaction of a miRNA with the 3' UTR of target mRNA. The mechanism of gene expression control depends on the degree of complementarity [101].

Most of the miRNAs are located in intergenic regions residing predominantly in introns, but they can also be found in exons on the antisense strand of defined transcription units. MiRNAs can also be located in intragenic regions, having their own promoter and being transcribed as independent units [102].

Deregulation of miRNAs expression occurs and can be due to genomic amplifications, deletions and mutations, epigenetic mechanism such as hyper methylation of the promotor and importantly due to changes in the tumor microenvironment [103-106].

Aberrant expression levels of miRNAs have been observed amongst others between breast cancer molecular subtypes [107,108] and related with prognosis [109-111] and with response to hormonal therapy [112,113].

Liquid biopsy

Almost all cancer biomarker and expression signature research discussed above, have been performed on tissue biopsies. The last decade, however, also liquid biopsies are evaluated as tumor diagnostics tool to enable a more improved and personalized cancer treatment.

Liquid biopsy is a less invasive method to detect in real-time the evolution of breast cancer. Liquid biopsies include mainly circulating tumor cells (CTCs) and cell-free DNA (cfDNA), although circulating endothelial cells (CECs) [114] or exosomes have also been described. Cell-free DNA originates from apoptotic or necrotic cells or viable cells which actively secret DNA fragments into the blood stream. Thus, cfDNA from normal cells or circulating tumor DNA (ctDNA) from tumor cells can be easily isolated and processed from plasma or serum. However, high-throughput and sensitive analyses are needed and now available to detect ctDNA. For the characterization of

ctDNA, next generation sequencing (NGS) and digital PCR (dPCR) are frequently used [115].

Genomic patterns in cell-free DNA such as somatic single nucleotide variants (SNVs), copy number alterations (CNA) and structural variants (SVs) derived from the tumor have been already effectively detected. Thus, cell-free DNA is a great tool to monitor patients during the course of treatment to improve their therapies based on genomic patterns [116-120]. CTCs are also available in the blood stream of cancer patients. Compared to cell-free DNA, characterization of CTCs, covers RNA and protein patterns besides DNA alterations [121-123]. Nevertheless, CTCs are less frequent present in blood compared to cell-free DNA [124].

Although the clinical utility of liquid biopsy still needs to be evaluated [125], its use is a crucial step towards a more individualized cancer therapy, which will revolutionize the ways to select and monitor cancer treatments [115].

9. Therapy resistance

Targeted therapies against the ER, such as tamoxifen or AI, and against HER2, such trastuzumab and lapatinib, are proposed to be successful because breast cancer patients are stratified based upon these molecular markers or based on subtypes. Unfortunately, not all patients respond (**de novo resistance**) while in the metastatic setting, patients who respond initially will eventually relapse (**acquired resistance**).

Approximately 40% of the metastatic patients with ER-positive primary breast tumors respond to hormonal therapy (antiestrogens or aromatase inhibitors) when given as first-line treatment [126]. In the adjuvant setting, tamoxifen therapy results in an 11% improvement of 10-year survival in lymph node-positive patients, independent of menopausal status or age [127]. Whereas, of the 60% of early stage breast cancer patients that receive chemotherapy in the adjuvant setting, only 2-15% will benefit, while all remain at risk of side-effects [127,128]. Thus ER as a biomarker is not perfect for prediction of treatment outcome. There is also substantial inter-individual variation in response to tamoxifen and Aromatase inhibitors.

10. Mechanisms of endocrine resistance: Overview

Several mechanisms have been described to contribute to hormonal resistance [60,129-131], but the (dis)function of ER-alpha plays a central role in endocrine therapy resistance.

Loss or modification of ER expression, is the main mechanism of the novo resistance to hormonal therapy. Different theories have been proposed to explain the loss of ER expression. Epigenetic changes, hypoxia and overexpression of EGFR or HER2 have shown to alter the ER transcription and to explain the reduced ER expression. Alterations in **DNA methylation at CpG islands of the ER promoter**, usually hypermethylation as well as **histone modifications** are the main epigenetic changes studied [130]. An example of histone modifications is the increased expression of EZH2, a histone methyl transferase that has been associated with downregulation of ER and tamoxifen resistance [132]. PR and CDK10 gene methylations have been also related to endocrine resistance [130]. Additional epigenetic changes have been

suggested for their relationship with tamoxifen resistance, including the lack of HOXB13 expression in especially a subset of ER-positive tumors seen after evaluation of the expression ratio of homeobox protein (HOXB13)/Interleukin-17B receptor (IL17BR) [133].

Mutations in the *ESR1* gene such as the A1587G, are also related to hormonal resistance and poor survival. This type of mutations present in only 1% of primary breast tumors [134], have been observed to arise especially after AI treatment [135,136].

Ligand-dependent but also independent activation of ERα can occur through **phosphorylation of the ER at specific amino acid sites** such as at Ser 118, 104, 106, 167 and Ser305 through different kinases including GSK-3, ERK1/2 MAPK, CDK2 and PKA have been associated with tamoxifen resistance. On the contrary, tamoxifen sensitivity has been related to ER phosphorylation at Ser167 and Ser282 through kinases such as ERK1/2 MAPK, p90RSK, CK2, Akt and mTOR/p70S6K. Phosphorylations including Ser118 and Tyr537 have shown a dual effect [137].

Lack of pioneer factors such FOXA1 have been also shown to be related to hormonal resistance in breast cancer. This pioneer factor is required for transcription of ER dependent genes, it is needed during the transcription process to let other binding proteins access the transcription binding site. Therefore, in the absence of FOXA1 cells do not respond to anti-estrogen treatment since the cell growth is not ER-alfa dependent [138,139].

Another mechanism directly related to ER are **ER splice variants**, which have been shown by Groenendijk et al. to explain hormonal treatment resistance. The dominant negative ERα variant ERΔ7 stains positive for ER by immunohistochemistry while it classifies Basal-like according to the molecular subtype classification. This splice variant lacks a functional response to estrogen and consequently may not respond to hormonal therapy [140].

Mechanisms related to **hormonal drug metabolism caused by genetic variants** might also explain tamoxifen resistance. It has been demonstrated in patients having different (*4, *5, *10, and *41) variant CYP2D6 genotypes, that they cannot or poorly metabolize tamoxifen into the active metabolite 4-OH-tamoxifen, and therefore those patients do not or poorly respond to the therapy [141-143]. Inhibition of metabolism of tamoxifen might also occur via co-administration of drugs that inhibit CYP2D6, such as the selective serotonin re-uptake inhibitors (SSRIs) [144].

Activation of proliferative kinase pathways can stimulate cancer growth alone or in concert with ER signaling and have been related to hormonal resistance. The PI3K/AKT/mTOR and the mitogen-activated protein kinase (MAPK) pathways are the most frequently altered in cancer and consequently the most studied. These pathways as well as its mutual intercommunication are explained below. Additional kinase pathways such as the protein kinase A (PKA) and p-21activated kinase-1 (PAK-1) have also been related to hormonal (tamoxifen) resistance.

11. PI3K complex, *PIK3CA* mutation and downstream resulting pathways

Since the phosphatidylinositol 3-kinase (PI3K) complex was first described, several studies have established the central role of PI3K signalling in diverse cellular processes critical for cancer progression, including proteins synthesis, growth, metabolism, proliferation, cell survival, apoptosis avoidance, motility and angiogenesis [145].

At genomic level, there are three classes of PI3K grouped according to structure and function. Class IA PI3K is the one most clearly implicated in human cancer [146]. This class consists of two main domains: the regulatory and the catalytic domain. The regulatory (R) domain is comprised of three genes: PIK3R1, PIK3R2 and PIK3R3 which encode for p85α, p85β, and p55γ respectively. p85α includes three different isoforms: p85α, p55α, and p50α. The catalytic (C) domain is comprised by the genes *PIK3CA*, *PIK3CB*, and *PIK3CD*, which encode for p110α, p110β, and p110δ respectively [146-148].

PIK3CA mutation

PIK3CA is the most frequent mutated gene in primary breast cancer up to 45%) [1]. Approximately 90% of *PIK3CA* mutations are clustered at 2 hotspot regions in exon 9 (E542K and E545K) and exon 20 (H1047R and H1047L) [149,150], encoding the helical and kinase domains, respectively.

PIK3CA mutations have been shown to phosphorylate and therefore activate AKT, the main component of the PI3K/AKT/mTOR pathway. The AKT phosphorylation is generally observed in exon 20 but not in exon 9 *PIK3CA* mutants [151]. Additional downstream evaluated markers such as mTORC1, pS6, p70S6K, p4EBP1 and GSK3 have not seen to be activated in *PIK3CA* mutated breast cancer cell lines nor tumors [1,152,153].

PIK3CA mutations have been shown to have different effects on therapy response depending on the molecular subtypes. For example, HER2-positive patients harboring the *PIK3CA* mutation show resistance to trastuzumab therapy [154-157]. On the other hand, the presence of the *PIK3CA* mutation in ER-positive breast cancer cell lines as well as in ER-positive, HER2-negative primary breast cancer patients have been associated with sensitivity to adjuvant tamoxifen [153].

Pathways related with *PIK3CA*

In breast cancer, hormonal resistance might also be related to activation of alternative proliferative pathways such as the PI3K/AKT/mTOR and the MAPK pathway, induced by upstream growth factors through their receptors including the insulin-like growth factor (IGF)-1 and IGF1R or the epidermal growth factor (EGF) and EGFR. Many of these pathways probably emerge as ER-independent drivers of tumor growth, survival and/or inhibition of apoptosis. [129,137,140,143].

PI3K/AKT pathway followed by mTOR resulting pathways

Probably the PI3K/AKT pathway is the one of the best studied. Several components of this pathway are often deregulated, including amplification of *HER2,* loss of *PTEN*

function and *PIK3CA* amplifications or mutations. Most of the players of this pathway are kinases (i.e. enzymes that transfer phosphate from ATP to a specific substrate). Here, the cascade of events known until today upon growth factor activation is described. The first step is the growth factor binding to its ligand at the extracellular level, followed by a dimerization of the receptor making it auto-phosphorylated. The dimerization triggers the recruitment of adapter proteins such as IRS1 activating the PI3K complex (previously described) to generate PIP3 which triggers AKT translocation to the cell membrane. PTEN and INPP4B reduce the levels of PIP3 in the membrane by dephosphorylating PIP3 and PIP2, respectively, thus inhibiting PI3K activation [158].

Once AKT is recruited to the cell membrane it can be phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) on threonine at position 308 (Thr308), followed by a second phosphorylation on serine at position 473 (Ser473) by the downstream mTORC2 complex. Upon activation, AKT dissociates from the membrane and then moves to the cytoplasm and the nucleus, where it phosphorylates multiple proteins involved in translation, metabolism, proliferation, survival, and angiogenesis [131,159].

When AKT is phosphorylated its downstream target mTOR will be activated [131]. This mTOR is a serine/threonine kinase which acts as the catalytic subunit of the two known mTOR complexes, mTORC1 and mTORC2. The mTORC2 complex is responsible for the AKT phosphorylation on Ser473 (previously described) [160-163].

AKT also activates mTORC1 through TSC2, consequently two mTORC1 main downstream target proteins, 4EBP1 and S6K, will be phosphorylated by mTORC1. 4EBP1, which is a repressor of mRNA translation, becomes inactive through its phosphorylation by mTORC1 [164]. 4EBP1 can be seen then as a tumor suppressor since it represses eIF4E, a molecule responsible for protein synthesis [165]. On the other hand, mTORC1 activates S6K, which is responsible for ribosomal biogenesis [164]. As a result, S6K represses IRS-1 via a feedback loop, resulting in a PI3K/AKT pathway inhibition [131].

12. Pathways, signatures and crosstalk

Different signatures related with the PI3K/AKT/mTOR pathway have been shown to predict hormonal (tamoxifen and letrazole) response in the adjuvant setting [153,166]. Previously, Loi et al. have shown that ER-positive tumors of breast cancer patients have high score of the *PIK3CA*-GS and are associated with longer MFS after adjuvant tamoxifen treatment. Their signature is based on the *PIK3CA* mutation status, mainly exon 20 mutations and grade, meaning that patients with a *PIK3CA* mutation pattern have high score of the mentioned signature. The *PTEN*-loss (Saal signature) was developed to represent IHC-detectable *PTEN* loss in breast cancer [167] while the PI3K signature by Creighton is based on a set of genes in which expression was induced or repressed by PI3K inhibitors [168].

The PI3K/AKT/mTOR pathway shares some features with other pathways i.e. the mitogen-activated protein kinase (MAPK), also known as the Raf/MEK/ERK pathway, both kinase pathways stimulate cell proliferation, through a signaling initiated on receptors located on the cell surface and resulting downstream in the activation of

nuclear transcription factors [169,170]. In human tissues, three major MAPK pathways are known, but the most relevant in breast cancer is the one involving MEK1/2 and ERK1/2 [171].

It has been shown that PI3K/AKT/mTOR and Raf/MEK/ERK pathways can collaborate to maintain cell viability [172]. The crosstalk can occur in both directions. For example, MAPK signalling can be either reduced by AKT through inhibition of Raf phosphorylation [173], or enhanced after inhibition of mTORC1 through a S6K-PI3K feedback loop [174]. In contrast, alterations in the PI3K/AKT pathway specifically to TSC2 and PTEN can be caused by the ERK and p90RSK [175,176] and by Ras respectively [177].

The following links show a general view of the crosstalk between the PI3K/AKT/mTOR and the Raf/MEK/ERK pathways:

http://www.sciencedirect.com/science/article/pii/S0305737213000728

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3291999/figure/f3-ijms-13-01886/

The complexity of molecular communication between pathways in breast cancer is further complicated by interactions of the PI3K/AKT/mTOR and MAPK pathways with other cascades, including the important ER pathway [138,169]. It has been observed that hormones such as estradiol, progesterone, and testosterone can act through G protein receptors activating the MAPK pathway [171].

Thus, estradiol can stimulate cell proliferation either through a non-genomic or genomic estrogen receptor effects (increasing the production of growth factors) which ultimately will result in more activation of both kinase pathways (PI3K/AKT/mTOR and MAPK). Moreover, transcription of growth factors can also be induced by these two pathways [170] leading to a feed-back in tumor cells to reactivate signaling cascades.

13. *In silico* modelling and data-bases

Considering the blooming data era, with millions of public available data, more researchers are choosing to work or at least to start their work with the analyses of in-silico data. It implies less costs and time when compared to wet lab work. Moreover it is cost effective and gives the opportunity to further explore others previous efforts. In the last decade, several research consortia such as The Cancer Genome Atlas (TCGA; https://cancergenome.nih.gov) and the International Cancer Genome Consortium (ICGC; https://icgc.org) have evaluated large series of cancers, including breast cancer using high-throughput and state-of-the-art technologies to profile each individual tumor for its genomic and epigenomic DNA alterations and for expressed mRNAs, microRNAs and proteins. All these data are publicly available for the research community for *in silico* exploration, modelling and/or validation.

14. Aims and outline of the thesis

The perception of breast cancer has changed in the last decades. The omics era has guided us to a better understanding of the heterogeneity of the disease, allowing us to make use of common features to better group patients and hopefully to improve patients' treatments.

This thesis is in line with the omics peers and is committed to improve diagnostic tools for breast cancer patients' treatment and thus aims to find potential biomarkers to predict hormonal treatment responsiveness and/or resistance in advanced ER-positive breast cancer patients.

For the mentioned purposes, the thesis firstly presents in **chapter II**, the effect of the *PIK3CA* mutation (the most common mutation in breast cancer) in relation to hormonal treatment (tamoxifen and AI) outcome in ER-positive metastatic breast cancer patients (MBCP), and additionally in relation to prognosis. In this study patients with the *PIK3CA* mutation are associated with longer time to progression (TTP) after first-line AI.

Consecutively, in **chapter III**, potential biomarkers for sensitivity to AI in ER-positive MBCP are proposed based on their relation to the *PIK3CA* mutation status by using *in silico* gene and microRNA expression profiles. *LRG1* expression is then proposed as a potential biomarker for AI treatment outcome independent of luminal A or B subtype.

In **chapter IV**, altered phosphorylation of proteins as well as altered protein expression related to the *PIK3CA* mutation status is presented in a subtype independent manner. In-silico data of cancer related proteins (including the ones in the PI3K/AKT/mTOR pathway) are used for this purpose. *PIK3CA* mutated breast tumors are furthermore characterized in an exon independent manner. In TMAs, a favorable prognosis is shown for lymph-node negative ER-positive patients with high MAPK1/3 phosphorylation in nuclei and in tumor cells.

Additionally, in **chapter V**, a different approach to the study the endocrine resistance is used. ER-positive patients with inflammatory breast cancer (IBC), an uncommon type of breast cancer (≅5%), are studied in relation to tamoxifen and AI response after adjuvant and first-line therapy. For this, ER-positive IBC patients are selected since their response to endocrine treatment is poorer compared to ER-positive non-IBC. Low expression of *ABAT* and *STC2* are proposed for adjuvant and/or first-line tamoxifen and/or AI resistance biomarkers.

Finally, in **chapter VI**, in an exploratory study of cell-free DNA mutations in MBCP treated with tamoxifen, different progression markers including *PIK3CA* mutations are identified to contribute to the understanding of tamoxifen response in ER-positive breast cancer patients. This study also shows the potential of using liquid biopsies as an alternative diagnostic tool to assess disease progression over time in a patient.

In conclusion, this thesis studies diagnostics/biomarkers involved in hormonal therapy resistance aiming at improved precision medicine for metastatic breast cancer patients.

ABBREVIATIONS

AI	Aromatase Inhibitors
AKT	protein kinase B
BC	Breast Cancer
BPA	Bisphenol A
CTC	Circulating Tumor Cells
CYP2D6	Cytochrome P450 family 2 subfamily D member 6
DDE	Dichloro-diphenyl-dichloroethylene
DDT	Dichloro-diphenyl-trichloroethane
4E-BP1	4E-binding protein 1
EGFR	*Epidermal growth factor receptor*
eIF4e	eukaryotic translation initiation factor 4E
EZH2	Enhancer of Zeste 2 polycomb repressive complex 2 subunit
FDA	Food and Drug Administration
FOXA1	Forkhead box A1
HER-2	Human epidermal growth factor 2
IBC	Inflammatory breast cancer
IGF1R	insulin-like growth factor Receptor
IHC	Immuno Histochemistry
INPP4B	*Inositol Polyphosphate-4-Phosphatase, type II*
IRS1	*Insulin Receptor Substrate 1*
MAPK=ERK	Mitogen-Activated Protein Kinase
MBCP	Metastatic Breast Cancer Patients
MEK	Extracellular signal-regulated kinases
MFS	Metastasis free survival
mTOR	*mammalian Target of Rapamycin*
mTORC1	mammalian Target of Rapamycin Complex 1
NCI	National Cancer Institute
OS	Overall Survival
PAH	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PFS	Progression-free survival
PIK3CA	Phosphatidylinositol-4,5-bisphosphate3-kinasecatalytic subunit alpha
PIK3CB	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta
PIK3CD	Phosphatidylinositol-4,5-bisphosphate3-kinase catalytic subunit delta
PIK3CA-GS	*PIK3CA* gene signature
PIP2	Phosphatidylinositol4.5-bisphosphate
PI3K	phosphatidylinositol 3-kinase
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PTEN	*Phosphatase and tensin homolog*
P90RSK	*Ribosomal protein S6 kinase A1*
S6K1	Ribosomal S6 kinase 1
TMA	TissueMicro Arrays
TSC2	Tuberous sclerosis complex 2

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CHAPTER II

Hotspot mutations in *PIK3CA* associate with first-line treatment outcome for aromatase inhibitors but not for tamoxifen

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ABSTRACT

PIK3CA mutations occur frequently in breast cancer, predominantly in exons 9 and 20. The aim of this retrospective study is to evaluate the *PIK3CA* mutation status for its relationship with prognosis and first-line endocrine therapy outcome. *PIK3CA* exon 9 and 20 were evaluated for mutations in 1,352 primary breast cancer specimens by SnaPshot multiplex analyses. The mutation status was studied for their relationship with metastasis-free survival (MFS) in 342 untreated lymph node-negative (LNN) patients and to time to progression (TTP) in estrogen receptor (ER)-positive patients with metastatic disease treated with first-line tamoxifen ($N = 447$) or aromatase inhibitors (AIs; $N = 84$). We detected in 423 patients hotspot mutations for *PIK3CA* (31 %). Mutations in exon 20 were detected in 251 patients (59 %), with H1047L and H1047R mutations in 37 (15 %) and 214 (85 %) cases, respectively. Mutations in *PIK3CA* exon 9 were discovered in 173 patients (41 %), with E542K and E545K mutations in 57 (32 %) and 104 (60 %) cases as most prevalent ones. Evaluation of the untreated LNN patients for prognosis showed no relationship between MFS and *PIK3CA* mutations, neither for exon 9 [HR = 1.04 (95 % CI 0.57–1.89), $P = 0.90$] nor for exon 20 [HR = 0.98 (95 % CI 0.63–1.54); $P = 0.94$] when compared to wild-type. The *PIK3CA* mutation status was also not associated with treatment outcome after first-line tamoxifen. On the other hand, patients treated with first-line AIs showed a longer TTP when having a *PIK3CA* mutation in exon 9 [HR = 0.40 (95 % CI 0.17–0.95); $P = 0.038$] or exon 20 [HR = 0.50 (95 % CI 0.27–0.91); $P = 0.024$] compared to wild-types, both significant in uni- and multivariate analysis including traditional predictive factors. All results remained when only HER2-negative patients were evaluated for each cohort. *PIK3CA* mutations in ER-positive tumors were significantly associated with a favorable outcome after first-line AIs, which needs further confirmation in other datasets. Mutations were not associated with prognosis in untreated LNN patients nor predictive outcome after first-line tamoxifen therapy in advanced disease patients.

I

The phosphatidylinositol-3-kinase (PI3K) pathway regulates several cellular processes critical for cancer progression, i.e., protein synthesis, growth, metabolism, proliferation, cell survival, apoptosis, motility and angiogenesis [1]. Since this pathway is frequently deregulated in breast cancer, it is an attractive pharmacologic target to investigate. The PI3K is a complex of regulatory and catalytic proteins and one of the mechanisms of abnormal PI3K pathway activation is through mutations in the 110 kDa catalytic protein encoded by *PIK3CA* or in the 85 kDa regulatory protein encoded by *PIK3* . *PIK3CA* is the most frequent (30 %) mutated oncogene in breast cancer (http://www.sanger.ac.uk/genetics/CGP/cosmic), especially in ER-positive tumors [2, 3]. Approximately 90 % of *PIK3CA* mutations [4], are clustered in two hotspot regions: exon 9 (E542K and E545K) encoding the helical domain and exon 20 (H1047R and H1047L) encoding the kinase domain [2, 5].

PIK3CA mutations may play an important role in the carcinogenesis and development of breast cancer and has been correlated with clinical and treatment outcome [6]. Several studies presented, however, contradicting results for the relationship between *PIK3CA* mutation status and clinical outcome. Some studies showed no correlation between *PIK3CA* mutations and clinicopathologic variables [4, 7], while others have shown a worse prognosis after treatment with *HER2*-inhibitors for patients with *HER2*+ tumors and *PIK3CA* mutations [8, 9]. Moreover, PIK3CA alterations are mainly present in invasive lobular (46 %) and ductal (22 %) breast carcinoma [10]. In lobular tumors, *PIK3CA* mutations have been found to be associated with tumor size, ER+ tumors and poor survival [6, 10], whereas other histological types with a low incidence of *PIK3CA* mutations have shown to be associated with a favorable prognosis [10]. Especially tumors bearing exon 20 *PIK3CA* mutations are related with poor prognosis compared to those having wild type *PIK3CA* and other *PIK3CA* mutations [11]. Finally, whole genome DNA analysis of a cohort of breast cancers revealed that *PIK3CA* mutations occur predominantly in ER-positive breast cancers of the molecular luminal subtype, whereas PI3K-pathway activation was especially observed in ER-negative basal breast cancers [3]. In this study, however, relationships with clinical and treatment outcome were not investigated.

Breast cancer patients with tumors expressing the ER are treated with endocrine therapies, i.e., with tamoxifen or AIs. Unfortunately, not all patients respond (de novo resistance) while in the metastatic setting, patients who do initially respond will eventually relapse (acquired resistance). Endocrine therapy resistance may occur through activation of the PI3K- and MAPK-pathways and/or their downstream targets AKT and mTOR [12, 13, 14, 15, 16]. In this respect it is of interest to note that phase III clinical trials showed substantial benefit when mTOR-inhibitors were added to AI treatment [17]. Also for survival after adjuvant tamoxifen therapy contradicting results have been published. Lai et al. [11] showed in a cohort of 152 patients that invasive ductal carcinoma with exon 20 *PIK3CA* mutation had a significant shorter survival after adjuvant tamoxifen therapy compared to wild type tumors. On the other hand, Loi et al. [18] observed no relation with *PIK3CA* mutation status but a beneficial outcome after adjuvant tamoxifen therapy when applying a PI3K exon 20 gene expression signature.

To address above contradicting findings, we examined retrospectively the *PIK3CA* mutations in a cohort of 1,352 breast cancer patients to establish the prognostic and predictive significance of these mutations in tumors of 342 untreated LNN patients as well as 532 ER-positive patients with advanced disease treated with first-line endocrine therapy, i.e., tamoxifen (N = 447) or aromatase inhibitors (N = 84). Moreover, we correlated patient and tumor characteristics and clinical outcome with the *PIK3CA* mutation status, stratified for helical (exon 9) or kinase (exon 20) domain hotspot mutations, respectively.

PATIENTS

The Erasmus University Medical Center (EMC; N = 1,031), at Rotterdam, the Netherlands Cancer Institute (NKI; N = 159), at Amsterdam, and the Radboud University Nijmegen Medical Centre at Nijmegen (N = 77) all located in the Netherlands, and the Sint Augustinus Hospital at Antwerpen (N = 85) in Belgium participated in this study. Primary breast cancer tissue specimens were collected from 1,352 female patients with primary or advanced breast cancer that entered the hospitals between 1978 and 2007. Patients and tumor characteristics are presented in Table 1 for all patients for which information was available and for the three different patient cohorts analyzed in this study.

Follow-up, tumor staging, and response to therapy was defined by standard International Union against Cancer (Geneva, Switzerland) classification criteria [19]. This retrospective study has been approved by the local medical ethics committees, performed in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.fmwv.nl) and Belgium, and reported following the REMARK recommendations [20]. DNA isolated from primary breast tumor specimens were evaluated for PIK3CA exon 9 and exon 20 hotspot mutation status to assess relationships with patient and tumor characteristics and with clinical and treatment outcome. ER, progesterone receptor (PR), and HER2 status of the primary tumor tissue specimens were established as described previously [21, 22, 23, 24].

The prognostic value of *PIK3CA* mutations was assessed in 342 LNN breast cancer patients. These patients had no metastatic disease at time of diagnosis and received no adjuvant systemic therapy. In addition, ER-positive patients with advanced disease treated with first-line tamoxifen (N = 447) or aromatase inhibitors (N = 84) were analyzed to determine the predictive value of PIK3CA mutations. Patients treated with endocrine therapy were selected based on the following inclusion criteria: Invasive ER-positive breast carcinoma; advanced disease deemed not curable by surgery and/or radiotherapy for which first-line tamoxifen or AI therapy had been given for at least 4 weeks; and frozen (N = 1,193) or paraffin-embedded (N = 159) primary tumor specimens were available.

Detailed patient characteristics for the cohort of tamoxifen treated patients have been previously described [25]. The cohort of 84 metastatic breast cancer patients treated with first-line AIs received either steroidal (15 exemestane) or non-steroidal AIs (43 anastrozole, 26 letrozole). Nine of these patients presented with metastatic disease at time of diagnosis, 52 patients had modified mastectomy and 23 patients underwent breast-conserving lumpectomy. Sixty-four patients received adjuvant endocrine

Table 1 *PIK3CA* mutation status and clinicopathological characteristics in all evaluated breast cancer patients and in 3 sub-cohorts for prognosis and treatment outcome

		All available data				342 untreated lymph node-negative patients				447 ER-positive patients treated with first-line tamoxifen				84 ER-positive patients treated with first-line aromatase inhibitors			
Factor analyzed	**Total**	**wild-type**	**Exon 9**	**Exon 20**	**P**	**wild-type**	**Exon 9**	**Exon 20**	**P**	**wild-type**	**Exon 9**	**Exon 20**	**P**	**wild-type**	**Exon 9**	**Exon 20**	**P**
PIK3CA status	1352	928	173	251		259	28	55		291	62	94		59	8	17	
Age (years)[a]	1134				0.231				0.127				0.304				0.079
≤55	498	350	67	81		132	18	25		102	18	24		8	4	6	
56-70	398	273	45	80		88	4	17		93	26	34		24	1	5	
>70	238	152	35	51		39	6	13		96	18	36		27	3	6	
Tumor size (cm)	1073				0.009				0.261				0.108[b]				0.184
≤2cm	369	233	62	74		112	16	28		81	24	27	(373)	13	4	6	
>2cm	704	500	76	128		147	12	27		162	25	54		46	4	11	
Nodal status	1109				0.537								0.616[b]				0.450[b]
LNN	500	349	61	90						132	26	39	(427)	13	3	6	(79)
LNP	609	406	83	120						144	33	53		41	5	11	
Disease-free interval (months)	1146				0.722				0.970				0.704				0.105[b]
0-24	352	244	42	66		49	5	9		107	21	38		24	1	3	(83)
>24	794	536	109	149		210	23	46		184	41	56		34	7	14	
Dominant site of relapse	696				0.071				0.399[b]				0.419[b]				0.272
bone	280	169	52	59		36	2	7	(114)	117	28	40	(443)	32	6	8	
LRR	76	54	6	16		13	1	1		25	3	12		2	1	1	
other	340	233	43	64		37	8	9		147	31	40		25	1	8	
ER protein	1201				<0.001				0.007								
ER-negative	209	176	14	19		71	3	6									
ER-positive	992	649	142	201		188	25	49									
PR protein	1085				<0.001				<0.001[b]				0.442[b]				0.004[b]
PR-negative	329	267	23	39		90	4	5	(332)	57	8	21	(367)	22	0	1	(82)
PR-positive	756	488	115	153		164	23	46		183	40	58		35	8	16	
HER2 amplification	995				0.071				0.041[b]				0.08[b]				0.107[b]
HER2 negative	860	594	108	158		204	23	48	(312)	121	18	43	(204)	38	7	8	(60)
HER2 positive	135	106	10	19		34	0	3		19	2	1		3	1	3	

The analyses of relationships between *PIK3CA* mutation status and age, TNM, dominant site of relapse, and ER, PR, HER2-status in 1,352 breast cancer patients and three sub-cohorts of patients. Patient follow-up and tumor characteristics were not available for part of the tumors in which the *PIK3CA* status was determined.

[a]Age was determined at time of diagnosis for the total cohort and the 342 LNN-patients and at time of therapy start for the 447 tamoxifen and 84 aromatase inhibitor patients

[b]Factors in the three sub-cohorts that lack information for part of the tumors. The numbers within brackets indicate the numbers of samples evaluated for those factors.

therapy and 17 patients were treated with adjuvant chemotherapy, however, all developed metastatic disease that was treated with first-line aromatase inhibitors.

METHODS

Multiplex PCR amplification and SnaPshot analysis

For the detection of mutations, stored DNA was amplified for exons 9 and 20 of *PIK3CA* using earlier published PCR primers [26]. The amplified exons were assessed for mutations at the following nucleotide positions (with corresponding amino acid changes) G1624 (E542K, Q), G1633 (E545K, Q), A1634 (E545G, A), A3140 (H1047R, L) using the SnaPshot® multiplex system (Life Technologies) as described previously [26, 27]. All specimens with a mutation or that failed initially, were re-analyzed with the SnaPshot to validate the mutation status of the tumor.

Statistics

The relationship of mutation status with patient and tumor characteristics was investigated using nonparametric methods, i.e., Spearman rank correlations for continuous variables and Wilcoxon rank-sum or Kruskal–Wallis and χ^2 square test for ordered variables. The Cox proportional hazards model was used to compute the hazard ratio (HR) in the analysis of metastasis-free survival (MFS) and time to progression (TTP). MFS was defined as the time elapsed between the surgery and the first distant metastasis. TTP was defined as the time elapsed between initiation of endocrine therapy and the first detection of disease progression. In multivariate analysis, the predictive value of *PIK3CA* mutation status was compared to the base model of traditional clinicopathological factors for metastatic breast cancer, i.e., age at start of therapy, disease free interval, dominant site of relapse and PR and HER2 status. The HR was presented with its 95 % confidence intervals (95 % CI). Survival curves were generated using the Kaplan–Meier method and a log rank test was used to test for differences. Computations were done with the STATA statistical package, release 12SE (STATA Corp., College Station, TX). All P-values were two-sided and $P < 0.05$ was considered statistically significant.

RESULTS

PIK3CA mutation frequencies and clinicopathological features

We evaluated the DNA of 1,352 primary breast tumor specimens for hotspot mutations in the *PIK3CA* gene using SnaPshot multiplex analysis (Fig. 1a). No hotspot mutations for *PIK3CA* were detected in 928 tumors, here referred to as wild-type, although these tumors may harbor mutations outside the analyzed hotspots. For 423 patients a mutation in exon 9 or exon 20 (31 %) was discovered. Exon 20 mutations were detected in 251 patients (59 %), with a H1047L in 37 (15 %) and a H1047R mutation in 214 (85 %) cases. Mutations in *PIK3CA* exon 9 were detected in 173 patients (41 %), with E542K and E545K mutations in 57 (32 %) and 104 (60 %) cases, respectively, as the most prevalent ones (Fig. 1b).

Clinicopathological characteristics in relation with *PIK3CA* status for all patients and the three distinguished sub-cohorts of patients are shown in Table 1 and in more detail in Supplemental Table S1. *PIK3CA* mutations, both in exon 9 and 20, are most

frequently observed in luminal ER-positive and/or in PR-positive tumors (all P < 0.001), in agreement with observations by others. Moreover, tumors with an exon 9 mutation appeared to be smaller (<2 cm; P = 0.009) and tend to metastasize preferentially to bone (P = 0.071) than those with a wild-type or exon 20 mutant *PIK3CA* gene. These cohorts included a cohort for prognosis of untreated LNN patients and two cohorts for treatment outcome of ER-positive patients with metastatic disease treated with endocrine therapy. For the prognostic cohort the *PIK3CA* mutations were predominantly observed in luminal (P = 0.001), ER-positive (P = 0.007), PR-positive (P < 0.001), and HER2-negative (P = 0.041) tumors. In the tamoxifen treated cohort, *PIK3CA* exon 20 mutations were especially detected in postmenopausal women (P = 0.003), whereas for the AI cohort exon 9 and 20 mutations were overrepresented in PR-positive tumors (P = 0.004). Since HER2 affects prognosis and response to endocrine therapy, survival analyses in the three sub-cohorts below were performed on all tumors (Table 1) and on HER2-negative tumors only (Supplemental Table S2).

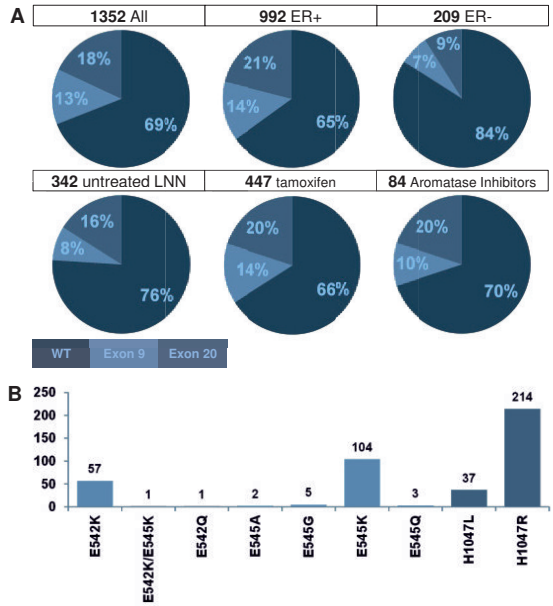

Figure 1 *PIK3CA* mutation frequencies. **A** The frequencies of *PIK3CA* exon 9 and 20 mutations as detected in the total cohort and in subsets of breast cancer patients. Mutations occur more frequently in estrogen receptor positive (ER+) compared to ER-negative (ER−) tumors. Lymph node-negative (LNN) breast cancer patients that received no adjuvant systemic therapy (N = 342) were investigated for the prognostic value of the *PIK3CA* mutation status. ER-positive breast cancer patients with metastatic disease treated with first-line tamoxifen (N = 447) or aromatase inhibitors (N = 84) were evaluated for a relation between *PIK3CA* mutation status with treatment outcome. WT are patients who have no *PIK3CA* hotspot mutation in exon 9 or exon 20, defined as wild-type. **B** The type and number of mutations detected in exon 9 (all starting with E) or in exon 20 (H1047R, H1047L) for the 423 patients with a *PIK3CA* mutation in their primary tumor.

PIK3CA mutation status and prognosis

To assess the prognostic value of *PIK3CA* mutation status, a subset of 342 LNN patients who received no adjuvant systemic therapy have been evaluated for the relation between mutation status and MFS. No significant differences were observed between wild-type and mutated *PIK3CA* tumors with regard to traditional prognostic factors age and menopausal status at diagnosis, tumor size, and grade. No association between *PIK3CA* mutation and MFS was found, neither for exon 9 [HR = 1.04 (95 % CI 0.57–1.90); P = 0.90] nor for exon 20 [HR = 0.98 (95 % CI 0.63–1.54); P = 0.94] when compared to wild-type (Fig. 2a). Since mutations occurred predominantly in luminal tumors, the prognostic value was also established in 262 ER-positive LNN patients of this sub-cohort. Again, for these 262 patients, no association

between *PIK3CA* mutation and MFS was found, neither for tumors with exon 9 mutations [HR = 1.03 (95 % CI 0.55–1.93); P = 0.94] nor for tumors with exon 20 mutations [HR = 1.04 (95 % CI 0.65–1.66); P = 0.88] when compared to wild-type tumors. Mutation status and MFS showed also no relationships in the subsets of (ER-positive) HER2-negative tumors only (Supplemental Table S2).

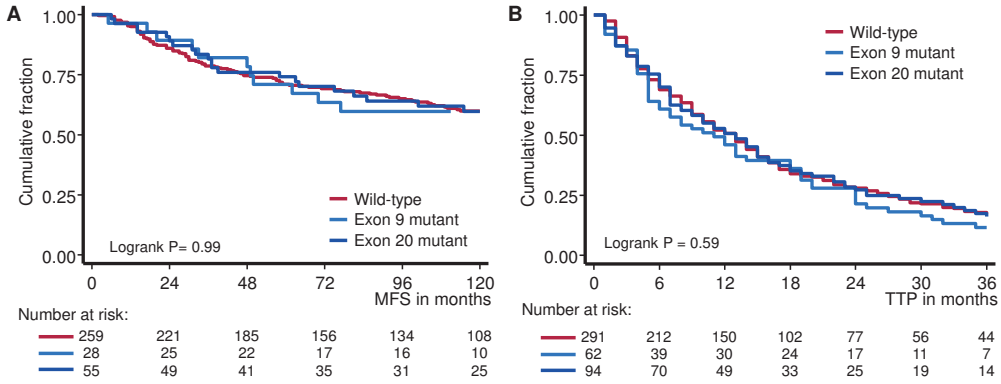

Figure 2 *PIK3CA* mutation status: Prognosis and treatment outcome after first-line tamoxifen.
A MFS analysis in 342 LNN breast cancer patients who had no adjuvant systemic therapy as a function of *PIK3CA* mutation status.
B TTP analysis in 447 ER-positive breast cancer patients with advanced disease treated with first-line tamoxifen as a function of *PIK3CA* mutation status.

PIK3CA mutation status and outcome after first-line tamoxifen therapy

Next, a subset of 447 ER-positive patients with advanced disease treated with first-line tamoxifen has been evaluated for the relationship between *PIK3CA* mutation status and treatment outcome. No significant differences were observed between *PIK3CA* wild-type and mutated tumors in relation to traditional predictive factors age at start of tamoxifen treatment, dominant site of relapse, disease-free interval and PR. The tumors with *PIK3CA* exon 9 and 20 mutations did not associate with TTP after tamoxifen, i.e., for exon 9 [HR = 1.17 (95 % CI 0.87–1.57); P = 0.30] and for exon 20 [HR = 1.01 (95 % CI 0.78–1.31); P = 0.93] (Fig. 2b). The relationship between *PIK3CA* mutation status and TTP was also not observed in the subset of HER2-negative tumors (Supplemental Table S2). The *PIK3CA* mutation status showed a relation with menopausal status at start of therapy in this subset (P = 0.003), which was also confirmed by a significant test of interaction (P = 0.010) for exon 20 mutation status and menopausal status. For this reason, the mutation status in relation to TTP was also evaluated independently for pre- and postmenopausal patients. No associations were observed for *PIK3CA* exon 9 mutations in premenopausal [N = 77; HR = 0.94 (95 % CI 0.50–1.78); P = 0.85] and postmenopausal [N = 369; HR = 1.22 (95 % CI 0.87–1.71); P = 0.25] women. The *PIK3CA* exon 20 mutations, on the other hand, showed a significant relation with TTP in premenopausal women [HR = 2.59 (95 % CI 1.08–6.25); P = 0.034] but not in postmenopausal women [HR = 1.00 (95 % CI 0.76–1.31); P = 0.99], although exon 20 mutations were especially detected in postmenopausal women.

PIK3CA mutation status and outcome after first-line aromatase inhibitor therapy

Finally, the *PIK3CA* mutation status was evaluated for a relationship with treatment outcome in a set of 84 ER-positive patients with advanced disease treated with first-line AIs. The PR status in this cohort was the only traditional predictive factor for metastatic disease that linked to the *PIK3CA* exon 9 and 20 mutation status (P = 0.004). Interestingly, patients with a *PIK3CA* mutation when compared with those being wild-type had a prolonged TTP after AI treatment, both for exon 9 [HR = 0.40 (95 % CI 0.17–0.95); P = 0.038] as well as for exon 20 [HR = 0.50 (95 % CI 0.27–0.91); P = 0.024] mutations (Fig. 3). These associations between mutation status and TTP were still significant when analyzed in the subset of HER2-negative tumors (Supplemental Table S2). An explanatory analysis in only patients treated with non-steroidal AIs (N = 69) demonstrated that the relation between *PIK3CA* mutation and prolonged TTP kept significant, i.e., for exon 9 [HR = 0.27 (95 % CI 0.08–0.88); P = 0.030] and for exon 20 [HR = 0.45 (95 % CI 0.23–0.87); P = 0.019]. The subset of steroidal AI treated patients (N = 15) was too small to draw conclusions. In multivariate analysis including age, disease-free interval, dominant site of relapse and PR and HER2 status as traditional predictive factors, the association with AI treatment outcome remained significant for both exon 9 and exon 20 mutants (see Table 2). These significant preliminary findings, however, need further validation in additional larger datasets.

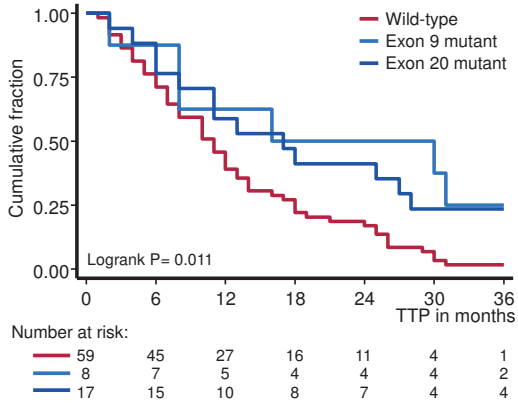

Figure 3 *PIK3CA* mutation status and treatment outcome after first-line aromatase inhibitors. TTP analysis in 84 ER-positive breast cancer patients with advanced disease treated with first-line aromatase inhibitors as a function of *PIK3CA* mutation status.

DISCUSSION

The purpose of this study was to evaluate *PIK3CA* exon 9 and exon 20 hotspot mutations in 1,352 primary breast tumor tissue specimens and to associate the mutation status with clinicopathological characteristics and with clinical outcome. Using SnaPshot analyses we identified *PIK3CA* mutations in 31 % of the patients with 13 % mutations in exon 9 and 18 % mutations in exon 20. The mutations were mainly detected in the ER-positive tumors (35 %) compared to the triple-negative tumors (11 %) (Supplemental Table S1). Moreover, fewer mutated tumors were observed for patients with LRR (29 %) compared to those patients with bone metastasis (40 %; N = 356, P = 0.056) and to those patients with bone or other distant metastases (35 %:

Table 2 Uni- and multivariate analysis for time to progression in 84 ER-positive breast cancer patients with metastatic disease treated with first-line aromatase inhibitors

Factor of base model	No. of patients	Univariate analysis			Multivariate analysis in 81 patients		
		HR	95% CI	P	HR	95% CI	P
Age at start therapy (years)							
≤55	18	1.00			1.00		
56-70	30	0.98	0.53-1.81	0.940	0.81	0.42-1.56	0.520
>70	36	0.95	0.52-1.73	0.860	0.87	0.45-1.66	0.660
Disease free interval (months)							
0-24	28	1.00			1.00		
>24	55	0.66	0.41-1.06	0.088	0.74	0.45-1.22	0.250
Dominant site of relapse							
LRR	4	1.00			1.00		
bone	46	3.50	0.84-14.53	0.085	3.28	0.77-13.96	0.110
other	34	2.66	0.63-11.18	0.182	2.49	0.58-10.68	0.220
PR status							
negative	23	1.00			1.00		
positive	59	0.63	0.38-1.06	0.080	0.64	0.35-1.16	0.140
HER2 status #							
negative	73	1.00			1.00		
positive	11	1.09	0.56-2.13	0.790	0.78	0.33-1.82	0.570
					Added to the base model		
PIK3CA mutation status							
wild-type	59	1.00			1.00		
Exon 9 mutant	8	0.40	0.17-0.95	0.038	0.40	0.16-1.00	0.051
Exon 20 mutant	17	0.50	0.27-0.91	0.024	0.50	0.26-0.98	0.045

For 81 of the 84 patients information was available for PIK3CA and all traditional predictive factors of the base model for metastatic disease, i.e., age, disease-free interval, dominant site of relapse, PR- and HER2-status.
HER2-status based on TargetPrint HER2 mRNA classification.

N = 696, P = 0.17). The frequency and distribution of *PIK3CA* hotspot mutations are comparable to those reported (Supplementary Figure S1) [3].

We evaluated exon 9 (helical) and exon 20 (kinase) domain *PIK3CA* mutations separately since exon 9 mutations have been reported mainly in lobular carcinomas and to associate with poor prognosis [28] and AKT-independent downstream signaling [29]. Although exon 9 mutations occur less frequent than exon 20 mutations, no significant differences were observed for almost all clinicopathological characteristics and clinical outcome in the sub-cohorts when stratified for *PIK3CA* exon 9 and 20 mutation status. We only observed in ER-positive tumors that *PIK3CA* exon 9 mutated tumors showed a trend to metastasize more often to bone and are smaller when compared to wild-type or exon 20 mutant tumors.

The *PIK3CA* mutant tumors in our analyses are mainly of the luminal subtype and PR-positive (80–83 %) in contrast to only 65 % PR-positive wild-type tumors. Other studies observed also significantly more PR-positive tumors in the *PIK3CA* mutant tumor group compared to the *PIK3CA* wild-type group, i.e., in a meta-analysis in 252 of the 333 mutant tumors (76 %) versus 374 of the 708 wild-type tumors (53 %) are PR-positive [5, 30, 31], respectively. However, presence of PR recently has shown to be only prognostic but not predictive for adjuvant tamoxifen therapy [32]. Moreover, the uni- and multivariate analyses for TTP after AI treatment in our cohort revealed no

significant relationship between PR and outcome and *PIK3CA* mutation status remained independently from PR predictive for response to AI. In line with this, whole-genome analysis of breast cancers of patients treated with AI in the neoadjuvant setting showed that mutations in the PI3K-pathway mainly occur in luminal A breast tumors with low recurrence risk [33]. Additionally, invasive ductal and lobular carcinomas did not differ in *PIK3CA* mutation frequencies and distributions in all our specimens (Supplemental Table S1).

Our study shows that patients with a *PIK3CA* mutation in their tumors do have a favorable outcome on first-line AI therapy, whereas *PIK3CA* mutations are not prognostic nor related to first-line tamoxifen outcome. These findings are unexpected since somatic mutations in *PIK3CA* have been shown to activate AKT and induce oncogenic transformation of breast cancer cells [34] in vitro [35] and in vivo [36]. Additionally, in vitro studies demonstrated that resistance to endocrine therapy might be due to activation of the PI3K-pathway and/or it downstream targets AKT and mTOR [13, 15, 37]. Moreover, recent phase II and III clinical trials of metastatic breast cancer patients treated with tamoxifen or exemestane showed benefit from addition of everolimus, an mTOR-inhibitor, further stressing the role of the PI3K-pathway activation in endocrine resistance [17, 38]. On the other hand, no improve in progression free survival after first-line letrozole plus temsirolimus, also an inhibitor of mTOR, was observed in a randomized phase III trial of postmenopausal metastatic breast cancer patients [39]. Additionally, mutations in the kinase domain of *PIK3CA* were associated with favorable relapse-free survival and weakly with clinical response in a neoadjuvant endocrine therapy trial [40]. Finally, comprehensive reviews of a large number of clinical studies regarding *PIK3CA* mutations in breast carcinomas [2, 41] indicate that *PIK3CA* mutations are associated with favorable prognosis in ER-positive breast cancer. All these studies, however, included lymph node-positive and/or (neo) adjuvant treated patients, which may explain why we could not confirm this association with prognosis in our study on untreated LNN patients. The contradicting findings might also be explained by recent views that *PIK3CA* mutations not always translate into a downstream activated PI3K pathway [3]. A system biological approach was applied in this study to reveal a mechanism of action in clinical samples, which showed that while *PIK3CA* mutations are predominantly present in luminal breast tumors the PI3K pathway activation mainly occurs in basal-like tumors. Luminal A *PIK3CA* mutated tumors in this study were low in PI3K pathway activation markers pAKT, pS6 and p4EBP1 and showed less PI3K-gene signature activity when compared to basal *PIK3CA* wild-type tumors. Analyses of down-stream PI3K pathway activation might be informative in our sub-cohorts for prognosis and tamoxifen, since no relationship with *PIK3CA* mutation status was observed for these cohorts, however, a system biological approach on these two cohorts was not applicable because genome wide RNA and/or protein data were not available.

We investigated primary tumor specimens and related their *PIK3CA* status with treatment outcome for advanced disease, nevertheless, it has been shown that the *PIK3CA* status can differ between primary tumor and metastatic lesions [42]. Biopsies of metastatic lesions, however, are often difficult to obtain due to their localization. Additionally, serum/plasma-derived circulating free DNA (cfDNA) from a subset of metastatic breast cancer patients contained *PIK3CA* mutations whereas no mutations were detected in cfDNA from patients with localized breast cancer [43]. These studies

also identified wild-type primary tumors with matched metastatic lesions or cfDNAs that harbored *PIK3CA* mutations [42, 43]. All this indicates that primary disease may differ sometimes from metastatic disease with regard to *PIK3CA* status. It may affect slightly our findings and might explain partially differences between our observations in metastatic disease from those obtained by others in the adjuvant setting [11, 18].

Interestingly, we found that patients with a *PIK3CA* mutation in primary tumors have longer TTP after AI therapy when compared to wild-type tumors whereas there is no association with treatment outcome after tamoxifen treatment. The association with TTP after aromatase inhibitor therapy remained significant in the subset of patients treated with non-steroidal AI (letrozole, anastrozole) and after multivariate analysis of the *PIK3CA* mutation status together with the traditional predictive factors. The discrepancy in *PIK3CA* mutation status relationship with treatment outcome after AI and tamoxifen therapy may be of relevance for the choice of treatment of ER-positive breast cancer patients. This discrepancy needs further evaluation since the AI-cohort is relatively small and because these sub-cohorts were not controlled for differences that could affect response to tamoxifen or AI for metastatic disease due to the retrospective design of the study. Our analyses indicate, however, that HER2 status does not affect the relationships between mutation status and outcome after AI and tamoxifen. Moreover, none of metastatic breast cancer patients treated with tamoxifen whereas 64 patients treated with AI received adjuvant endocrine therapy, but Cox regression analyses for adjuvant tamoxifen in the AI-cohort revealed no significant relationship with TTP [HR = 1.66, (95 % CI 0.95–2.89); P = 0.074]. Based on these observations we do not believe that adjuvant endocrine therapy could explain the differences in response for metastatic disease for our analyzed cohorts of patients.

In a neoadjuvant study on ER-positive breast tumors, it has been reported that everolimus increases the efficacy of letrozole [44]. Tumors with exon 20 *PIK3CA* mutations in this study showed less proliferation based upon Ki67-measurements after letrozole therapy compared to wild-type tumors and tumors carrying exon 9 mutations. Unfortunately, for analyses of overall survival, patients were not stratified for *PIK3CA* mutation status. Endocrine therapy combined with mTOR inhibitors, such as everolimus, has also been studied in cell line models. Cell lines harboring the *PIK3CA* mutation are sensitive to this combined therapy, however, cell lines with an active PI3K pathway due to *PTEN* deletions or activating mutations in *KRAS* or *BRAF* have no additional benefit from this combined therapy [45, 46]. Further studies are needed to evaluate treatment opportunities for ER-positive breast cancer patients with and without a *PIK3CA* mutation and PI3K/AKT/mTOR pathway activation.

CONCLUSIONS

This study is the first to report that metastatic breast cancer patients with *PIK3CA* mutations in ER-positive primary tumors show favorable outcome after first-line aromatase inhibitor treatment. This significant preliminary association should be verified in randomized prospective clinical trials to establish predictive significance [47]. Moreover, *PIK3CA* mutations in luminal ER-positive tumors have no prognostic value and are not predictive for first-line tamoxifen treatment.

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CHAPTER III

LRG1 mRNA expression in breast cancer associates with *PIK3CA* g pe and with aromatase inhibitor therapy outcome

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HIGHLIGHTS

- Expression of 9 mRNAs and 3 miRs relates to *PIK3CA* genotype in 2 breast cancer cohorts.
- All 9 mRNAs and 2 miRs were upregulated in tumors with *PIK3CA* mutations.
- *LRG1* and *PLCL1* mRNA levels relate to *PIK3CA* status irrespective luminal subtype.
- *LRG1* and *PLCL1* mRNA levels associate with aromatase inhibitor therapy outcome.
- *LRG1* expression is decreased after neo-adjuvant letrozole treatment.

ABSTRACT

Background

PIK3CA is the most frequent somatic mutated oncogene in estrogen receptor (ER) positive breast cancer. We previously observed an association between *PIK3CA* genotype and aromatase inhibitors (AI) treatment outcome. This study now evaluates whether expression of mRNAs and miRs are linked to *PIK3CA* genotype and are independently related to AI therapy response in order to define potential expressed biomarkers for treatment outcome.

Materials and methods

The miR and mRNA expression levels were evaluated for their relationship with the *PIK3CA* genotype in two breast tumor datasets, i.e. 286 luminal cancers from the TCGA consortium and our set of 84 ER positive primary tumors of metastatic breast cancer patients who received first line AI. BRB Array tools class comparison was performed to define miRs and mRNAs whose expression associate with *PIK3CA* exon 9 and 20 status. Spearman correlations established miR–mRNA pairs and mRNAs with related expression. Next, a third dataset of 25 breast cancer patients receiving neo-adjuvant letrozole was evaluated, to compare expression levels of identified miRs and mRNAs in biopsies before and after treatment. Finally, to identify potential biomarkers miR and mRNA levels were related with overall survival (OS) and progression free survival (PFS) after first-line AI therapy.

Results

Expression of 3 miRs (miR-449a, miR-205-5p, miR-301a-3p) and 9 mRNAs (*CCNO, FAM81B, LRG1, NEK10, PLCL1, PGR, SERPINA3, SORBS2, VTCN1*) was related to the PIK3CA status in both datasets. All except miR-301a-3p had an increased expression in tumors with *PIK3CA* mutations. Validation in a publicly available dataset showed that *LRG1, PGR*, and *SERPINA3* levels were decreased after neo-adjuvant

AI-treatment. Six miR–mRNA pairs correlated significantly and stepdown analysis of all 12 factors revealed 3 mRNAs (*PLCL1, LRG1, FAM81B*) related to PFS. Further analyses showed *LRG1* and *PLCL1* expression to be unrelated with luminal subtype and to associate with OS and with PFS, the latter independent from traditional predictive factors.

Conclusion

We showed in two datasets of ER positive and luminal breast tumors that the expression of 3 miRs and 9 mRNAs associate with the *PIK3CA* status. Expression of *LRG1* is independent of luminal (A or B) subtype, decreased after neo-adjuvant AI-treatment, and is proposed as potential biomarker for AI therapy outcome.

Abbreviations

AI, aromatase inhibitors; AKT also known as Protein kinase B (PKB); BC, breast cancer; ER, estrogen receptor; *FAM81B,* family with sequence similarity, 81 member B; HER2, human epidermal growth factor receptor 2; *LRG1,* leucine rich alpha 2 glycoprotein 1; MBC, metastatic breast cancer; mTOR, mechanistic target of rapamycin; PFS, progression free survival; *PLCL1,* phospholipase C like 1; SNP, single nucleotide polymorphism.

INTRODUCTION

Breast cancer (BC) is a heterogeneous disease with different clinical, biological and phenotypical features (Koren and Bentires-Alj, 2015). Targeted therapies against two critical pathways in BC, the ER and HER2, such as tamoxifen or aromatase inhibitors and trastuzumab respectively, are successful when BC patients are stratified based upon their ER and HER2 status. Unfortunately, not all patients respond (de novo resistance) while in the metastatic setting, patients who do respond will eventually relapse (acquired resistance).

Resistance to ER and HER2 targeted therapies may occur through activation of the PI3K pathway and/or their downstream targets AKT and mTOR (Clarke et al., 2015). Activation of the PI3K pathway was especially seen in ER negative tumors with predominantly amplified but hardly mutated (7%) *PIK3CA*, whereas mutations in this gene are most frequently found in ER positive tumors, up to 52% in luminal BC according to Ma et al. (2015) and The Cancer Genome Atlas Network (2012). Furthermore, primary tumors of BC patients with *PIK3CA* mutations were unexpected associated with favorable prognosis (Volinia and Croce, 2013) and clinical benefit from endocrine treatment (Ramirez-Ardila et al., 2013). Interestingly, *PIK3CA* mRNA was the most prominent gene in a prognostic signature of 30 mRNAs and 7 miRs (Volinia and Croce, 2013) established in one of two genome wide integrated transcriptome studies performed in BC until now (Buffa et al., 2011; Volinia and Croce, 2013). The prognostic value of the mRNA component of this signature was confirmed on eight BC cohorts and outperformed several well-known RNA predictors, however, the predictive value of this signature remains to be established.

The aim of our current study was to investigate mRNAs and miRs as potential predictive biomarkers correlated with *PIK3CA* genotype and AI therapy outcome. We evaluated the genome wide transcriptome of two BC datasets, and established overlapping mRNAs and miRs whose expression was related to the genotype of *PIK3CA*. The levels of these mRNAs and miRs were correlated with response to neo-adjuvant and first-line AI therapy.

MATERIALS AND METHODS

Patients and datasets

This study evaluated two previously published cohorts of breast cancer specimens for which *PIK3CA* genotype and mRNA expression profiles were available. It included a cohort of 286 luminal breast cancer patients (TCGA dataset) (The Cancer Genome Atlas Network, 2012) and a cohort of 84 ER positive breast cancer patients with metastatic disease who received first line aromatase inhibitors (AI dataset) (Ramirez-Ardila et al., 2013). The TCGA transcriptome data for mRNA and miR were uploaded from their database portal (https://tcga-data-nci-nih-gov.eur.idm.oclc.org/), and evaluated for only luminal specimens (based on PAM50 classification) with wildtype *PIK3CA* or with an exon 9 or exon 20 mutation for this gene.

The AI dataset included ER positive breast cancer patients from 3 institutes (Erasmus University Medical Center (EMC), Rotterdam; Netherlands Cancer Institute (NKI), Amsterdam; Sint Augustinus Hospital, Antwerpen). Patient characteristics, medical

ethics board approval, code of conduct, report criteria, and therapy response criteria for this cohort have been described previously (Ramirez-Ardila et al., 2013). Briefly, the patients received either steroidal (15 exemestane) or non-steroidal AI (43 anastrozole, 26 letrozole). *PIK3CA* status and mRNA expression profiles were established by SnaPshot® multiplex assays (Life technologies) (Ramirez-Ardila et al., 2013) and 44k mRNA oligoarrays (Agilent Technologies) (Jansen et al., 2013), respectively. The miR expression profiles were obtained for 768 probes (671 unique miRs) with Human MicroRNA Array v2.0 fluidic cards (Taqman Low Density Arrays, TLDAs) from Life Technologies according to the manufacturer's protocol. Expression data are deposited at NCBI GEO, with accession number GSE41994 for mRNAs (Jansen et al., 2013) and GSE78870 for miRs.

In addition, we evaluated a third dataset of 25 breast cancer patients who received neo-adjuvant letrozole, available at NCBI GEO with accession code GSE59515 (Turnbull et al., 2015). This neo-adjuvant dataset contains the transcriptome of primary tumor biopsies taken before and after 2 weeks and after 3 months treatment.

Data analyses

The TCGA set mRNA levels were median centered by gene while the mature/star miR strand levels were normalized to reads per million mapped miRNAs. The AI set mRNA levels were quantified and normalized using Agilent Feature Extraction software (Agilent, Santa Clara, CA, US). The miRNA TLDAs generated Ct values which were normalized against the median Ct value of the TLDA. All mRNA and miR expression data were log2 transformed and evaluated using BRB ArrayTools, Version 4.5.0-Beta_1 (June 2015) (http://linus.nci.nih.gov.eur.idm.oclc.org/BRB-ArrayTools.html). BRB class comparison was performed when at least 80% expression data points were available in the AI set. The expression data in the TCGA set were available for all the samples. A P value <0.005 and <0.05 after 100,000 permutations was considered significant for mRNAs and miRs, respectively. The integrated evaluation of miR–mRNA pairs was performed using the following databases: miRTarbase, mirSel, miRecords (V.4 – Apr 27-2013), Ingenuity Pathway Analysis (IPA®) integrated with TarBase V.5.0. Pathway analyses were performed using (IPA®) and DAVID (https://david.ncifcrf.gov).

Statistics

Computations were performed with the STATA statistical package, release 13 (STATA Corp., College Station, TX). All P values were two sided and $P < 0.05$ was considered statistically significant. Bonferroni multiple test corrections were also used. Briefly, relationships between *PIK3CA* genotype, clinic-pathological factors and miR/mRNA expression levels were investigated using nonparametric methods, i.e. Spearman rank correlations for continuous variables and Wilcoxon rank sum or Kruskal Wallis and chi square test for ordered variables. The Cox proportional hazard model was used in univariate analysis to compute the hazard ratio (HR) for PFS. The HR was presented with its 95% confidence intervals (95% CI). Survival curves were generated using the Kaplan–Meier method and a log rank test was applied to test for differences. PFS was defined as the time elapsed between initiation of first line AI therapy and the first detection of disease progression. miRs and mRNAs considered statistically significant in univariate analysis were included in a multivariate stepdown

analysis to determine the strongest independent predictive biomarkers. Finally, these potential predictive biomarkers were added to the base model of traditional clinic-pathological factors for metastatic breast cancer, i.e., age at start therapy (≤55, 56–70 or >70 years), disease-free interval (0–24 or >24 months), dominant site of relapse (Local regional relapse vs bone vs visceral), PR-status (negative or positive), and HER2-status (negative or positive, based on Target-Print HER2 mRNA classification).

RESULTS

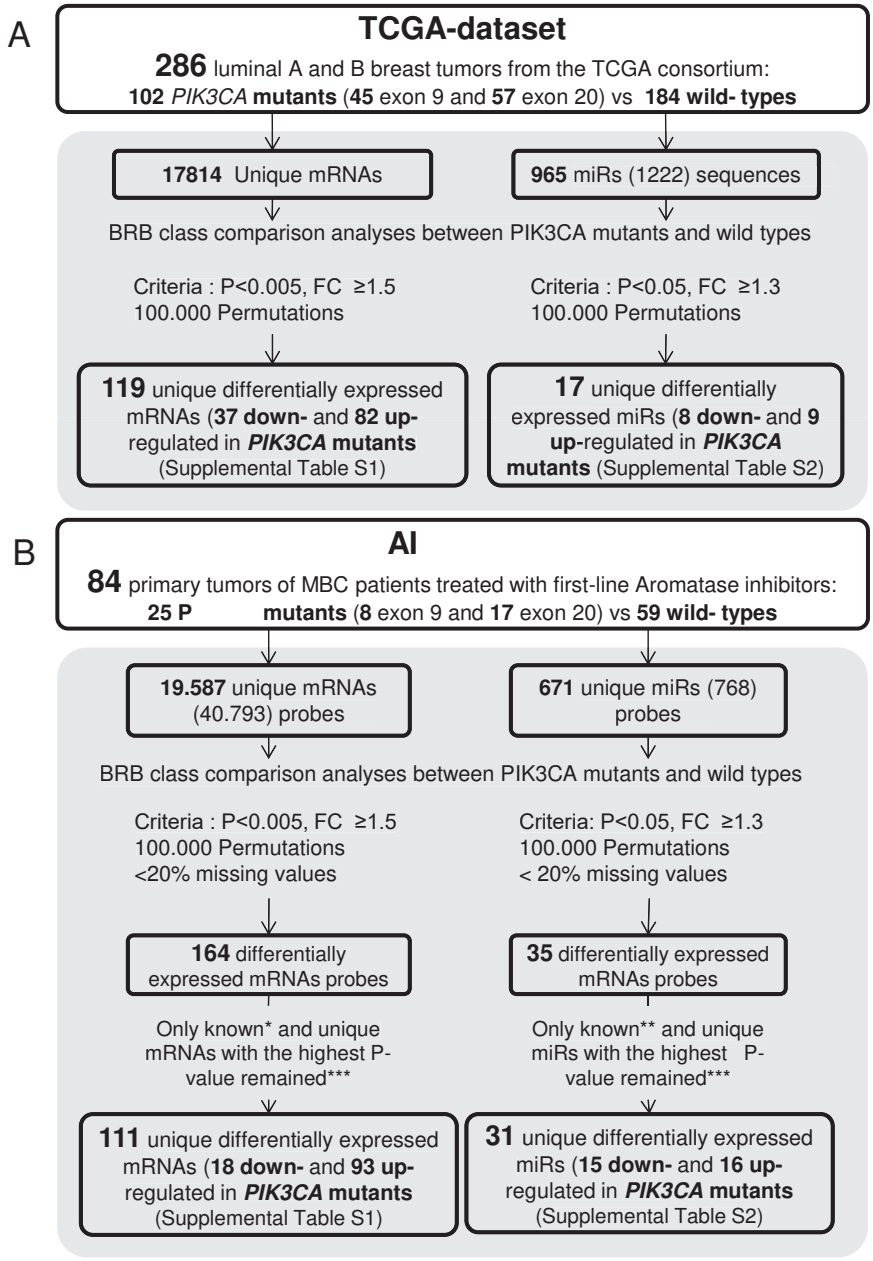
PIK3CA genotype and transcriptome profiles

The class comparison algorithm of BRB Array tools was used to identify differentially expressed mRNAs and miRs between *PIK3CA* wildtype and *PIK3CA* mutated ER positive (luminal) primary breast cancer specimens. This analysis revealed 119 and 111 unique differentially expressed mRNAs ($P < 0.005$) in the TCGA and AI dataset, respectively (Figure 1, Supplemental Table S1). The majority of genes showed increased expression in tumors with a *PIK3CA* mutation in both datasets, i.e. 69% (82/119) in the TCGA dataset and 84% (93/111) in the AI dataset. Likewise, 17 and 31 unique miRs were differentially expressed between *PIK3CA* wild type and mutated tumors ($P < 0.05$) in the TCGA and AI datasets (Figures 1, Supplemental Table S2). About half of the miRs showed increased expression in *PIK3CA* mutated tumors for the TCGA, i.e. 9 miRs in TCGA [53% (9/17)] and 16 miRs [52% (16/31)] in the AI dataset were upregulated.

The 119 and 111 identified mRNAs were examined with Ingenuity® Pathway Analysis (IPA) to establish regulated signaling pathways in both datasets. Not surprising IPA revealed Estrogen as the only common significant up stream regulator for a subset of genes both in the AI dataset (P value = 7.09E−03) and the TCGA dataset (P value = 1.43E−06) (Supplemental Figure S1). Additional pathways were not found to be enriched (based on the IPA and DAVID analyses).

Breast cancer *PIK3CA* mutation specific transcriptome

To obtain a *PIK3CA* mutation specific transcriptome independent of the cohort studied, we selected the overlapping potential biomarkers between both the TCGA- and AI-dataset (Supplemental Figure S2). In total 9 mRNAs (*CCNO, FAM81B, LRG1, NEK10, PGR, PLCL1, SERPINA3, SORBS2 and VTCN1*; Supplemental Table S3) and 3 miRs (hsa-miR-205-5p, hsa-miR-301a-3p and hsa-miR-449a) were related with *PIK3CA* genotype in both datasets. Interestingly, all biomarkers except one (miR-301a-3p) showed increased expression in *PIK3CA* mutated ER positive (luminal) breast cancer (Figure 2). Since the TCGA dataset contains both luminal A and B samples, the 12 potential biomarkers associated with *PIK3CA* status were further evaluated for the relationship between their expression and luminal A or B subtype. The expression of *LRG1, PLCL1, SERPINA3, CCNO* and miR-449a were confirmed after multiple testing correction to be independent of subtype (Table 1). Moreover, the levels of these 5 biomarkers were in both TCGA- and AI-dataset not correlated with MKI67 levels, a gene significantly upregulated in luminal B compared to luminal A subtype. This again indicates that *LRG1, PLCL1, SERPINA3, CCNO* and miR-449a expression are independent from luminal subtype



Fi 1 Workfl used to di r di zed by *PIK3CA* mutati **A** TCGA dataset (publicly available data from the TCGA consortium). **B** AI dataset. *Known mRNAs indicates mRNAs with gene names annotated according to platform GPL6480 updated in Nov 16, 2014 (http://www-ncbi-nlm-nih-gov.eur.idm.oclc.org/geo/query/acc.cgi?acc=GPL6480); **known miRs indicates miRs annotated according to miRbaseV20; ***unique mRNAs or miRs with the highest P-value indicate those cases in which at least two probes for an annotated mRNA or miR name were found differentially expressed. We selected the probe with the highest P-value.

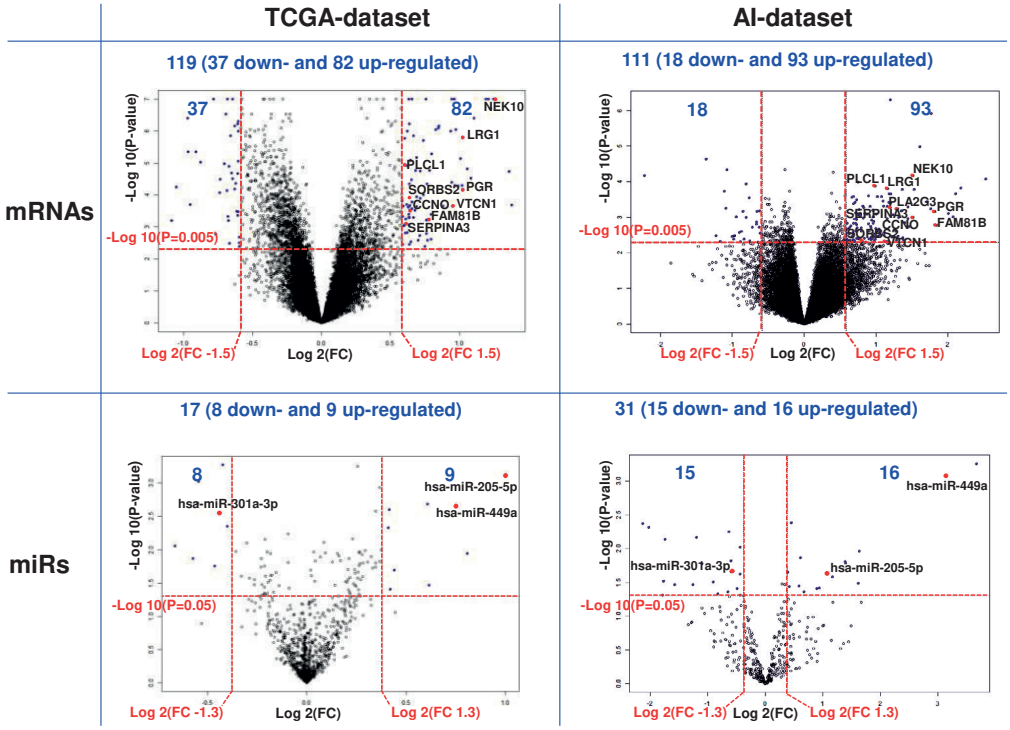


Figure 2 Differentially expressed mRNAs and miRs when analyzed by *PIK3CA* mutation status. Volcano plots of differentially expressed mRNAs (top side) and miRs (bottom side) in the TCGA dataset (left side) and in the AI dataset (right side) analyzed by *PIK3CA* genotype. The vertical dotted lines indicate the threshold for a relative expression fold change (FC) of breast cancer *PIK3CA* exon 9 or exon 20 mutated tumors compare to *PIK3CA* wild types. The horizontal dotted lines represent the threshold of a P value. The blue dots in the upper sides are significantly upregulated (on the right) and down regulated (on the left) in *PIK3CA* mutants. Overlapping mRNAs and miRs in both datasets are labeled and indicated in red dots. The volcano plots are based on number of probes (see Figure 1 for details).

Integrated analyses of common miRs and mRNAs

Since miRs can regulate expression of specific target genes, the expression of the common 9 mRNAs and 3 miRs were checked for their statistical relationship by spearman correlation in both the TCGA- and AI-dataset. This exploratory analysis resulted in 14 miR–mRNA pairs with a $P < 0.05$, including 6 pairs still significant after multiple testing correction (Supplemental Table S4). These 6 miR–mRNA pairs were explored in different miR databases to identify putative mRNAs targeted by our miRs, and demonstrated only *PGR* as target of miR-205-5p in the miRSel database (Sempere et al., 2007). Highest correlations in the TCGA and AI dataset, i.e. $r_s = 0.69$ and 0.78, were observed between subtype independent expression of *CCNO* and miR-449a, possibly due to their chromosomal co-localization on 5q11.

Additionally, expression levels of the 9 mRNAs were also correlated with each other (Supplemental Table S5), and showed coregulatory expression for 12 mRNA pairs in both datasets after multiple testing correction. Ingenuity identified *TP73* for the *LRG1-SERPINA3* pair and *STAT3* for the *PGR-SERPINA3* pair as their common transcription regulator, respectively.

Table 1 Overview: Identification of potential biomarkers related to the PIK3CA status in relation to luminal subtype, overall survival and progression-free survial after first line AI therapy

Factor	TCGA-set									AI-set					
	Relationships with *PIK3CA* status and Luminal subtype for 286 patients						Relationships with Overall Survival available for 272 patients			Relationships with Progression-Free Survival in 75 patients who received first-line AI therapy					
	PIK3CA status			Subtype			Univariate analysis			Univariate analysis			Mutivariate analysis: Each factor added to the base model of traditional factors [c]		
	Wild-type	Mutated	P MW[a]	Luminal A	Luminal B	P MW[a]	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Median expression levels for:															
LRG1	0.10	1.06	<0.001	0.65	0.35	0.139	0.83	0.70-0.99	0.033	0.69	0.55-0.87	0.002	0.70	0.54-0.90	0.006
PLCL1	-0.11	0.76	<0.001	0.45	-0.03	0.013	0.71	0.52-0.97	0.029	0.61	0.46-0.81	0.001	0.58	0.43-0.79	<0.001
FAM81B	0.27	1.64	<0.001	1.19	0.00	<0.001[b]	0.88	0.71-1.08	0.216	0.82	0.73-0.92	< 0.001	0.83	0.73-0.94	0.004
CCNO	0.11	0.67	<0.001	0.41	0.23	0.319	0.97	0.75-1.27	0.832	0.84	0.73-0.96	0.010	0.84	0.73-0.97	0.018
NEK10	0.21	1.66	<0.001	1.29	-0.21	<0.001[b]	0.88	0.72-1.06	0.172	0.76	0.65-0.90	0.001	0.79	0.65-0.95	0.012
PGR	0.73	2.03	<0.001	1.81	0.45	<0.001[b]	0.91	0.77-1.06	0.221	0.84	0.74-0.95	0.006	0.85	0.72-0.99	0.039
SERPINA3	0.17	0.77	0.001	0.53	0.32	0.069	0.81	0.66-0.98	0.031	0.82	0.70-0.97	0.020	0.85	0.70-1.04	NS
SORBS2	-0.37	0.33	<0.001	0.30	-0.86	<0.001[b]	0.82	0.63-1.07	0.142	0.69	0.56-0.86	0.001	0.71	0.56-0.89	0.004
VTCN1	-0.90	0.37	<0.001	0.30	-1.44	<0.001[b]	0.91	0.77-1.08	0.284	0.84	0.73-0.97	0.021	0.87	0.73-1.03	NS
hsa-miR-449a	0.93	1.59	0.011	1.13	1.21	0.817	0.84	0.66-1.07	0.166	0.93	0.87-0.99	0.022	0.93	0.87-0.99	0.030
hsa-miR-301a-3p	3.36	2.88	0.003	2.84	3.74	<0.001[b]	1.38	1.00-1.89	0.047	1.30	1.01-1.68	0.043	1.21	0.91-1.61	NS
hsa-miR-205-5p	9.46	10.82	<0.001	10.49	9.04	<0.001[b]	1.00	0.86-1.16	0.988	0.93	0.84-1.04	0.209			

[a] Mann Whitney (MW) nonparametric test was applied for the evaluation of the expression levels in relationship to PIK3CA status (wild type vs mutant) or luminal subtype (A vs B).

[b] Significant after the Bonferroni correction for multiple testing (P = 0.05/12 = 0.004).

[c] Each factor added to the base model of traditional factors including age, disease free interval, dominant site of relapse, PR and HER2 status.

PIK3CA mutation specific transcriptome before and after neo-adjuvant AI therapy

Next, above identified potential biomarkers were analyzed on the third neo-adjuvant dataset to establish whether their expression levels change after letrozole treatment (Figure 3). Expression in the before treatment biopsies were only significantly decreased for *VTCN1* in the 8 clinical non-responders when compared to the 17 clinical responders. On the other hand, expression levels for *LRG1, PGR*, and *SERPINA3* were decreased after 2 weeks treatment and attenuated up to 3 months therapy when compared to the pre-treatment levels (Figure 3A). The downregulation for these 3 genes during neo-adjuvant letrozole therapy was only observed in patients with clinical response but not in those without clinical response, as exemplified for *LRG1* (Figure 3B).

A

Evaluation of biomarkers in primary tumors biopsies of patients receiving neo-adjuvant letrozole (GSE59515)									
Factor	median expression levels measured in biopsies before treatment		Mann Whitney P-values	median expression levels measured at 3 time-points for all patients			Kruskal-Wallis P-values		
	No clinical response	Clinical response	All	Before	After 2 weeks treatment	After 3 months treatment	All	Specimens with clinical response	Specimens with no clinical response
Number of specimens:	8	17	25	25	25	25	75	51	24
LRG1	28.93	38.98	0.0805	35.48	23.90	27.21	0.0060	0.0131	0.3688
PLCL1	8.00	5.05	0.7708	7.15	4.81	7.37	0.3725	0.7002	0.4056
FAM81B	0.33	0.51	0.9072	0.51	1.51	4.40	0.2943	0.2918	0.7171
CCNO	7.08	14.95	0.7267	10.8	12.61	2.83	0.1440	0.0951	0.6080
NEK10	15.06	64.19	0.0709	43.61	18.95	25.60	0.1275	0.0874	0.7965
PGR	22.26	43.44	0.9072	40.61	6.49	6.04	0.0002	0.0010	0.1197
SERPINA3	279.69	433.96	0.2684	429.7	196.57	151.85	0.0157	0.0236	0.3706
SORBS2	53.31	47.98	0.4845	48.99	54.41	57.72	0.7600	0.6809	0.9975
VTCN1	18.65	151.17	0.0020	70.72	91.63	121.34	0.4368	0.5399	0.3337
hsa-miR-449a	-7.55	-3.77	0.1621	-4.01	-2.41	-3.60	0.6592	0.9492	0.4415
hsa-miR-301a-3p	-4.35	3.06	0.0709	-0.59	-1.86	-1.80	0.8445	0.5449	0.4242
hsa-miR-205-5p	-2.73	4.95	0.1157	3.13	1.81	-1.72	0.0811	0.1955	0.1878

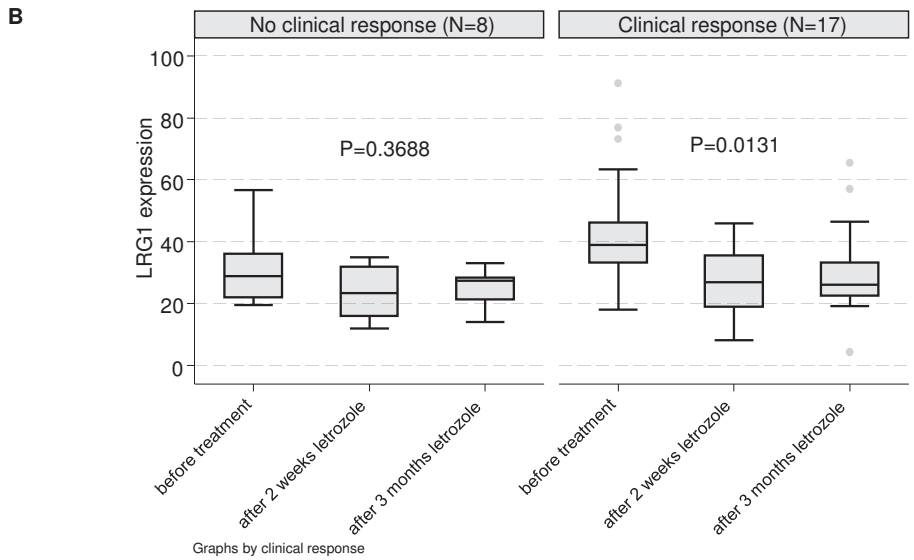


Figure 3 Gene expression alterations in primary tumor biopsies before and after AI treatment from patients who received neo-adjuvant AI therapy. The figure illustrates the results after validation in the publicly available NCBI GEO-dataset GSE59515 including 25 breast cancer patients who received neo-adjuvant letrozole therapy. The cohort contained a subset of 17 patients with and a subset of 8 patients without clinical response after AI-treatment. The dataset contains the expression profiles of primary tumor biopsies before and after 2 weeks and 3 months treatment from these patients. **A** presents the median expression levels and P-values measured before treatment for the 2 patient subsets (Mann–Whitney P-values) and before and after 2 weeks and 3 months AI-treatment for all patients (Kruskal Wallis P-values). The Kruskal Wallis test was also performed on each subset of patients separately. **B** is a boxplot illustrating *LRG1* expression levels before and after treatment in both patients with and without clinical response to neo-adjuvant letrozole.

PIK3CA mutation specific transcriptome and hormonal therapy outcome

The *PIK3CA* genotype linked potential biomarkers were further evaluated for their relationship with OS for 272 patients of the TCGA-dataset and with PFS for 75 MBC patients who received first line AI and for whom both expression and clinical data were available. Expression of *LRG1, PLCL1, SERPINA3* and miR-301a-3p was related with OS, based on all therapies that the patients had received (Table 1). In addition, expression levels of all biomarkers except miR-205-5p were significantly ($P < 0.05$) related to PFS (Figure 4A). Only the expression of miR-301a-3p was linked to therapy resistance whereas expression of all other biomarkers was associated with response to AI. The 11 potential biomarkers that associated with therapy outcome, were each further evaluated along with traditional predictive factors, the base model. In this multivariable analyses eight factors were independent from the base model (Table 1) and these were combined for a stepdown analysis. Since we know that *PIK3CA* mutations correlate with PFS in our cohort, as reported by us (Ramirez-Ardila et al., 2013), a stepdown including *PIK3CA* status was analyzed to reveal variables that outperformed the *PIK3CA* status correlation with PFS, i.e. are more strongly correlated with PFS. Performing the stepdown analysis with and without *PIK3CA* status resulted in similar findings, i.e. only *FAM81B* ($P < 0.001$), *LRG1* ($P = 0.001$) and *PLCL1* ($P = 0.013$) were independent biomarkers associated with AI therapy outcome in MBC patients (Figure 4B). Interestingly, *LRG1* and *PLCL1* expression was

shown to be related to *PIK3CA* independently of the luminal subtype. The observed association with PFS for these 2 potential biomarkers is illustrated in Kaplan Meier survival curves in Figure 4C–D. Finally, we investigated the expression of *LRG1* and *PLCL1* in our dataset of 101 MBC patients who received first line tamoxifen, however, no significant relationship with tamoxifen outcome was observed for both genes (data not shown).

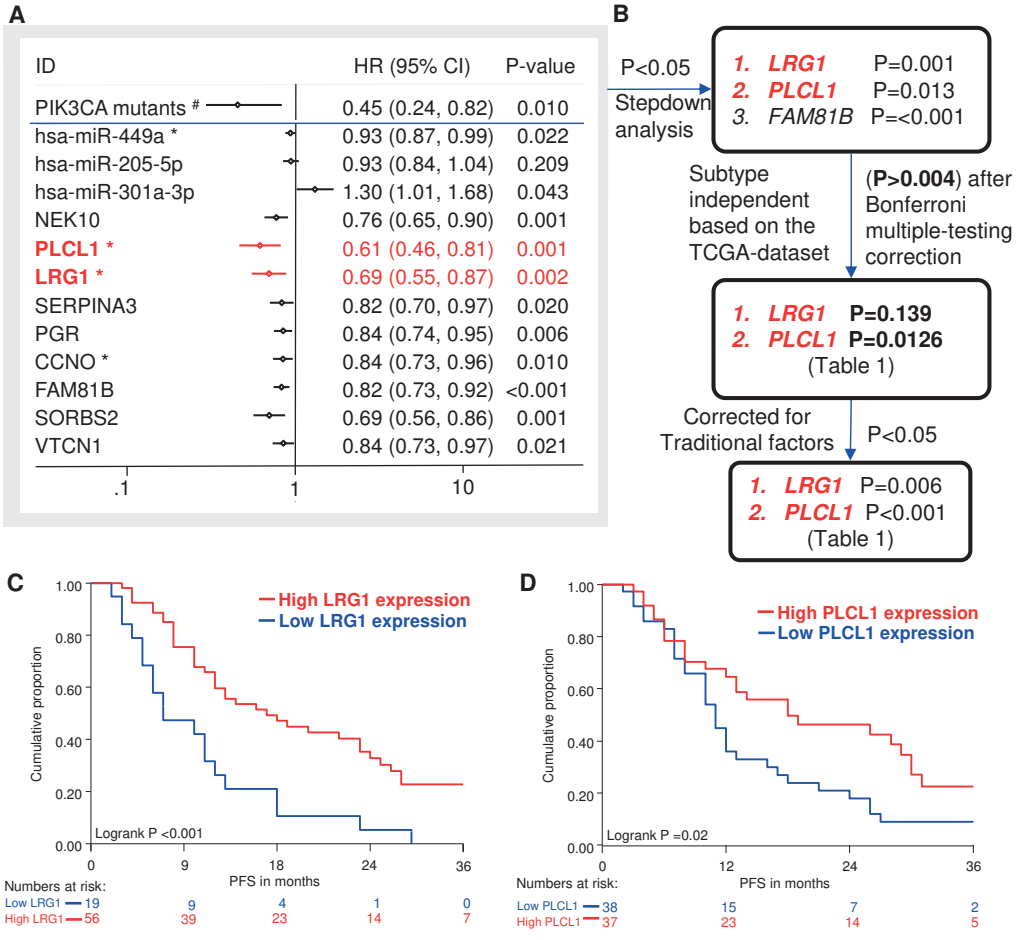


Figure 4 Relation to PFS after first line AI treatment. **A** Forest plot summarizing univariate analyses in 75 MBC patients in relation to PFS after first line AI treatment. Traditional factors are not included here. The variables in red remained significant in multivariate analysis after stepdown analyses and corrected for traditional factors. (#) indicates dichotomized variable, all others are continuous variables. All variables are up-regulated in *PIK3CA* mutants except for miR-301a-3p. (*) indicates variables which are subtype independent, i.e. had no significant relationship (P > 0.004) with subtypes after Bonferroni multiple-testing correction. **B** Steps followed to identify independent potential biomarkers related to *PIK3CA* status and independent of subtype **C–D**. PFS analyses in 75 ER positive breast cancer patients with advanced disease treated with first line AI as function of **C** *PLCL1* expression and **D** *LRG1* expression.

DISCUSSION

In this study, the transcriptome of a TCGA cohort of 286 luminal A and B tumors from BC patients and our cohort of 84 ER positive primary breast tumors from patients who developed metastatic disease were stratified by *PIK3CA* genotype. In both datasets we demonstrated 1 miR with decreased and 2 miRs and 9 mRNAs with increased expression in tumors with a *PIK3CA* hotspot mutation. Evaluation of these biomarkers in the neo-adjuvant dataset showed that expression of 3 mRNAs decreased after letrozole therapy. Multivariate analyses, including clinico-pathological factors and molecular tumor subtypes, revealed *LRG1* and *PLCL1* as independent potential biomarkers for PFS after first line AI therapy. All our findings indicate that high *LRG1* expression levels hallmark primary breast cancer with *PIK3CA* mutations and treatment response after neo-adjuvant and first-line AI-therapy.

The majority of differentially expressed mRNAs in both datasets had an increased expression in *PIK3CA* mutated tumors. In addition, we found 6 miR–mRNA pairs with significant related expression levels in both datasets after Bonferroni multiple correction test. Negative correlations have been generally reported since miRs control gene expression by mRNA degradation and/or translation inhibition (Bartel, 2004). However, most of our miR–mRNA pairs showed a positive correlation. Dvinge et al. indicated that the BC miRNA–mRNA landscape was dominated by positive connotations, and suggested co-transcriptional modules, especially in ER+ samples (Dvinge et al., 2013).

The established *PIK3CA* mutation specific potential biomarkers were previously linked to breast cancer, PI3K pathway and/or endocrine therapy. For example, *PGR* belonged to the regulated genes after PI3K inhibition (Bosch et al., 2015), whereas *SERPINA3* was part of activated PI3K signatures (Loi et al., 2010). *SERPINA3* was identified as biomarker for estrogen regulation in response to neoadjuvant AI therapy (Miller and Larionov, 2010), whereas SNPs for *NEK10* (Milne et al., 2014) and *VTCN1* (Tsai et al., 2015) were linked to BC susceptibility. Moreover, miR-449a expression was increased in luminal BC compared to the other subtypes (Dvinge et al., 2013) and its tumor suppressor activity was regulated by PI3K (Liu et al., 2015). The upregulated expression of miR-205 and miR-449a in tumors with a *PIK3CA* mutation was shown to suppress the epithelial mesenchymal transition (EMT) in BC and liver cancer, respectively (De Cola et al., 2015) (Chen et al., 2015a). Interestingly, miR-205-5p directly targets HER3 receptor, involved as well in EMT, inhibiting the PI3K pathway activation respectively (De Cola et al., 2015). In contrast, the down regulated miR-301a-3p in tumors with *PIK3CA* mutation, was shown to increase the EMT in laryngeal neoplasm (Lu et al., 2015) and to target in BC especially genes involved in T cell related processes (Dvinge et al., 2013). Combining results in BC cell lines on *PIK3CA* status obtained from the COSMIC database (http://cancer.sanger.ac.uk/cell_lines) and on miR-301a-3p expression described by Ma et al. (2014) suggest that BC cell lines with *PIK3CA* mutations have decreased miR-301a-3p expression compared to wild types.

Although all 9 mRNAs and 2 miRs associated with *PIK3CA* genotype and PFS in MBC, only *PLCL1, LRG1, CCNO, SERPINA3* and miR-449a were not related with luminal subtypes A and B. Furthermore, mRNA expression of *FAM81B, PLCL1*, and

LRG1 remained as independent potential biomarkers for PFS after first line AI treatment once stepdown analyses and multivariate analysis with well-known clinical traditional factors are performed.

Thus, we propose *PLCL1* and *LRG1* as potential luminal subtype independent biomarkers that associate with *PIK3CA* genotype and first line AI treatment outcome.

PLCL1 and *LRG1* have so far not been linked with BC and *PIK3CA* genotype. *PLCL1* encodes for phospholipase C like 1, also known as PRIP 1, which has been suggested to be involved in insulin induced GABA(A) receptor functioning (Fujii et al., 2010) and in gonadotropin secretion (Matsuda et al., 2009). Interestingly, CA2+ and phospholipase C signaling was recently indicated as one of the over represented canonical networks in ER positive BC (Ellis et al., 2012). *LRG1* (encodes for leucine rich alpha 2 glycoprotein 1) was proposed as a metastasis suppressor in hepatocellular carcinoma (HCC) since exogenous recombinant human LRG1 protein inhibited migration and invasion of HCC cells in vitro (Zhang et al., 2015). Moreover, LRG1 was shown as potential biomarker for detection of cancer in urine and/or serum such as epithelial ovarian, lung and colon cancer (Chen et al., 2015b; Ivancic et al., 2014; Smith et al., 2014; Wu et al., 2015). In endometrial carcinoma patients LRG1 expression was related to poor prognosis (Wen et al., 2014). Our transcriptome analyses of tumor biopsies before and after neo-adjuvant letrozole showed decreased expression upon treatment for *LRG1, PGR*, and *SERPINA3*. These 3 genes were also reported within the top 150 differentially expressed genes between pre- and 2 weeks post anastrozole neo-adjuvant treatment (Dunbier et al., 2013). All this suggest that alterations in *LRG1* levels may be used as readout for a response to AI. Further studies in BC are needed to resolve the relationship of *PLCL1* and *LRG1* with *PIK3CA* mutation and AI therapy response.

In conclusion, we used mRNA and miR expression and identified 9 mRNAs and 3 miRs overlapping between ER positive and luminal A and B tumors in relation to *PIK3CA* mutation status. The expression of 2 genes, *LRG1* and *PLCL1*, was shown to be independent of subtype and related to PFS after first line AI treatment in MBC patients. Expression levels of *LRG1*, but not *PLCL1*, were shown to decrease significantly after treatment in patients with clinical response to neo-adjuvant letrozole. Since our analyses were based on *PIK3CA* genotype, and given the high frequency of *PIK3CA* mutations, this exploratory study provides potential novel biomarkers for PFS after first line AI treatment. These results need to be validated in future studies.

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CHAPTER IV

Increased MAPK1/3 phosphorylation in luminal breast cancer related with *PIK3CA* hotspot mutations and prognosis

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ABSTRACT

Introduction

While mutations in *PIK3CA* are most frequently (45%) detected in luminal breast cancer, downstream PI3K/AKT/mTOR pathway activation is predominantly observed in the basal subtype. The aim was to identify proteins activated in *PIK3CA* mutated luminal breast cancer and the clinical relevance of such a protein in breast cancer patients.

Materials and methods

Expression levels of 171 signalling pathway (phospho-)proteins established by The Cancer Genome Atlas (TCGA) using reverse phase protein arrays (RPPA) were *in silico* examined in 361 breast cancers for their relation with PIK3CA status. MAPK1/3 phosphorylation was evaluated with immunohistochemistry on tissue microarrays (TMA) containing 721 primary breast cancer core biopsies to explore the relationship with metastasis-free survival.

Results

In silico analyses revealed increased phosphorylation of MAPK1/3, p38 and YAP, and decreased expression of p70S6K and 4E-BP1 in *PIK3CA* mutated compared to wild-type luminal breast cancer. Augmented MAPK1/3 phosphorylation was most significant, i.e. in luminal A for both *PIK3CA* exon 9 and 20 mutations and in luminal B for exon 9 mutations. In 290 adjuvant systemic therapy naïve lymph node negative (LNN) breast cancer patients with luminal cancer, high MAPK phosphorylation in nuclei (HR= 0.49; 95% CI, 0.25-0.95; P=0.036) and in tumor cells (HR= 0.37; 95% CI, 0.18-0.79; P=0.010) was related with favorable metastasis-free survival in multivariate analyses including traditional prognostic factors.

Conclusion

Enhanced MAPK1/3 phosphorylation in luminal breast cancer is related to *PIK3CA* exon-specific mutations and correlated with favorable prognosis especially when located in the nuclei of tumor cells.

Keywords: *PIK3CA* mutations, MAPK1/3 phosphorylation, luminal breast cancer, tissue microarray, Metastasis free survival (MFS).

INTRODUCTION

The Cancer Genome Atlas (TCGA) data on breast cancer have shown that the *PIK3CA* mutation frequency could rise to 45% in luminal A tumors compared to only 9% in basal tumors [1]. Remarkably, the reverse protein phase array (RPPA) data of this study demonstrated that phosphorylation of AKT, S6 and 4EB-P1, typical markers of PI3K pathway activation (PI3K-AKT signalling pathway in KEGG: http://www.genome.jp/kegg-bin/show_pathway?hsa04151) were highly expressed in basal-like and HER2 molecular subtypes and correlated strongly with INPP4B and PTEN loss, and with *PIK3CA* amplification. Moreover, protein and mRNA signatures of PI3K pathway activation were enriched in basal-like over luminal A breast cancers [1].
Mutations in *PIK3CA* occur predominantly in two hotspot regions, i.e. in the helical and kinase domains encoded by exon 9 and 20, respectively. COSMIC, the catalogue of somatic mutations in cancer (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) reported so far 2745 missense mutations for *PIK3CA* in breast carcinomas, which were located in exon 9 and more frequently in exon 20.

It has been reported that the *PIK3CA* mutation status between primary and corresponding metastatic disease can be discordant [2]. Interestingly, single cell *PIK3CA* mutational analyses revealed heterogeneity in circulating tumor cells and metastases compared to primary breast tumors [3]. Additionally, different treatment responses have been observed for the distinct molecular subtypes in relation to *PIK3CA* mutation status. For example, prolonged survival was observed in ER-positIve early disease patients treated with adjuvant tamoxifen and in advanced disease patients treated with first-line aromatase inhibitors for those with *PIK3CA* mutant tumors [4], [5]. In contrast, *PIK3CA* mutations in HER2+ patients were linked to trastuzumab therapy resistance [6], [7], [8]. This resistance might be due to co-occurrence of both oncogenes or to tumor cell heterogeneity with underrepresented trastuzumab responsive HER2+ clones.

In order to understand the apparent disconnection in breast cancer between genetic activation through *PIK3CA* oncogenic driver mutations and its downstream PI3K/AKT/mTOR signalling cascade, we evaluated in especially luminal tumors pathway proteins for their relation with *PIK3CA* mutations. Therefore, TCGA *in silico* RPPA-database of 171 cancer related (phospho) proteins from 361 breast cancer specimens were stratified by type of *PIK3CA* mutation and molecular subtypes. Expression or phosphorylation of seven proteins correlated significantly with *PIK3CA* status in luminal breast cancer. Phosphorylated MAPK1/3, as most significant upregulated protein, was further explored on TMAs containing 721 primary breast cancer specimens, including a subset of luminal tumors from 290 systemic untreated LNN patients, to address the prognostic value of this phosphorylated kinase (Figure 1).

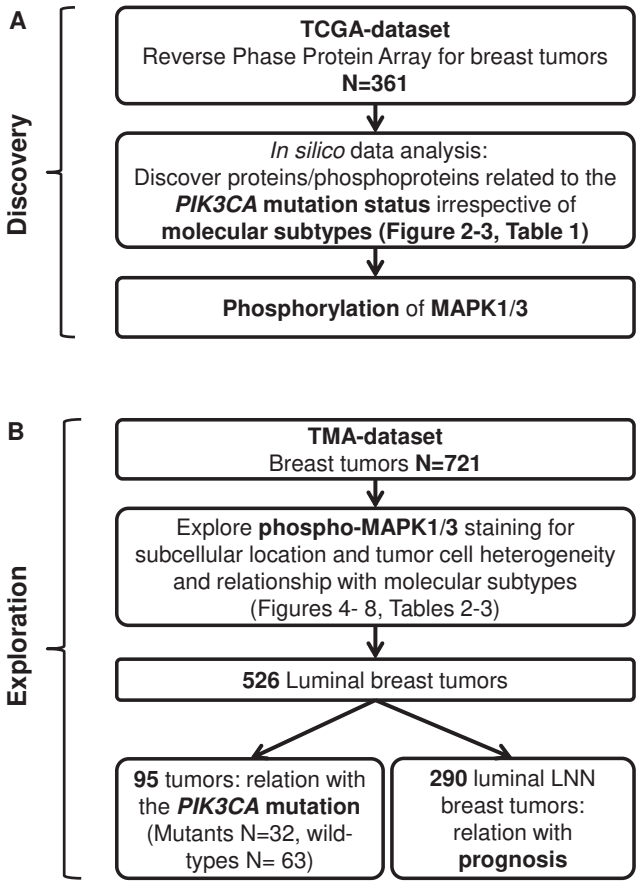

Figure 1 Overview of the study design. Figure 1A. In-silico data used from the TCGA consortium to identify potential biomarkers (protein expression and/or phosphorylation patterns related to the *PIK3CA* mutation status adjusted for subtype. Figure 1B. Tissue microarrays data used to explore the subcellular localization of the discovered potential biomarker phospho-MAPK1/3 and its correlation with subtypes, *PIK3CA* mutation status and prognosis. Abbreviations: IHC: Immunohistochemistry; TMA: Tissue microarrays; LNN: Lymph Node Negative.

MATERIALS AND METHODS

Databases and software packages

We have used the quantified expression of 171 cancer-related proteins and phosphoproteins by RPPA of 403 primary breast cancer specimens available from TCGA (see Supplemental table S1). The data used by us were deposited [1] and can be retrieved at TCGA portal at https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm. The tumors were selected based on their known *PIK3CA* mutation status and molecular subtype [9]. Of the 403 tumors, 366 specimens with a single *PIK3CA* mutation within the hotspot in exons 9 or 20 or wild-type were selected for further analyses, excluding tumors with *PIK3CA* double mutations. Five additional specimens were excluded, because 4 samples belonged only to the normal-like subtype and one specimen lacked subtype classification. In the remaining 361 tumors, either mutant (N=94) or wild-type (N=267) for *PIK3CA*, the protein expression and phosphorylation levels were analyzed irrespective of molecular subtype as well as stratified by subtype (using the PAM50 classification) (Figure 2).

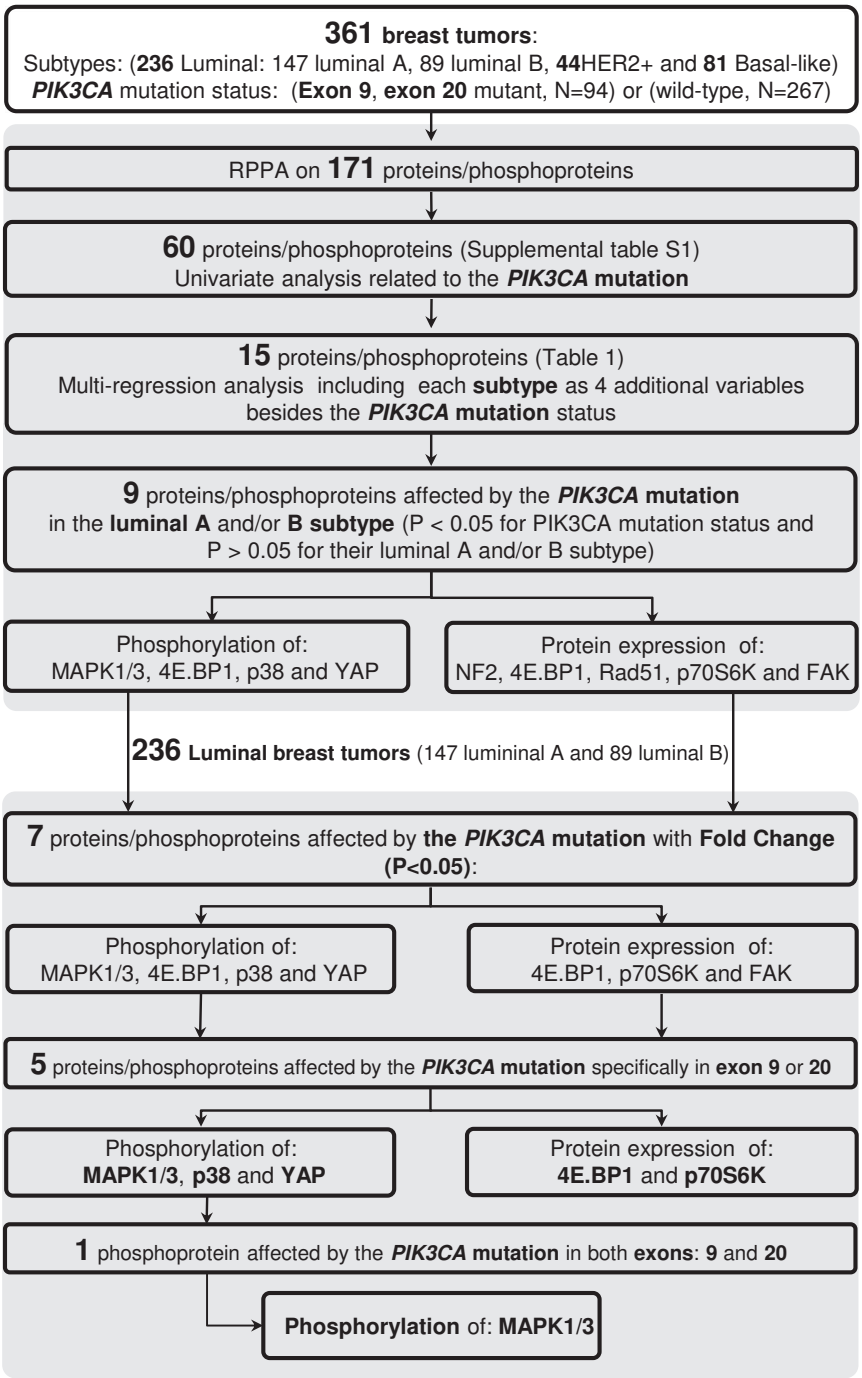

Figure 2 Data analysis flowchart to discover potential protein biomarkers for breast cancer. The figure shows the detailed criteria used to identify the association of the *PIK3CA* mutation with the protein expression and phosphorylation patterns using Reverse Phase Protein Array (RPPA) *in silico* data from the TCGA consortium on 361 breast tumors and 171 proteins or phosphoproteins.

Data analyses and statistics

Differences between groups were examined with a two-tailed student's t-test or when not normally distributed with the Mann-Whitney test. Multiple linear regression analyses were performed on the protein expression and phosphorylation levels to establish their relationships with the *PIK3CA* genotype and molecular subtypes. The residuals of the regression analyses were afterwards checked for their constant variance by the Breusch-Pagan/Cook-Weisberg test for heteroscedasticity and for their normality. In multiple linear regression analyses, the *PIK3CA* wild-type basal subtype was used as reference to which the other subtypes and their *PIK3CA* status were compared. Proteins significant after these regression analyses were subsequently investigated for their relationship with *PIK3CA* genotype in luminal A and B specimens and also for their associations with exon 9 and exon 20 mutations, separately. The protein expression and phosphorylation levels were visualized with boxplots to illustrate the levels in luminal A and B *PIK3CA* wild-type or mutant with the levels obtained in basal wild-types (Figure 3).

The Cox proportional hazard model was used in univariate analysis to compute the hazard ratio (HR) for metastasis-free survival (MFS). The HR was presented with its 95% confidence intervals (95% CI). Survival curves were generated using the Kaplan–Meier method and a log rank test was applied to test for differences. MFS was defined as the time elapsed between the date of diagnosis and the date of distant metastatic relapse. In multivariate analysis, the biomarker was added to the base model of traditional prognostic factors for early breast cancer disease, i.e., age at diagnosis, menopausal status, tumor size, and tumor grade. Analyses were executed in STATA statistical package, release 14 (STATA Corp., College Station, TX). All P-values were two-sided, $P<0.05$ were considered statistically significant.

Immunohistochemistry and evaluation of MAPK1/3 phosphorylation

Tissue microarrays (TMAs) of formalin-fixed, paraffin-embedded primary breast cancer specimens were described previously [11], and were immunocytochemically stained according to the procedures previously applied by us [10], [11]. Briefly, tissue samples were used from patients with primary operable breast cancer between 1985 and 2000. This TMA contained a cohort of 817 patients. Tumors were included for analyses if histologic subtype, Bloom-Richardson score for tumor differentiation grade, estrogen and progesterone receptor status (ER, PgR), HER2/neu, epidermal growth factor receptor (EGFR) and cytokeratin 5 (CK5) status were available. Above selection criteria resulted finally in the evaluation of triplicate tissue core biopsies from 721 patients with primary breast cancer. These included 526 luminal breast tumors amongst other subtypes, of which 290 were from lymph node negative patients who received no (neo)adjuvant systemic therapies as described previously (van der Willik et al., AJCR 2016). This retrospective study with coded tumor tissues was approved by the Erasmus MC medical ethics committee at Rotterdam, The Netherlands (MEC 02.953). TMAs were stained with the primary rabbit polyclonal antibody for MAPK1/3 phosphorylation at 1:200 dilution (Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody #9101 from Cell Signaling

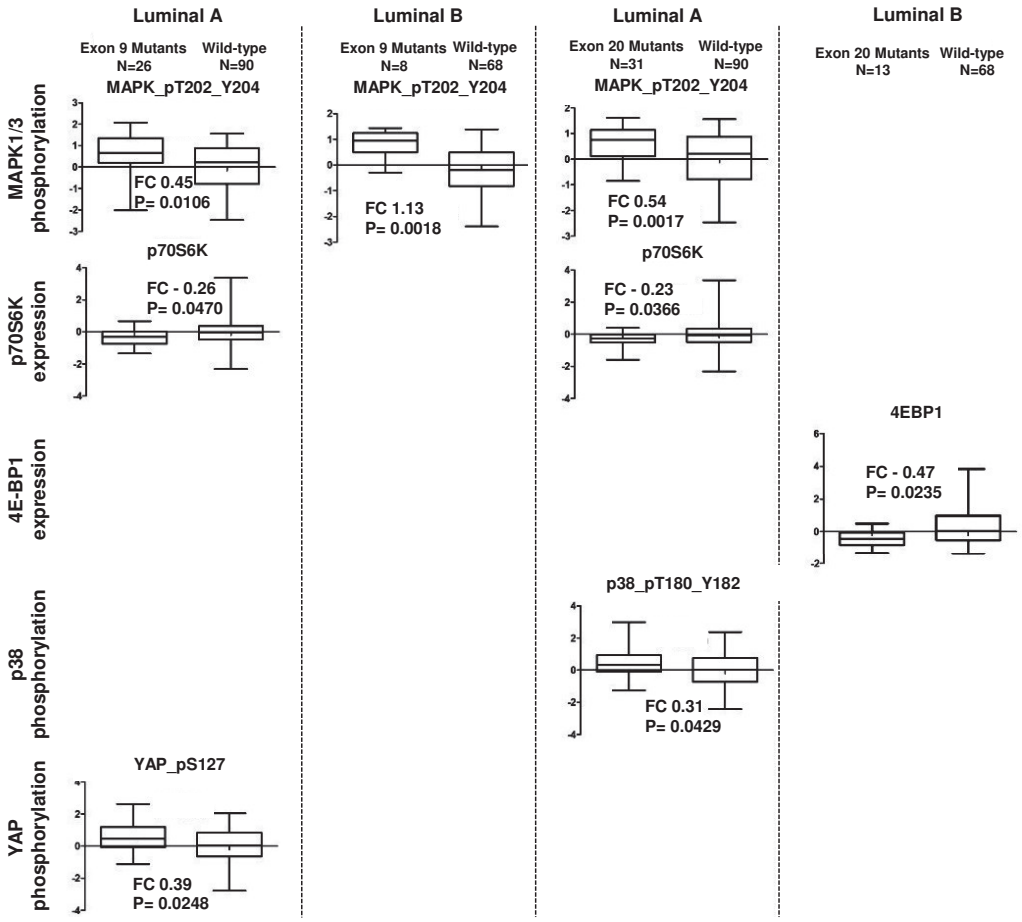

Figure 3 Protein alterati ns in *PIK3CA* exon-specific mutated luminal breast cancer. Differential phosphorylation and expression of 5 proteins due to *PIK3CA* mutation status but not molecular subtype as established in the multi-regression analysis (Table 1). The figure presents fold changes (FC) and P-values of non-parametrical Mann Whitney tests for MAPK1/3, p38 and YAP phosphorylation and expression of p70S6K and 4E-BP1 in luminal A and B breast cancer with exon 9 or exon 20 *PIK3CA* mutations.

Technology). The antibody was incubated overnight at 4 °C after 20 minutes antigen retrieval at pH6.0. Subsequently, the TMA-slides were incubated with a secondary antibody (Rabbit Envision+ System, HRP (DAKO)) and staining was visualized using diaminobenzidine (DAB).

Both nuclei and cytoplasm were scored for MAPK1/3 phosphorylation by two independent observers with regard to staining intensity and to the estimated proportion of tumor cells with positive nuclei and/or cytoplasm within a core biopsy. These scores were categorized to explore staining pattern differences. Negative versus positive staining was evaluated for both nuclei and cytoplasm separately, and combined since a subset of specimens showed heterogeneity in tumor cell staining (see figure 4). Positive staining was specified for its intensity (weak, moderate, strong) and/or proportion of staining. To have enough cases for proper evaluation, groups

were taken together to obtain more robust categories. For that reason, negative and weak staining were combined into one category (weak), and moderate and strong staining into another category (strong). In a similar way, the categories for proportions were dichotomized into less than 20% positivity (low) versus more than 20% positive nuclei or cytoplasm (high).

PIK3CA mutation analysis

PIK3CA status were established by SnaPshot® multiplex assays (Life technologies) as described previously [5]. For only 105, of which 95 luminals, out of the 721 cases, (sufficient) DNA was available from the TMA core biopsies to identify *PIK3CA* exon 9 and exon 20 mutations.

RESULTS

Protein expression and phosphorylation related to *PIK3CA* genotype and molecular subtypes

Student t-test or Mann-Whitney test analyses of proteins in 361 tumors, showed that 60 proteins had significant differences in expression or phosphorylation levels between tumors mutant (N=94) or wild-type (N=267) for *PIK3CA* (Supplemental Table S1). Of these 60 proteins, 15 protein changes were significant ($P<0.05$) in multiple linear regression analyses for *PIK3CA* status when molecular subtypes were taken into account (Figure 2, Table 1).

To identify protein alterations correlated with mutation and not with subtype, only proteins were studied further for which expression level or phosphorylation status was affected by the *PIK3CA* mutation status and not significantly by subtype. Of the 15 proteins, the residuals of 11 proteins showed that the assumptions for constant variance (4 failed test for heteroscedasticity) were valid allowing extrapolation of findings (Table 1). Subsequently, 9 proteins had P values <0.05 for *PIK3CA* mutation status and $P>0.05$ for luminal A and/or B subtypes (Table 1). Of these, MAPK1/3 phosphorylation and NF2 expression had significant different levels between *PIK3CA* wild-types and mutants irrespective of molecular subtype.

The other proteins were differentially expressed or phosphorylated due to *PIK3CA* status and subtype when compared to the wild-type basal subtype. Only significant for *PIK3CA* mutation status and independent of subtype in luminal B were the altered levels for Rad51 expression and 4E-BP1 expression and phosphorylation, and in luminal A the p38 and YAP phosphorylation and expression of p70S6K and FAK. The applied (non)parametric tests have demonstrated significant proteins alterations in the TCGA dataset. For the findings on p70S6K, however, these may not be extrapolated due to test limitations and/or assumptions.

Table 1 Protein alterations in breast cancer related with *PIK3CA* genotype irrespective of molecular subtype.

P-values in statistical analysis for protein alterations in relation to PIK3CA mutation status alone or combined with molecular subtypes in 361 tumor specimens						
		Student t-test or Mann-Whitney	Multiple linear regression (Basal *PIK3CA* wild-types as reference)			
Protein	Alteration	*PIK3CA*	*PIK3CA*	HER2	Luminal A	Luminal B
MAPK1/3	Phosphorylation of T202/Y204	<0.001 [a]	<0.001 [c]	0.883	0.054	0.933
4E.BP1	Expression	<0.001	0.001	0.155	0.002	0.679
p70S6K	Expression	0.010 [a]	0.009 [b,c]	0.014	0.137	<0.001
p38	Phosphorylation of T180/Y182	<0.001	0.001	0.053	0.580	0.001
YAP	Phosphorylation of S127	0.003	0.001	0.003	0.120	<0.001
4E.BP1	Phosphorylation of T70	<0.001	0.049 [c]	0.734	<0.001	0.591
FAK	Expression	0.021	0.048	0.018	0.394	0.007
NF2	Expression	0.014	0.025	0.391	0.684	0.751
Rad51	Expression	<0.001	0.016 [b,c]	0.195	<0.001	0.268
PR	Expression	<0.001 [a]	<0.001 [b]	0.974	<0.001	<0.001
Akt	Phosphorylation of S473	0.012	0.002	0.138	0.013	<0.001
Bcl.xL	Expression	0.018 [a]	0.002 [b,c]	0.836	0.011	0.003
MIG.6	Expression	0.002	0.017 [c]	0.002	0.043	<0.001
Cyclin_E1	Expression	<0.001	0.008 [c]	<0.001	<0.001	<0.001
S6	Expression	<0.001	0.039	0.004	<0.001	<0.001

Phosphorylation and expression levels of 171 proteins measured with reverse phase protein arrays (RPPA) obtained from the TCGA database, were evaluated for their relationship with *PIK3CA* genotype and molecular subtype. Student t-test and Mann-Whitney test analyses defined protein alterations that are significantly related to *PIK3CA* status in all 361 tumor specimens. Multiple linear regression evaluated protein levels in relation to *PIK3CA* status combined with molecular subtype and used the wild-type basal-like subtype as reference. Only 15 protein alterations were significant for *PIK3CA* mutation status after linear regression. Of these, 9 proteins were of interest because their levels were altered due to *PIK3CA* ($P<0.05$) but not by luminal subtype ($P>0.05$).

[a] P-value based on Mann-Whitney test.

[b] residuals failed test for heteroskedasticity.

[c] residuals failed test for normality.

PIK3CA genotype related protein alterations in luminal breast cancer

The altered expression and phosphorylation levels of the 9 selected proteins were further investigated in the luminal subsets, i.e. 147 luminal A and 89 luminal B tumors (Supplemental Table S2). Two proteins, i.e. NF2 and RAD51, showed no significant fold changes in this luminal subset when evaluated for all mutants together, and were excluded from further analyses. In *PIK3CA* mutated specimens compared to wild-type specimens significant fold changes were observed for the remaining 7 proteins with increased MAPK1/3 phosphorylation seen in luminal A and B cancers, decreased 4E-BP1 expression and phosphorylation in luminal B tumors, and decreased p70S6K and FAK expression and increased p38 and YAP phosphorylation in luminal A typed specimens.

Protein expression and phosphorylation related to *PIK3CA* exon 9 and 20 mutations

Five of the 7 proteins altered in the luminal subset, all but FAK expression and phosphorylation of 4E-BP1, were significantly related to exon specific mutations when compared to wild-type counterparts (Figure 3, Supplemental Table S2).
Tumors with *PIK3CA* exon 9 hotspot mutations had increased MAPK1/3 and YAP phosphorylation and decreased p70S6K expression in luminal A, and only increased MAPK1/3 phosphorylation in luminal B (Figure 3). Tumors harboring exon 20 mutations had increased MAPK1/3 and p38 phosphorylation in luminal A and decreased expression of p70S6K in luminal A while only 4E-BP1 was decreased in luminal B. The differences in phosphorylation observed in our *in silico* analyses for p38 and YAP in relation to exon-specific *PIK3CA* mutations, have not been yet reported by others to our knowledge.

Tumor cell heterogeneity and subcellular localization of MAPK1/3 phosphorylation

Protein functioning is not only defined by their expression and phosphorylation status, but may also depend on (sub)cellular localization. The RPPA findings, as described above, however, do not reveal which cell types and cellular compartments express proteins. To address this disadvantage, we examined MAPK1/3 phosphorylation for tumor cell heterogeneity and its subcellular localization in TMAs containing core biopsies of 721 primary tumor tissue specimens (Figures 4 & 5). Immunohistochemical staining for MAPK1/3 phosphorylation was performed, since it was the most significant protein alteration seen in *PIK3CA* exon 9 mutant luminal A and B tumors and in exon 20 mutant luminal A tumors (Figure 3).
Our TMA biopsies showed heterogeneity in staining patterns for MAPK1/3 phosphorylation as illustrated by the examples of figure 4 and quantified in figure 5. Moreover, MAPK1/3 phosphorylation was especially seen in the luminal and HER2 intrinsic subtypes, whereas tumors of the basal subtype had somewhat less staining of especially nuclei (Figure 6).

The MAPK1/3 phosphorylation staining revealed besides tumors with no and >80% positive tumor cells, also tumors with 11% to 80% tumor cells with MAPK1/3 phosphorylation in their cytoplasm (18% in Figure 5D) and in their nuclei (32% in Figure 5B). These findings suggest heterogeneity in a subset of specimens containing both tumor cell populations positive as well as negative for MAPK1/3 phosphorylation. Additionally, Figure 4 indicates heterogeneity in subcellular phosphorylation of MAPK1/3, which is confirmed by the staining distributions for proportions and intensities between nuclei and cytoplasm, respectively, as illustrated in Figures 5E and 5F. For example, cases with no cytoplasmic MAPK1/3 had weak to strong nuclear MAPK1/3 staining (Figure 5F) in up to 100% of the nuclei (Figure 5E). Overall, the staining proportion and intensity for MAPK1/3 phosphorylation was significantly heterogeneous (both Chi-square test $P<0.001$) when compared between nuclei and cytoplasm.

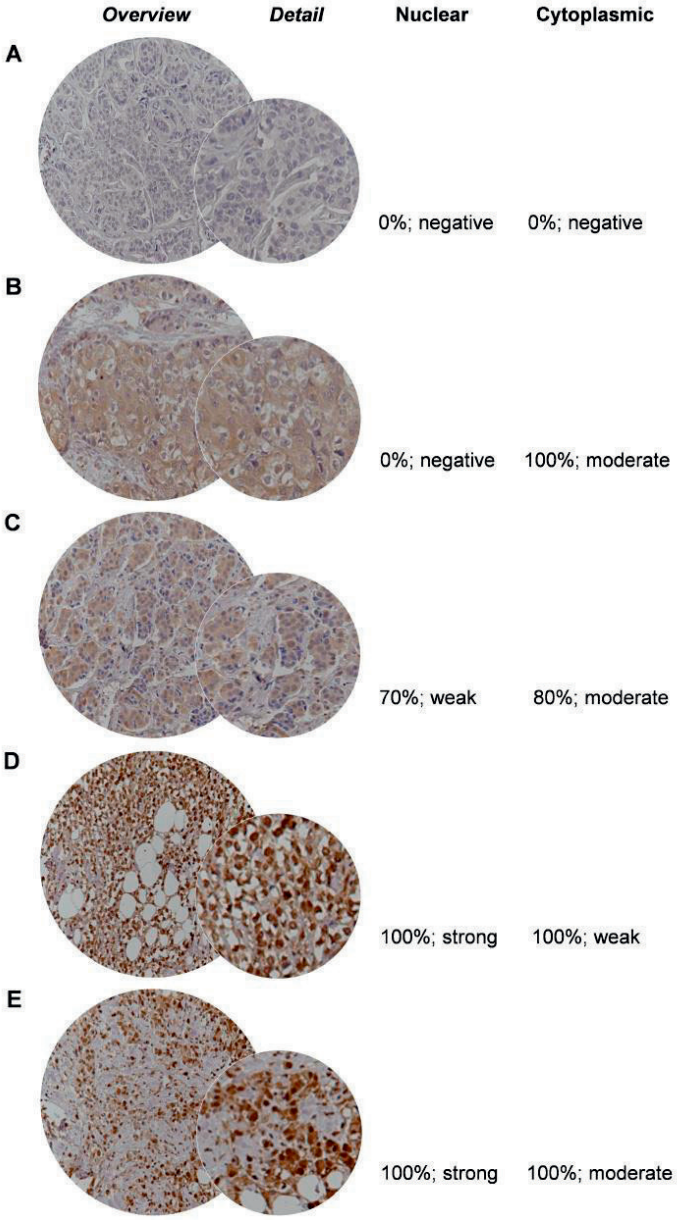

Figure 4 Phosphorylated MAPK1/3 staining patterns examples defined by intensity and proportion of MAPK1/3 phosphorylated tumor cells. Figure 4**A** Negative for both nuclear and cytoplasmic. Figure 4**B** Negative for nuclear and 100% moderate cytoplasmic intensity. Figure 4**C** 70% weak nuclear and 80% moderate cytoplasmic intensity. Figure 4**D** 100% strong for both nuclear and cytoplasmic intensity. Figure 4**E** 100% strong nuclear and 100% moderate cytoplasmic intensity.

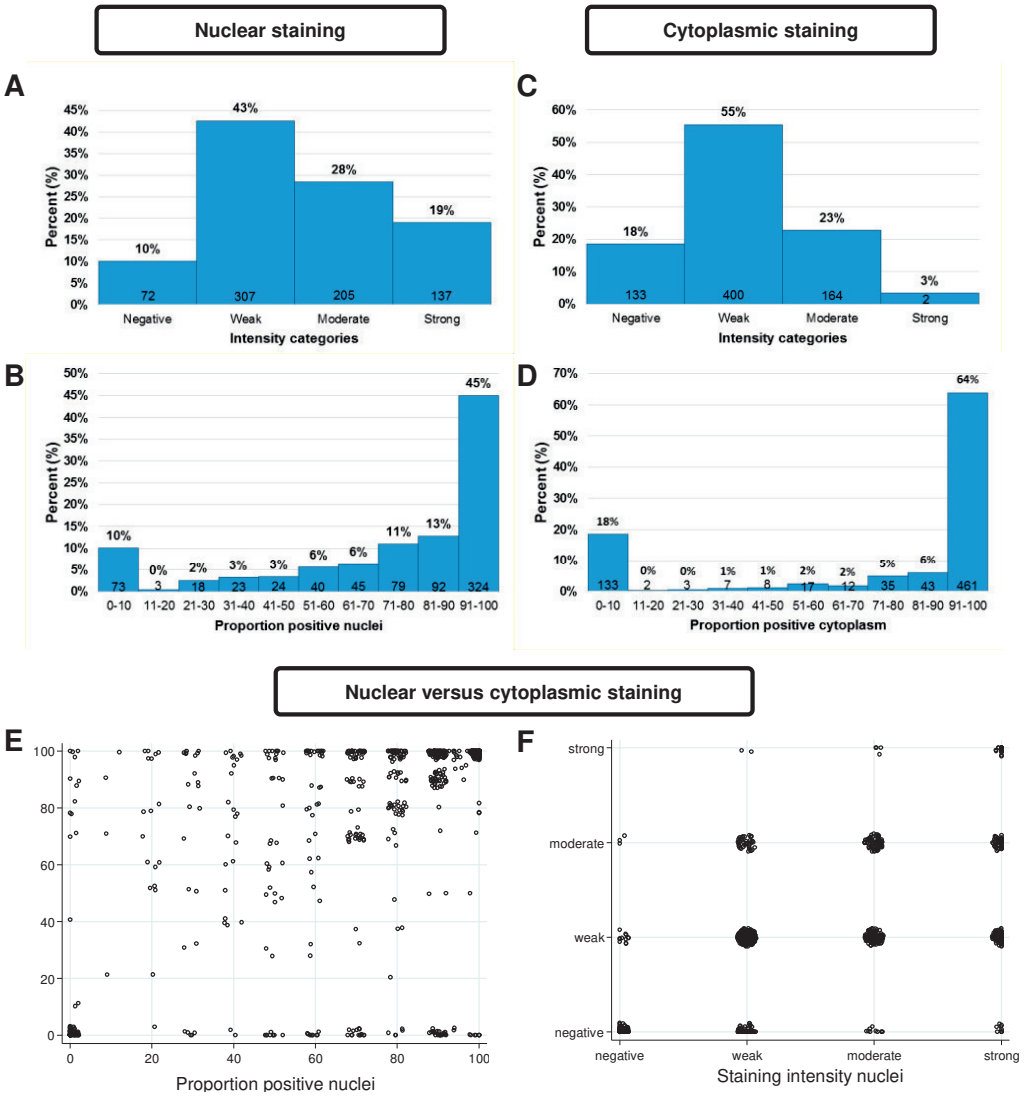

Figure 5 MAPK1/3 phosphorylation immunohistochemical staining evaluation. Tissue microarrays containing primary breast tumor core biopsies from 721 breast cancer patients were evaluated for nuclear, cytoplasmic and tumor cell MAPK1/3 phosphorylation. The figures present the percentage (above each bar) and number of cases (bottom values) for nuclear (Figure 5**A**, 5**B**) and cytoplasmic staining (Figure 5**C**, 5**D**) defined by intensity (Figure 5**A**, 5**C**) and by proportion (Figure 5**B**, 5**D**).
Additionally, it illustrates the number of cases comparing MAPK1/3 phosphorylation between cytoplasmic and nuclear staining for proportion (Figure 5**E**) and for intensity (Figure 5**F**).

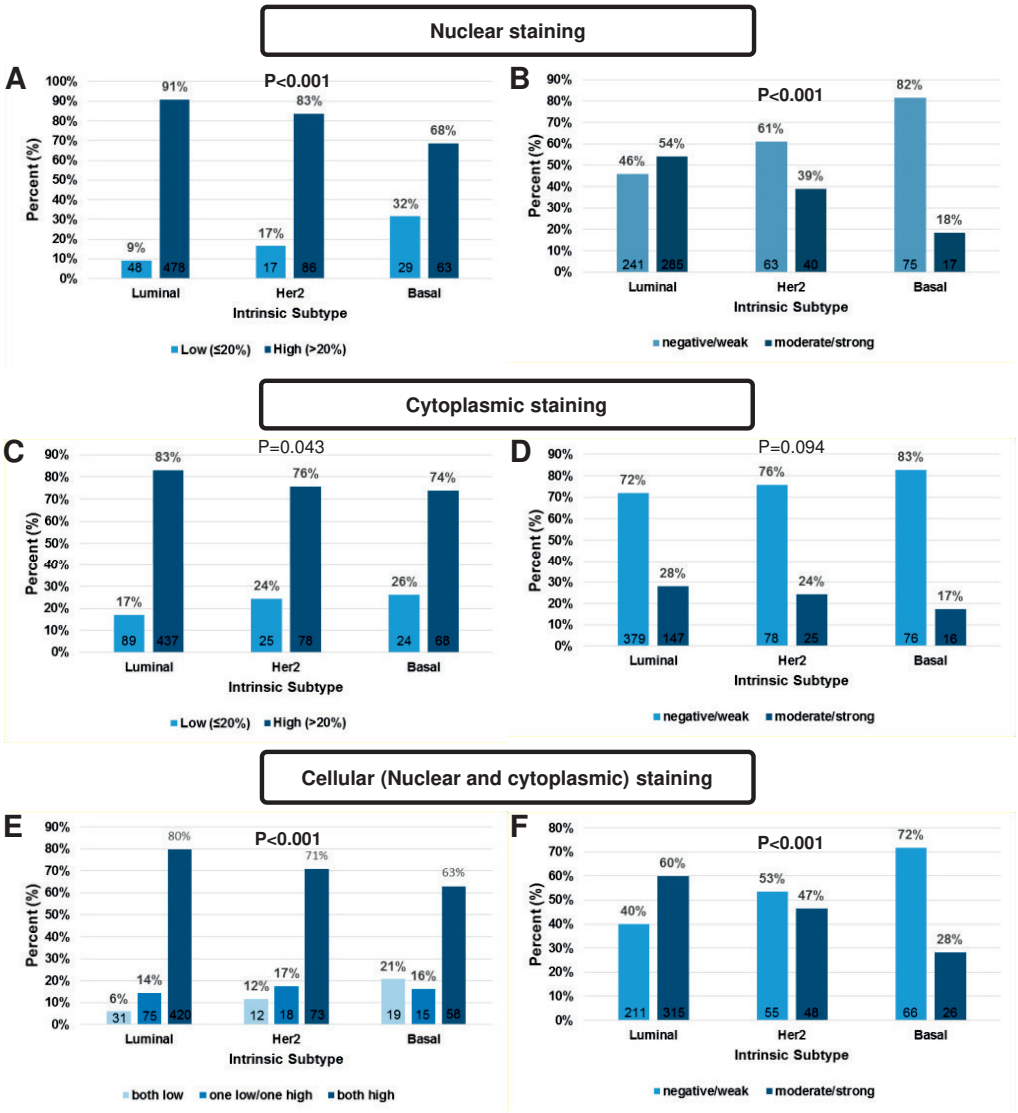

Figure 6 MAPK1/3 phosphorylation in relation with intrinsic subtypes analysed in 721 primary breast tumors. The figures present the percentage (above each bar) and number of cases (bottom values) for nuclear (Figure 6**A**, 6**B**), cytoplasmic (Figure 6**C**, 6**D**) and cellular staining (Figure 6**E**, 6**F**) defined by proportion (Figure 6**A**, 6**C**, 6**E**) and by intensity (Figure 6**B**, 6**D**, 6**F**). The intrinsic subtypes were defined by ER, PgR, HER2/neu, EGFR and Cytokeratin 5 staining and classified as described previously by us (PMID: 27186402) as luminal (positive for ER and/or PgR, negative for HER2/neu), Her2 (positive for HER2/neu) and basal (positive for EGFR and/or Cytokeratin 5, negative for ER, and HER2/neu). P-values are based on Chi-square test.

MAPK1/3 phosphorylation staining and *PIK3CA* mutation

MAPK1/3 phosphorylation was also evaluated in a subset 95 luminal breast cancers with known *PIK3CA* genotype, including 32 tumors with *PIK3CA* exon 9 (n=9) or exon 20 mutations (n=23). No significant differences between *PIK3CA* mutants and

wildtypes were observed for staining proportion and intensity of nuclei and cytoplasm, when analysed separately or combined (Figure 7).

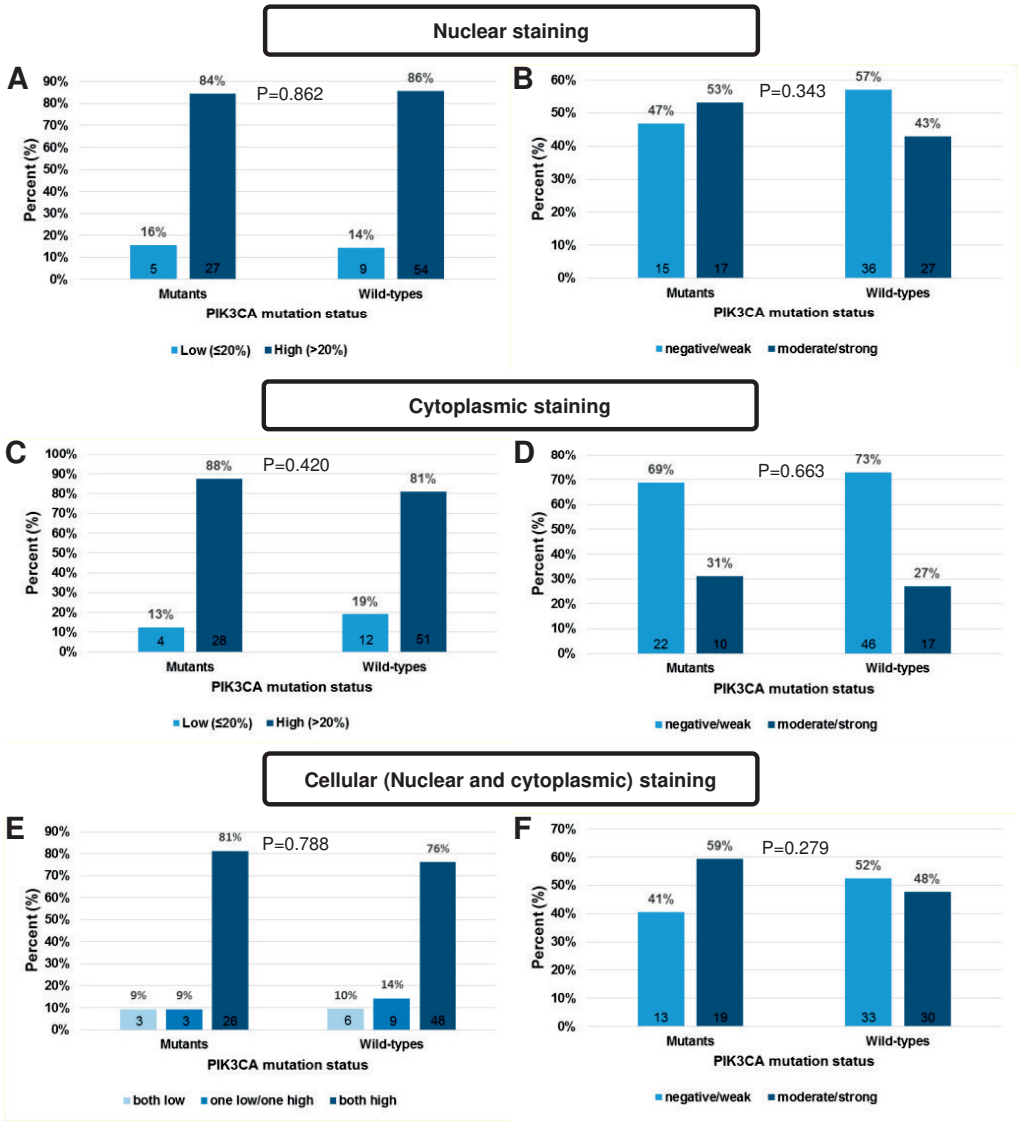

Figure 7 MAPK1/3 phosphorylation in relation to *PIK3CA* mutation status analyzed in 95 available luminal breast tumors. The figures present the percentage (above each bar) and number of cases (bottom values) for nuclear (Figure 7**A**, 7**B**), cytoplasmic (Figure 7**C**, 7**D**) and cellular staining (Figure 7**E**, 7**F**) defined by proportion (Figure 7**A**, 7**C**, 7**E**) and by intensity (Figure 7**B**, 7**D**, 7**F**). No association was found for subcellular localization of MAKP1/3 phosphorylation with *PIK3CA* mutation status. P-values are based on Chi-square test.

Clinical relevance of subcellular localization of MAPK1/3 phosphorylation in breast cancer tissue specimens

Logrank tests for trend were evaluated to determine whether MAPK1/3 phosphorylation staining intensity and proportion were related with MFS (Table 2). These tests demonstrated a relationship with MFS in especially luminal tumors for nuclear and tumor cell MAPK1/3 phosphorylation for both staining intensity and proportion. In contrast, cytoplasmic staining was associated with MFS in only 2 of 9 analyses.

MAPK1/3 phosphorylation staining patterns were evaluated for their prognostic value in a cohort of luminal primary breast tumors from 290 LNN patients who did not receive (neo)adjuvant systemic therapy. Kaplan-Meier survival curve analyses and the logrank tests for trend showed that only nuclear and tumor cell staining were related with MFS in this cohort (Table 2 & Figure 8). The Kaplan-Meier analyses demonstrated that high (>20% in Figure 8A) as well as strong (Figure 8D) nuclear MAPK1/3 phosphorylation associate with favorable survival. Similar results were obtained for tumor cell staining (Figures 8C and 8F) but not for cytoplasmic staining (Figures 8B and 8E).

Uni- and multivariate analyses for MAPK1/3 phosphorylation staining patterns were performed for this cohort of 290 LNN patients (Table 3). Staining patterns in multivariate analyses were compared to the base model of traditional prognostic factors which included age, menopausal status, tumor size, Bloom-Richardson differentiation grade and progesterone receptor status. Only high nuclear and tumor cell stain staining proportions were independent from the traditional factors and associated with favorable survival. MAPK1/3 phosphorylation in more than 20% of nuclei was associated with longer MFS compared to no or less than 20% of positive nuclei (HR=0.49; 95% CI: 0.25-0.95;P=0.036). Similarly, in specimens with staining in more than 20% of both nuclei and cytoplasm, MAPK1/3 phosphorylation was also related with favorable MFS (HR=0.37; 95% CI: 0.18-0.79; P=0.010).

Table 2 Staining intensity and proportion for MAPK1/3 phosphorylation in nuclei and cytoplasm of tumor cells and its relationship with metastasis free survival in breast cancer: P-values for logrank test for trends

Staining	Categories	primary breast cancer (N=721)		Luminal breast cancer (N=526)		LNN luminals (N=290)	
		Number of cases per category	P-value	Number of cases per category	P-value	Number of cases per category	P-value
Nuclei							
Intensity:	Negative versus Positive	72; 649	0.023	34; 492	0.016	18; 272	0.038
	Weak versus Strong	379; 342	0.053	241; 285	0.003	131; 159	0.046
Proportion:	Low (≤20%) versus High (> 20%)	94; 627	0.093	48; 478	0.017	23; 267	0.005
Cytoplasm							
Intensity:	Negative versus Positive	133; 588	0.096	87; 439	0.020	42; 248	0.101
	Weak versus Strong	533; 188	0.091	379; 147	0.073	205; 85	0.209
Proportion:	Low (≤20%) versus High (> 20%)	138; 583	0.155	89; 437	0.018	43; 247	0.056
Tumor cells (nuclei and cytoplasm combined)							
Intensity:	Negative versus Positive	57; 664	0.011	28; 498	0.000	14; 276	0.006
	Weak versus Strong	332; 389	0.040	211; 315	0.001	110; 180	0.028
Proportion:	Both Low versus Low/High versus Both High	62; 108; 551	0.069	31; 75; 420	0.004	15; 36; 239	0.006

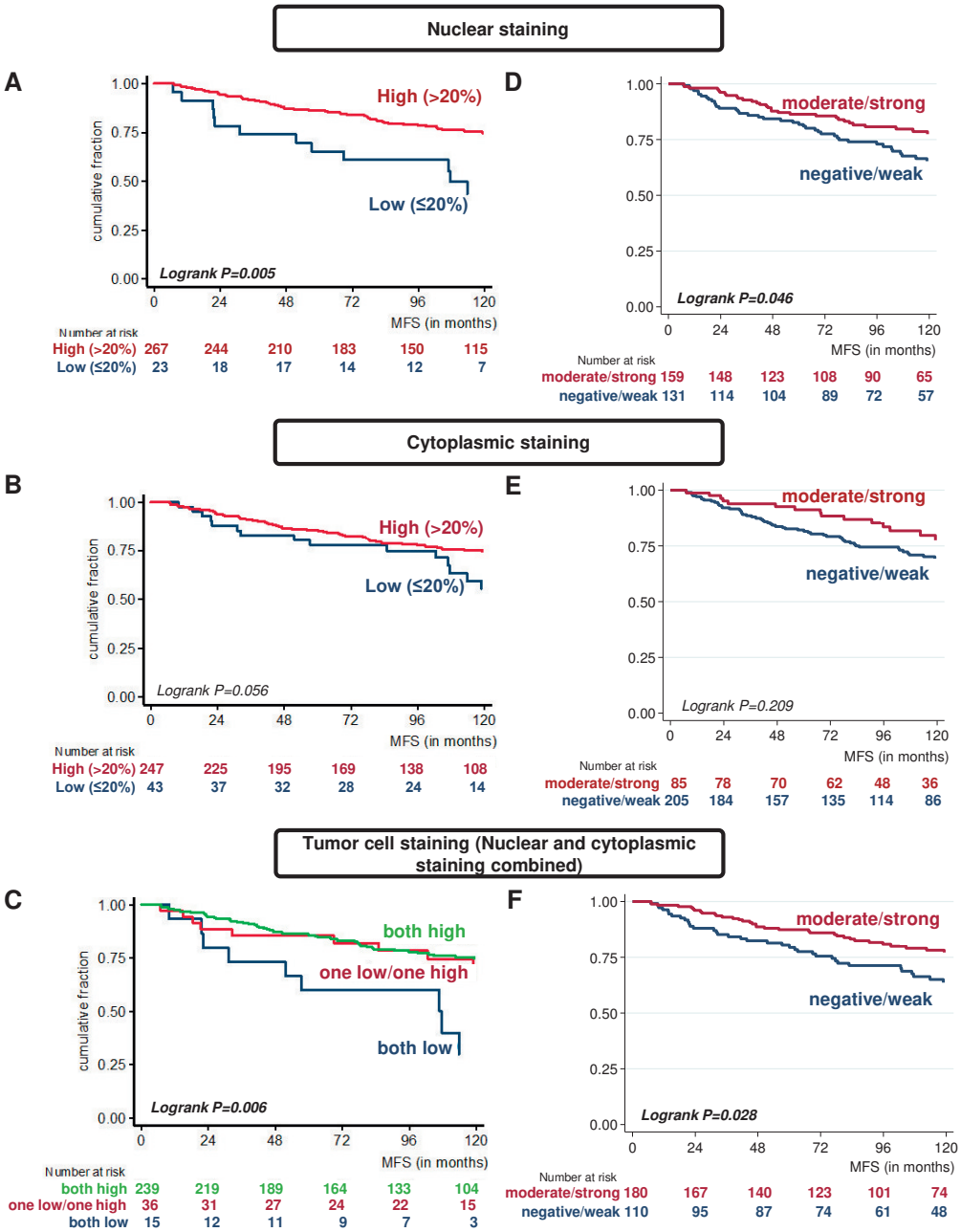

Figure 8 Subcellular localization of phosphorylated MAPK1/3 and its prognostic significance. Kaplan Meier curve analyses for MFS in luminal primary breast tumors of 290 (neo) adjuvant systemic therapy naïve LLN patients shows that high (Figure 8**A**) and strong (Figure 8**D**) nuclear MAPK1/3 phosphorylation correlates with favorable prognosis. No correlation was observed for cytoplasmic phosphorylated MAPK1/3 regarding staining proportion (Figure 8**B**) and intensity (Figure 8**E**). Finally, nuclear and cytoplasmic staining combined (cellular staining) was associated with favorable prognosis when defined by proportion (Figure 8**C**) as well as by intensity (Figure 8**F**). Abbreviations: MFS: Metastasis-free survival; LNN: Lymph Node Negative.

Table 3 Univariate and multivariate analyses for metastasis-free survival of MAPK1/3 phosphorylation

Factor	Luminal LNN , (neo)adjuvant systemic therapy naïve							
	univariate analysis					multivariate analysis		
	No. of patients	%	HR	95% CI	P-value	HR	95% CI	P-value
Age at diagnosis (in years):								
<40	26	9%	1.00			1.00		
41-55	116	40%	0.75	0.37-1.53	0.432	0.73	0.34-1.55	0.412
56-70	101	35%	0.56	0.26-1.20	0.135	0.26	0.09-0.79	0.018
>70	47	16%	0.81	0.34-1.92	0.634	0.31	0.09-1.03	0.057
Menopausal status:								
premenopausal	126	43%	1.00			1.00		
postmenopausal	164	57%	0.99	0.63-1.57	0.995	2.32	1.03-5.20	0.042
tumor size (cm):								
<2cm	196	68%	1.00			1.00		
≥2cm	94	32%	2.37	1.51-3.73	<0.001	2.34	1.47-3.74	<0.001
Differentiation grade:								
1	68	23%	1.00			1.00		
2	153	53%	2.12	1.07-4.23	0.032	1.79	0.89-3.60	0.104
3	69	24%	3.05	1.46-6.37	0.003	2.67	1.26-5.68	0.011
PgR status:								
negative	63	22%	1.00			1.00		
positive	227	78%	0.82	0.48-1.40	0.462	0.82	0.46-1.43	0.477
Staining of MAPK1/3 phosphorylation						*added to base model*		
Nuclei								
Intensity:								
Negative	18	6%	1.00			1.00		
Positive	272	94%	0.49	0.24-0.97	0.042	0.58	0.27-1.25	0.165
Weak	131	45%	1.00			1.00		
Strong	159	55%	0.63	0.40-0.99	0.048	0.77	0.47-1.25	0.293
Proportion:								
Low (≤20%)	23	8%	1.00			1.00		
High (> 20%)	267	92%	0.42	0.23-0.79	0.006	0.49	0.25-0.95	0.036
Cytoplasm								
Intensity:								
Negative	42	14%	1.00			1.00		
Positive	248	86%	0.63	0.36-1.10	0.104	0.69	0.39-1.22	0.203
Weak	205	71%	1.00			1.00		
Strong	85	29%	0.71	0.42-1.21	0.211	0.78	0.46-1.34	0.377
Proportion:								
Low (≤20%)	43	15%	1.00			1.00		
High (> 20%)	247	85%	0.59	0.34-1.02	0.059	0.64	0.36-1.13	0.121
Tumor cells (nuclei and cytoplasm combined)								
Intensity:								
Negative	14	5%	1.00			1.00		
Positive	276	95%	0.37	0.18-0.77	0.008	0.42	0.19-0.92	0.029
Weak	110	38%	1.00			1.00		
Strong	180	62%	0.61	0.39-0.95	0.030	0.78	0.49-1.27	0.319
Proportion:								
both low	15	5%	1.00			1.00		
low/high	36	12%	0.40	0.16-0.99	0.047	0.39	0.15-1.02	0.056
both high	239	82%	0.33	0.16-0.67	0.002	0.37	0.18-0.79	0.010

Analyses performed in 290 patients with luminal LNN breast tumors .The 290 patients did not receive adjuvant systemic therapy. Abbreviations: LNN: Lymph Node Negative; HR: Hazard ratio; PgR: Progesterone receptor.

DISCUSSION

This study aimed to reveal proteins downstream PI3K with altered phosphorylation or expression levels in luminal breast cancer with *PIK3CA* exon 9 and/or exon 20 mutations. Our *in silico* analyses of publically available RPPA data showed in luminal A and B tumors with *PIK3CA* mutations an increased phosphorylation of MAPK1/3, p38 and YAP and decreased expression of p70S6K and 4E-BP1. We showed that MAPK1/3 phosphorylation was the most significant protein upregulated in *PIK3CA* exon 9 mutated luminal A and B tumors and in *PIK3CA* exon 20 mutated luminal A tumors. Our immunohistochemical staining of MAPK1/3 phosphorylation on TMAs demonstrated tumor cell heterogeneity in subcellular location, staining intensity and proportion. We demonstrated that high MAPK phosphorylation in especially the nuclei of tumor cells correlated with favorable prognosis.

We investigated protein changes detected by RPPA to identify alterations specific for luminal breast cancer specimens with a *PIK3CA* exon 9 or exon 20 mutation. Mutations in *PIK3CA*, next to TP53, occur most frequently in breast cancer. TP53 mutations and PI3K/AKT/mTOR pathway activation are particularly observed in breast cancer of the basal subtype [1]. The *PIK3CA* mutations, however, are predominantly observed in ER-positive tumors and have been shown to be present in approximately 27% of luminal B up to 45% in luminal A specimens [1]. Above considerations were taken into account in our *in silico* analyses using a multi-regression model combining *PIK3CA* status and molecular subtype. These analyses helped to identify alterations related to the *PIK3CA* mutation status in a (luminal) subtype independent way. Although 15 protein changes had a significant relation with *PIK3CA* mutation status, only 9 of these were not correlated with luminal A and/or B subtypes. These 9 proteins were further explored within the subset of 236 luminal specimens for the relation with exon-specific *PIK3CA* mutations. This subsequent *in silico* evaluation demonstrated just 5 of these 9 protein alterations to be *PIK3CA* exon mutation specific in luminal A and/or B tumors, including increased phosphorylation of MAPK1/3, p38 and YAP and decreased expression of p70S6K and 4E-BP1. Future investigations are needed to validate our *in silico* results in other subsets.

Differences in protein phosphorylation patterns in relation to *PIK3CA* mutations have been reported for human cancer cell line models. It was shown that somatic knock-in of both KRAS and *PIK3CA* mutations in human breast epithelial cells was accompanied by increased MAPK1/3 phosphorylation, MAPK pathway activation, and tumor formation in immune-comprised mice [12]. Moreover, it was shown that phosphorylation of MAPK1/3 resulted in an activation of the protein and translocation from the cytoplasm to the nucleus [13]. We could not evaluate this translocation of MAPK1/3 upon phosphorylation with the available RPPA data from the TCGA consortium, since the RPPA was performed on protein lysates of cancer tissue and not on protein lysates of nuclear and cytoplasmic fractions. Therefore, we performed an immunohistochemical staining for MAPK1/3 phosphorylation on TMAs containing core biopsies of breast cancer specimens. These stainings enabled us to establish the subcellular location of MAPK1/3 phosphorylation and to examine tumor cell heterogeneity. Our results detected in 32% of cases nuclear MAPK1/3 phosphorylation for only a subset of tumor cells (ranging from 20% to 80%). This

suggests that MAPK activation is not always seen in all tumor cells for a substantial subset of breast cancers.

Importantly, high MAPK1/3 phosphorylation in nuclei was related with favorable prognosis in our cohort of LNN luminal breast cancer specimens. Others have stained tissue microarrays for MAPK1/3 phosphorylation in breast cancer to determine either the relation with PI3K or with clinical outcome. Nuclear MAPK1/3 phosphorylation was investigated on 563 estrogen receptor positive primary tumors from patients participating in a randomized trial of 1 to 3 years adjuvant tamoxifen [14, 15]. One study showed that at baseline both *PIK3CA* exon 9 and 20 mutated specimens have higher MAPK1/3 phosphorylation levels compared to wild-types [14]. Although this association with *PIK3CA* mutations was not confirmed in our TMA cohort, this is explained by the small number of cases for which the *PIK3CA* status could be determined. The other study demonstrated a trend between tamoxifen response and MAPK1/3 phosphorylation [15]. Additionally, nuclear and cytoplasmic MAPK1/3 phosphorylation were also studied in large well-characterized breast cancer series to investigate its biological and clinical significance [16, 17]. This study showed that nuclear MAPK1/3 phosphorylation was associated with better outcome, especially in tamoxifen-treated cases. All these immunohistochemical studies in large cohorts of (luminal) breast cancer specimens confirm in part our in silico findings in relation to *PIK3CA* mutation [18] and our TMA findings that nuclear MAPK1/3 phosphorylation correlates with favourable outcome [16, 17]. Future prospective studies are needed to verify above retrospective findings and to establish MAPK1/3 phosphorylation as biomarker.

In conclusion, we have explored several methods combining in silico multivariate analyses and immunocytochemistry on luminal breast cancer specimens. We have shown that *PIK3CA* mutated breast tumor specimens are characterized in an exon dependent manner by increased MAPK1/3 phosphorylation. The observed relationships in clinical specimens indicate that the phosphorylation of MAPK1/3 and its subcellular localization might associate with clinical outcome. Considering the high prevalence of *PIK3CA* mutations in breast cancer, our findings will aid in the design of future studies to evaluate and/or target downstream effectors of the *PIK3CA* mutation. Our findings indicate that MAPK1/3 phosphorylation may play an important role as a target downstream effector in luminal breast cancer patients with a *PIK3CA* mutation.

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CHAPTER V

Decreased expression of *ABAT* and *STC2* hallmarks ER-positive inflammatory breast cancer and endocrine therapy resistance in advanced disease

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HIGHLIGHTS

- ER+ inflammatory breast cancer (IBC) can be used as endocrine therapy resistance model.
- Molecular characterization revealed *ABAT* and *STC2* as ER+ IBC discriminatory genes.
- *ABAT* and *STC2* are novel biomarkers for tamoxifen resistance in advanced breast cancer.

ABSTRACT

Background

Patients with Estrogen Receptor α-positive (ER+) Inflammatory Breast Cancer (IBC) are less responsive to endocrine therapy compared with ER+ non-IBC (nIBC) patients. The study of ER+ IBC samples might reveal biomarkers for endocrine resistant breast cancer.

Materials & methods

Gene expression profiles of ER+ samples from 201 patients were explored for genes that discriminated between IBC and nIBC. Classifier genes were applied onto clinically annotated expression data from 947 patients with ER+ breast cancer and validated with RT-qPCR for 231 patients treated with first-line tamoxifen. Relationships with metastasis-free survival (MFS) and progression-free survival (PFS) following adjuvant and first-line endocrine treatment, respectively, were investigated using Cox regression analysis.

Results

A metagene of six genes including the genes encoding for 4-aminobutyrate aminotransferase (*ABAT*) and Stanniocalcin-2 (*STC2*) were identified to distinguish 22 ER+ IBC from 43 ER+ nIBC patients and remained discriminatory in an independent series of 136 patients. The metagene and two genes were not prognostic in 517 (neo)adjuvant untreated lymph node-negative ER+ nIBC breast cancer patients. Only *ABAT* was related to outcome in 250 patients treated with adjuvant tamoxifen. Three independent series of in total 411 patients with advanced disease showed increased metagene scores and decreased expression of *ABAT* and *STC2* to be correlated with poor first-line endocrine therapy outcome. The biomarkers remained predictive for first-line tamoxifen treatment outcome in multivariate analysis including traditional factors or published signatures. In an exploratory analysis, ABAT and STC2 protein expression levels had no relation with PFS after first-line tamoxifen.

Conclusions

This study utilized ER+ IBC to identify a metagene including *ABAT* and *STC2* as predictive biomarkers for endocrine therapy resistance.

Keywords

Inflammatory breast cancer; Endocrine therapy resistance; Metastatic disease; ABAT; STC2.

INTRODUCTION

Breast cancer is the most prevalent form of cancer in women in the United States and Europe (Torres-Arzayus et al., 2010). The majority of patients with breast cancer bear tumors expressing detectable levels of the Estrogen Receptor (ER). For these patients, targeted therapies are available including strategies directed at the receptor itself, such as tamoxifen and fulvestrant. In addition, estrogen deprivation offers another therapeutic strategy that can be achieved by ovarian ablation, or LHRH analogs, in the premenopausal patient, or with aromatase inhibitors (AIs) in the postmenopausal setting. These therapies are highly effective; adjuvant endocrine therapy has been shown to reduce mortality from ER+ breast cancer to the same degree as adjuvant chemotherapy (Early Breast Cancer Trialists' Collaborative, G, 2005). Unfortunately, part of the patients with ER+ breast cancer show de novo resistance to endocrine therapy, whereas others initially benefit but ultimately relapse due to acquired endocrine resistance (Leary et al., 2010). Predicting, modulating and/or restoring endocrine responsiveness remain important clinical priorities for which molecular targets are urgently needed.

Inflammatory breast cancer (IBC) is a rare (~5%) but aggressive form of locally advanced breast cancer. At time of diagnosis, virtually all patients with IBC have lymph node metastases and 1/3 of the patients have metastases in distant organs. As a consequence, the prognosis for patients with IBC is dismal (Dawood et al., 2011; Dirix et al., 2006). Analysis of the Surveillance, Epidemiology and End Results (SEER)-database revealed that IBC is characterized by atypical clinicopathological features (Dawood et al., 2011), including frequent absence of ER protein expression (Hance et al., 2005). Our research group and others have shown that this IBC-specific clinicopathological profile is corroborated at the molecular level by a distinct gene expression profile (Bertucci et al., 2004; Van Laere et al., 2007a; Van Laere et al., 2005). Exploration of this gene expression profile led to the discovery of pronounced activation of the transcription factor NFkB in IBC (Lerebours et al., 2008; Van Laere et al., 2006) and more recently to the observation that TGFβ-signaling is repressed (Van Laere et al., 2008). Furthermore, we demonstrated that the IBC-specific expression profile harbors the molecular traits of aggressive tumor cell behavior in general (Van Laere et al., 2008), including stem cell biology (Van Laere et al., 2010). As such, we consider IBC, although occurring rarely, as a suitable example to elucidate mechanisms responsible for tumor cell dissemination, metastasis and drug resistance in breast cancer in general.

The majority (depending on the reference up to 66%) of patients with IBC lack ER protein expression, but ER+ tumor samples from patients with IBC exist. Clinically, patients with ER+ IBC are less responsive to endocrine treatment as compared to patients with other forms of ER+ breast cancer. In light of molecular heterogeneity and our previous results, we reasoned that studying ER+ IBC focusing on endocrine treatment response might provide new insights into molecular resistance mechanisms of endocrine therapy. In the current study, we evaluated expression profiles from patients with ER+ IBC and nIBC. The purpose of this study was 1) to identify differentially expressed genes between IBC and nIBC, 2) assess their accuracy to predict ER+ IBC, and 3) to define their relationship with endocrine therapy response in clinical samples. Discriminatory genes were identified by gene expression arrays,

of which two genes remained deregulated in an independent series of ER+ samples between patients with and without IBC. When applied onto clinically annotated expression series from patients with ER+ breast cancer treated with endocrine therapy either in the adjuvant or advanced setting, decreased expression of these two genes were linked with poor responsiveness to endocrine therapy. These two genes when validated with quantitative real-time PCR for mRNA expression and with immunohistochemistry for protein expression, demonstrated predictive value only at the mRNA level.

MATERIALS AND METHODS

Study design and patient samples

The present study describes a retrospective analysis performed in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands, Belgium and France, and is reported following the REMARK recommendations (McShane et al., 2006). The local medical ethics committees have approved the study. Follow-up, tumor staging, and response to therapy was defined by standard International Union Against Cancer (Geneva, Switzerland) classification criteria (Hayward et al., 1978). Samples were recruited from the Translational Cancer Research Unit (TCRU, Antwerp, Belgium), the Institut Poali-Calmettes (IPC, Marseille France), the Erasmus Medical Center (EMC, Rotterdam, the Netherlands) and the Netherlands Cancer Institute (NKI, Amsterdam, the Netherlands). The ER-status of the tumors was established by immunohistochemistry (≥10% positive tumor cells) or EIA (≥10 fmol/mg protein) and together with additional clinicopathological characteristics have been described before for each of the series (Bekhouche et al., 2011; Desmedt et al., 2007; Jansen et al., 2013; Kok et al., 2009; Loi et al., 2008; Reijm et al., 2014; Schmidt et al., 2008; Van der Auwera et al., 2010; Van Laere et al., 2007b; Wang et al., 2005).

The mRNA-datasets used in this study are presented in Table 1 and includes ER+ IBC-subsets (I–II), endocrine treated subsets (III–VI), and untreated lymph node-negative (LNN) patients (VII–IX).

Table 1 Datasets. Detailed information on the datasets used in this study

Series	Analysis	Subset	Reference	GEO / ArrayExpress	Platform	Total number of cases
I	IBC	Discovery	Van Laere et al.	E-MTAB-1006	Affymetrix	65
II		Validation	Bertucci et al.	NA	Affymetrix	136
III	Treatment Outcome	Tamoxifen Adjuvant	Loi et al.	GSE6532	Affymetrix	250
IV		Tamoxifen Advanced	Kok et al.	NA	Agilent	96
V		Aromatase Inhibitors	Jansen et al. (2013)	GSE41994	Agilent	84
VI		Tamoxifen Advanced	Jansen et al. (2007)	NA	qRT-PCR	231
VII	Clinical Outcome	LNN ER+, untreated	Wang et al.	GSE2034	Affymetrix	221
VIII		LNN ER+, untreated	Desmedt et al.	GSE7390	Affymetrix	134
IX		LNN ER+, untreated	Schmidt et al.	GSE11121	Affymetrix	162

The datasets are numbered as series I to IX and the subset provides information about its study subject (IBC, treatment outcome, clinical outcome). For each dataset the author and array reference are presented next to the array-platform.

The discovery and test phase incorporated 2 patient series with ER+ IBC: a) a discovery series (I) of 65 samples from patients with and without ER+ IBC retrieved from the TCRU (E-MTAB-1006) and b) an independent test series (II) of samples from 136 patients with and without ER+ IBC that received adjuvant treatment, retrieved from IPC. Samples from patients with ER+ IBC in series I (N = 22) and series II (N = 39) were selected by strictly adhering to the consensus diagnostic criteria (Dawood et al., 2010).

To evaluate discovered genes for their relationship with endocrine treatment outcome, 4 additional data sets (III–VI) were incorporated of patients with ER+ breast cancer treated with endocrine therapy for primary and advanced disease: 3 data sets (IV–VI) of 411 metastatic breast cancer patients in total from EMC, NKI and TRCU; and 1 data set with primary breast cancers of 250 patients (III). The data set of 411 patients with advanced ER+ breast cancer treated with first-line therapy contained three subsets, one of 96 patients treated with tamoxifen (IV) and one of 84 patients treated with aromatase inhibitors (AIs) (V), and one of 231 patients treated with tamoxifen and profiled using RT-qPCR for dedicated genes (VI). The RT-qPCR data set was used as an independent validation series for the genome-wide expression series. All samples in these cohorts were classified according to the Recurrence Score (RS) (24), the Genomic Grade Index (GGI) (25). For each of these patient series tumor size and histological grade were recorded, in addition to age and menopausal status at start of therapy, dominant site of relapse and disease-free interval for the RT-qPCR data set. To assess the prognostic value of the discovered genes, we incorporated also 3 series (VII-IX) of ER+ tumors from 517 LNN breast cancer patients, who did not receive any type of adjuvant systemic therapy. Details regarding the application of the above classifiers are provided in the Supplementary data file, Tables A.1 and A.2.

The discovered predictive genes were also evaluated for their protein expression pattern in a tissue microarray including cores of ER-positive primary tumor specimens from a cohort of advanced breast cancer patients who have been treated with first-line tamoxifen previously described (Reijm et al., 2014). A subset of 110 ER-positive tumors were explored for their protein staining, i.e. the number of positive cells and the staining intensity, and a staining IHC-score was calculated to evaluate the relationship between IHC-score and progression-free survival.

METHODS

RNA isolation and (genome-wide) expression profiling

RNA isolation for the samples retrieved from each of the participating centers (TCRU, EMC, NKI and IPC) and quality control was done as described before (Bekhouche et al., 2011; Jansen et al., 2005, 2013; Kok et al., 2009; Van Laere et al., 2007a). Genome-wide expression profiles were available from Affymetrix HGU133A or HGU133plus2 platforms (I–III, VII–IX) and 44k mRNA oligoarrays of Agilent Technologies (IV–V).

Expression analyses were verified by RT-qPCR (series VI) and were performed for the "IBC-like" genes (i.e. *ABAT, ADAMDEC1, CLEC7A, ETS1, ITK* and *STC2*) to discriminate between IBC and nIBC, for the Recurrence Score genes (i.e. *AURKA, BAG1, BCL2, BIRC5, CCNB1, CD68, CTSL2, ERBB2, ESR1, GRB7, GSTM1, MKI67, MMP11, MYBL2, PGR* and *SCUBE2*) and for a panel of reference genes (i.e. *HMBS, HPRT1, TBP* and *B2M*). Assay details are provided in Supplementary Table A.1. The cDNA synthesis, quantification and the methodology to ensure PCR specificity have been described previously (Sieuwerts et al., 2005; van Agthoven et al., 2009). RT-qPCR was performed in a Mx3000P™ Real-Time PCR System (Agilent, Amsterdam, The Netherlands) using the TaqMan-based gene expression assays from Applied Biosystems/Life Technologies and SYBR-based intron-spanning forward and reverse primer combinations for the other genes. Levels of the target genes, expressed relative to the reference genes were quantified as follows: mRNA target = $2^{(\text{mean Ct reference genes} - \text{mean Ct target genes})}$.

Expression levels of each series (Supplementary Table A.2) were normalized and subsequently harmonized for cross-platform evaluation and robust regression analyses. To accomplish harmonization of series, Hampel'S M-Estimators were calculated in SPSS (version 20) for all series and applied to establish the harmonization factor for the genes in each series when using series I as reference (Supplementary Table A.3).

Comparative analysis or ER+ IBC and nIBC expression profiles

Global differences in gene expression between samples from patients with and without ER+ IBC in the discovery series were analyzed using the global test (Goeman et al., 2004) and Principal Component Analysis (PCA). Using the PAM50-algorithm, each sample in the discovery series was classified according to the molecular subtypes, ER activity, and Risk-Of-Relapse (ROR) models based on the molecular subtypes alone (ROR-S) or in combination with cell proliferation (ROR-P), as described before (Ellis et al., 2011). In addition, the Recurrence Score (RS) (Paik et al., 2004) and the HOXB13/IL17RB gene expression ratio (Jansen et al., 2007; Ma et al., 2006) were calculated.

Biomarker discovery analysis

We performed Prediction Analysis of Microarrays (PAM) (Tibshirani et al., 2002) to identify a series of biomarkers able to discriminate ER+ IBC from ER+ nIBC samples. The discovery series were randomly divided into training sets of 40 samples and test sets of 25 samples to obtain 10 gene signatures, which were compared to identify common classifiers. In total six classifier genes were shared between these 10 gene signatures, i.e. *ABAT, ADAMDEC1, CLEC7A, ETS1, ITK* and *STC2*. PCA was performed onto the ER+ IBC discovery and validation series to evaluate the discriminatory performance of all genes and of the six common classifier genes together. Mutual relationships between these common classifier genes and ER were investigated using the Ingenuity Pathway Analysis (IPA) software.

Construction of an ER+ IBC-like metagene

The thus identified six genes *ABAT, ADAMDEC1, CLEC7A, ETS1, ITK* and *STC2* were combined into an ER+ IBC-like metagene. The regression coefficients of each of the genes obtained within the discovery series were used to calculate a score for the metagene in all other series. This metagene score was evaluated as biomarker representing the signature of above six genes.

Diagnostic evaluation of biomarkers and classifiers

The IBC discovery and test sets (series I & II) were used to assess the predictive potential of the biomarkers to identify IBC and nIBC. The biomarkers were evaluated as continuous variables with Receiver Operator Characteristic (ROC) analyses using the STATA statistical package. The ROC analyses were performed to define Area Under Curves (AUC) and assess the discriminatory potential of the biomarkers. Next, ROC analyses were used to select cutoffs with optimal sensitivity and specificity. These cutoffs generated dichotomized biomarkers which were subsequently explored as classifiers using distribution dot-plots created in STATA and evaluated for their diagnostic effectiveness by SISA (http://www.quantitativeskills.com/sisa/). The distribution dot-plots illustrated the performance and the number of false positives/negatives when applying the cutoffs. The SISA tool established for the classifiers their accuracy, sensitivity, specificity and Youden's Index of the predictions.

Survival analysis of biomarkers and classifiers

The biomarkers were evaluated for their relationship with survival using Cox regression analyses in two ways: as a continuous variable or dichotomized to a threshold as classifier to distinguish “IBC-like” and “nIBC-like”. For the assessment of the relationship with first-line therapy outcome in advanced disease, Progression-Free Survival (PFS), defined as the time elapsed between initiation of endocrine therapy and the first detection of disease progression, was considered as endpoint. PFS was censored at 36 months. For the assessment of the relationship with prognosis and adjuvant therapy in early disease, Metastasis-Free Survival (MFS) was used as endpoint and defined as the time elapsed between the date of diagnosis and the date of distant metastatic relapse. Multivariate Cox regression analyses were performed on each of the endocrine treated advanced disease subsets for PFS (series **IV–VI**). The models included the biomarkers as continuous variable on the one hand and the published signatures for the Recurrence Score or the GGI on the other hand. Additional multivariate analyses were performed with the base model of clinic-pathological factors including age and menopausal status at start of therapy, dominant site of relapse, disease free interval (DFI), and the mRNA expression levels of ER (*ESR1*), PR (*PGR*), and HER2 (*ERBB2*). All data computations were done with the STATA statistical package version 12.0. All P-values are two-sided and $P < 0.05$ was considered statistically significant.

Tissue microarrays and immunohistological and evaluation

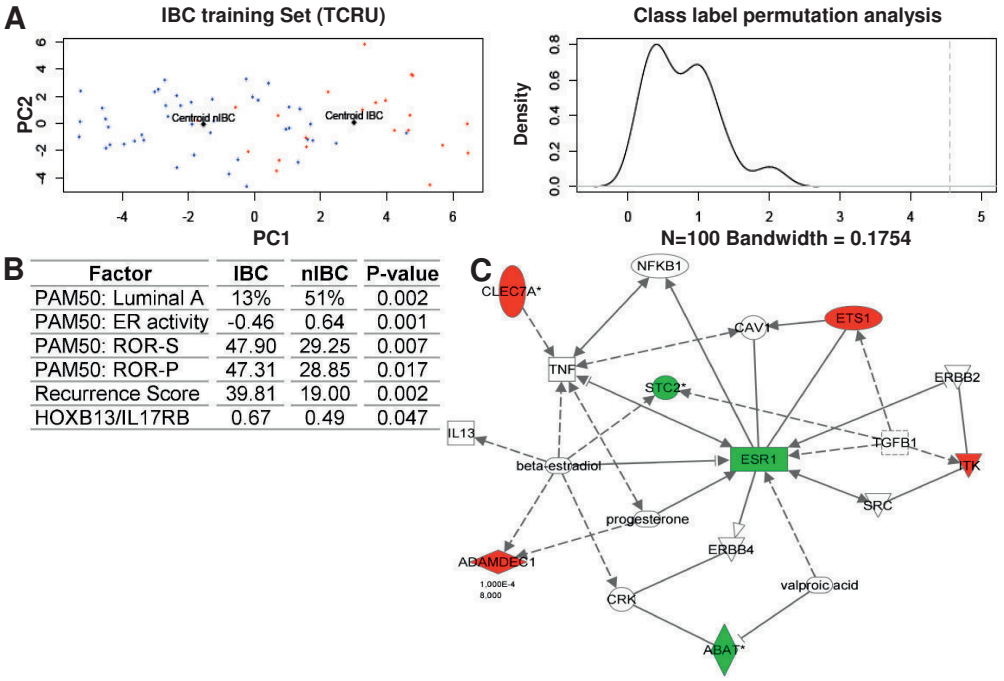
Tissue microarrays (TMAs) of formalin-fixed, paraffin-embedded primary breast tumor specimens were prepared and immunohistochemically stained according to the procedures described previously (Reijm et al., 2014). The staining was performed with

the primary monoclonal antibody against ABAT (HPA041690) and STC2 (HPA045372; Atlas Antibodies AB, Stockholm, Sweden). The antibodies were incubated for 1 h (1:100 dilution) after 20 min antigen retrieval at pH6.0. Subsequently, the TMA-slides were incubated with a secondary antibody and staining was visualized using diaminobenzidine (DAB). ABAT and STC2 protein staining was scored for quantity and intensity. Staining was grouped into standardized categories for the percentage of staining positive cells (0%, 1–20%, 21–50%, 51–75%, or 76–100% of positive cells) and for staining intensity (negative, weak, moderate, strong). Subsequently, the scores for quantity and intensity were multiplied to generate a staining IHC-score. Based on the IHC-score, a specimen was classified as negative or positive for ABAT and STC2 protein expression.

RESULTS

Comparative analysis of ER+ IBC and nIBC expression profiles

Using global test analysis and PCA, we observed that ER+ samples from patients with and without IBC exhibited significant differences in their expression profiles that led to segregation of IBC and nIBC samples in the PCA plot of the discovery series (Figure 1A). Evaluation of the Recurrence Score, the HOXB13/IL17RB expression ratio and PAM50-derived scores for molecular subtypes, ER activity, risk of relapse models (ROR-S and ROR-P), indicated decreased ER signaling and sensitivity to endocrine treatment for ER+ samples from IBCs compared to those of nIBCs (Figure 1B).



Factor	IBC	nIBC	P-value
PAM50: Luminal A	13%	51%	0.002
PAM50: ER activity	-0.46	0.64	0.001
PAM50: ROR-S	47.90	29.25	0.007
PAM50: ROR-P	47.31	28.85	0.017
Recurrence Score	39.81	19.00	0.002
HOXB13/IL17RB	0.67	0.49	0.047

Figure 1 IBC discriminatory genes. Evaluation of IBC and nIBC in the discovery series I (Figure 1**A**–**B**) and of ER+ IBC discriminatory genes (*ABAT, ADAMDEC1, CLEC7A, ETS1, ITK, STC2*) (Figure 1**C**). Figure 1**A** illustrates the Principle Component Analyses of the tumor samples by their gene expression profiles. Red dots denote ER+ IBC samples, blue dots denote ER+ nIBC samples. The centroids for both tumor

phenotypes are indicated in black and labeled respectively “Centroid IBC” and “Centroid nIBC”. PCA for the common 6 classifier genes showed an expected segregation of ER+ samples from patients with and without IBC on the 2D scatter plot representation of the 1st (X-axis) and the 2nd (Y-axis) principal component. Class label permutation analyses (applying 100 class label permutations) demonstrated that the centroids of the ER+ samples from patients with and without IBC are significantly segregated (Observed Euclidean distance = 4.555, average expected Euclidean distance = 0.890; $P < 0.010$). Figure 1**B** presents the results on PAM50 analyses, Recurrence score, and HOXB13/IL17RB. For PAM50, the percentage Luminal A-type tumors in IBC and nIBC is provided in addition to the ROR-S, ROR-P and ER activity scores. The ER activity score ranges from negative to positive, with negative values indicating repressed ER activity. In addition, the RS and the HOXB13/IL17RB gene expression ratio are provided for both tumor types. The reported P-values result from the comparison of the IBC and nIBC groups with respect to these variables. Figure 1**C** depicts the network obtained for the 6 IBC discriminatory genes together with the estrogen receptor-α (*ESR1*) when evaluated with Ingenuity Pathway Analyses. This exploratory analysis revealed interactions with hormone receptor signaling, inflammation, cell survival, epidermal growth factor signaling, stem cell signaling and TGFβ signaling, indicating a potential involvement for each of these biological features in endocrine resistance. The molecules are color-coded red if the corresponding gene is overexpressed in ER+ IBC samples and green if the corresponding gene is repressed in ER+ IBC samples. Uncolored nodes are added by the software. Solid lines signify direct gene–gene interactions, whereas broken lines represent indirect relationships that may require secondary effectors not depicted in the network. All connections are supported by at least one published report or from canonical information stored in the Ingenuity Pathway Knowledge Base.

Biomarker discovery analysis

As shown above, ER+ IBC samples exhibited molecular characteristics of resistance to endocrine therapy, making their gene expression profiles a potential source for biomarker discovery. Using PAM on 10 alternatively composed training sets of 40 randomly selected ER+ samples from the discovery set (series I), we generated 10 distinct gene signatures distinguishing IBC from nIBC samples. Application of these gene signatures onto corresponding series of the 25 left-out samples revealed an average sensitivity of 89% (range 71%–100%), specificity of 80% (range 67%–100%) and test error rate of 18% (range 0%–28%). These 10 gene signatures had 6 overlapping genes: *ABAT, ADAMDEC1, CLEC7A, ETS1, ITK1* and *STC2*. Relative to nIBC, *ABAT* and *STC2* expression levels were decreased in IBC whereas the levels of the remaining 4 genes were increased (Table 2 and Figure 2). Exploratory IPA analysis was performed to investigate mutual relationships between the 6 classifier genes and ER (Figure 1C) and suggested their potential involvement in endocrine therapy resistance.

Table 2 ER+ IBC discriminatory genes. The expression levels in IBC and nIBC, significance and regression coefficients for the genes identified in the biomarker discovery analysis

ER+ IBC Metagene				
Genes	**Median Expression level**		**P-value**	**Regression Coefficient**
	nIBC (N=43)	IBC (N=22)		
ABAT	10.63	8.64	9.19E-08	-0.295
ADAMDEC1	6.26	9.07	1.31E-03	0.566
CLEC7A	7.24	8.12	9.55E-03	0.270
ETS1	4.09	4.99	7.09E-05	0.112
ITK	5.27	7.09	2.49E-04	0.332
STC2	10.80	7.64	1.70E-04	-0.630

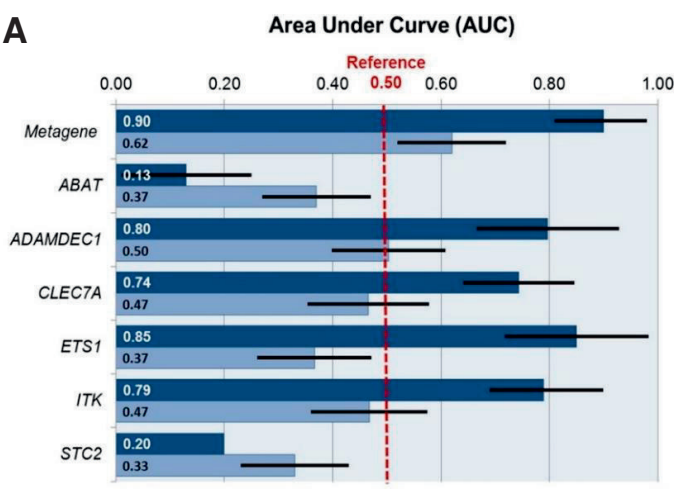
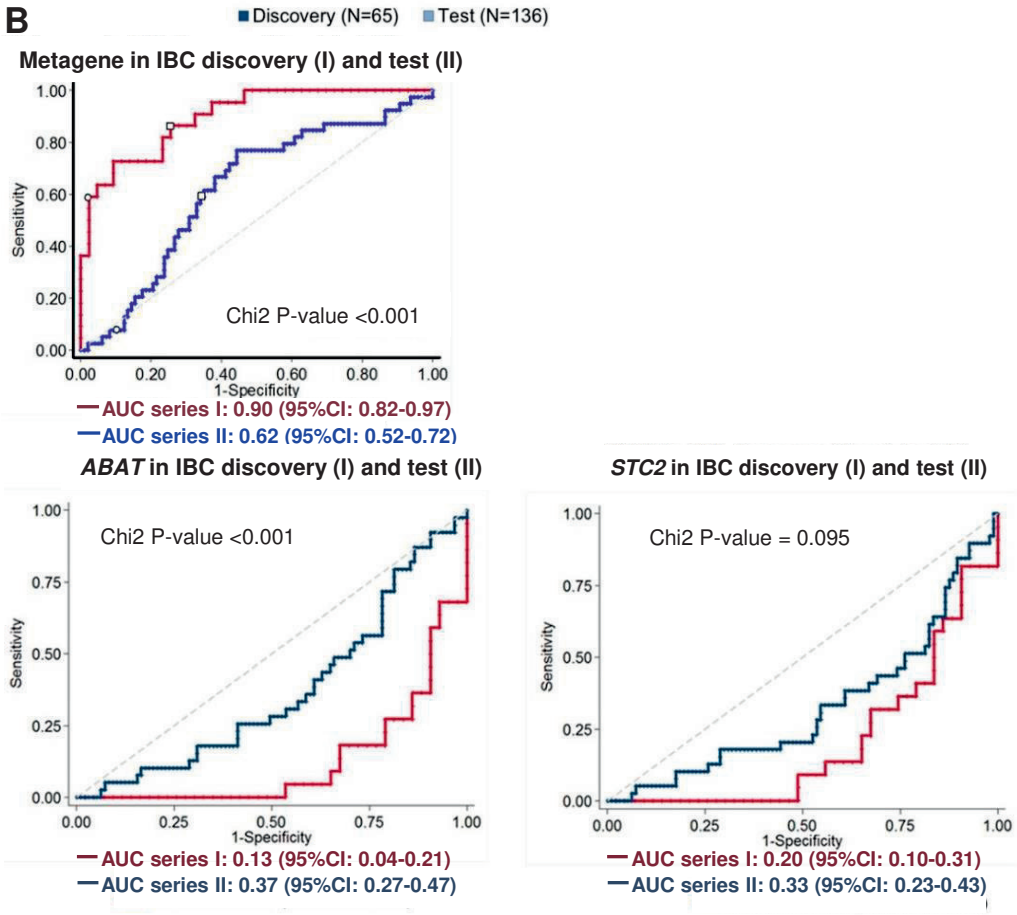


Figure 2 Receiver Operator Characteristics (ROC) Analyses. The ROC-analyses generate Area Under Curve (AUC) values presented in Figure 2**A** as measure for the discriminatory potential of the individual genes to predict IBCs and nIBCs correctly within the test (series II) compared to the discovery (series I). Factors with AUC (or their intervals) value 0.5 are not informative. The results show as illustrated with ROC plots in Figure 2**B** that AUCs for only the metagene, *ABAT* and *STC2* are discriminatory and comparable for the discovery and test.



Diagnostic effectiveness of biomarkers and classifiers

Next, these six genes were evaluated for their potential to distinguish IBC from nIBC in an independent subset of patients with and without IBC (series II). Only the AUCs for the metagene score, *ABAT*, and *STC2* were discriminatory and comparable in both the discovery and test cohort (Figure 2). The ROC AUCs of these three biomarkers were further explored to establish optimal classifier cutoffs. Distribution dot-plots in the discovery cohort were used to verify the ER+ IBC classification thresholds, which were set for the metagene score at ≥0.0 and for both *ABAT* and *STC2* at ≤ 10.0 (Figure 3A, C and E). The classifiers, however, exhibited moderate diagnostic effectiveness with regard to ER+ IBC prediction, since only a maximum of 64% accuracy was achieved within the test (Figure 3B, D and F).

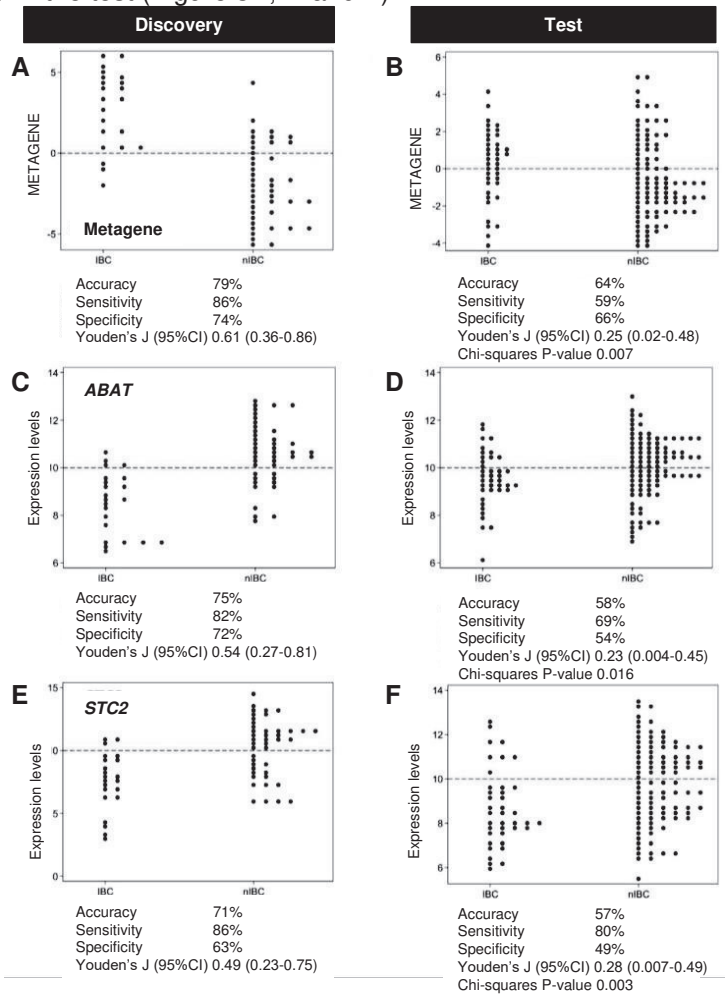


Figure 3 Dot-plots and diagnostic effectiveness. This figure represents dot-plots and the diagnostic performance of the biomarkers in the discovery and test series for IBC and nIBC (series I and II). The metagene scores and expression levels of *ABAT* and *STC2* measured in the discovery (series I) were evaluated in dot-plots to explore the defined thresholds that classify samples as IBC-like or nIBC-like. The diagnostic effectiveness of the biomarker IBC classification were evaluated in the independent test (series II).

Biomarkers in early disease: prognosis and adjuvant tamoxifen

The metagene score, *ABAT*, and *STC2* were subsequently evaluated as continuous variable for their relationship with MFS with regard to prognosis and outcome after adjuvant tamoxifen (Table 3). The prognostic value was determined on three series of in total 517 (neo)adjuvant systemic treatment naïve patients with ER+ LNN breast cancer (series VII–IX). None of the biomarkers, assessed by microarrays, were prognostic when evaluated for all 517 patients. The biomarkers were also evaluated in 250 ER+ patients treated with adjuvant tamoxifen (series III). Decreased expression of *ABAT* (HR = 0.73; 95% CI = 0.61–0.87; P = 0.001) showed a significant correlation with poor MFS in these tamoxifen treated patients, whereas *STC2* and the metagene score had no association with MFS after tamoxifen.

Table 3 Biomarkers and outcome. This table provides the results of the univariate Cox regression analyses performed for MFS and PFS to determine the prognostic and predictive value of the metagene, *ABAT* and *STC2* in the different patient series

				Biomarkers as continuous variable:					
				Metagene score		*ABAT*		*STC2*	
Clinical setting	Series	N	outcome[a]	HR (95%CI)[b]	P	HR (95%CI)	P	HR (95%CI)	P
Early Disease									
Prognosis	VII-IX	517	MFS	0.99 (0.92-1.08)	0.967	0.93 (0.82-1.06)	0.298	1.01 (0.92-1.11)	0.867
Adjuvant tamoxifen	III	250	MFS	1.09 (0.99-1.20)	0.084	0.73 (0.61-0.87)	0.001	0.93 (0.83-1.04)	0.216
Advanced Disease									
First-line tamoxifen	IV	96	PFS	1.13 (1.03-1.25)	0.012	0.80 (0.69-0.93)	0.004	0.90 (0.79-1.03)	0.117
First-line aromatase inhibitors	V	84	PFS	1.28 (1.13-1.45)	<0.001	0.74 (0.60-0.91)	0.004	0.76 (0.66-0.88)	<0.001
First-line tamoxifen	VI	231	PFS	1.09 (1.04-1.14)	<0.001	0.85 (0.80-0.92)	<0.001	0.93 (0.88-0.98)	0.004

[a] Outcome defined by metastasis free survival (MFS) or by progression free survival (PFS).
[b] Hazard Ratio (HR) and its 95% confidence interval (CI) for MFS in early disease and for PFS in advanced disease.

Biomarkers and first-line endocrine therapy for advanced disease

The metagene score, *ABAT*, and *STC2* were also evaluated for their relation with PFS on microarray based series of patients with advanced disease treated with first-line tamoxifen (IV, N = 96) or aromatase inhibitors (V, N = 84), and validated with RT-qPCR on an independent series of patients treated with first-line tamoxifen (VI, N = 231). As continuous variables, increased metagene scores and decreased expression of *ABAT* and *STC2* were correlated with poor treatment outcome in all three series of patients, except for *STC2* in series IV (Table 3). As classifiers, apart from *STC2* in series IV, all three biomarkers showed significant associations with PFS for all series in the Kaplan–Meier survival analyses (Figure 4).

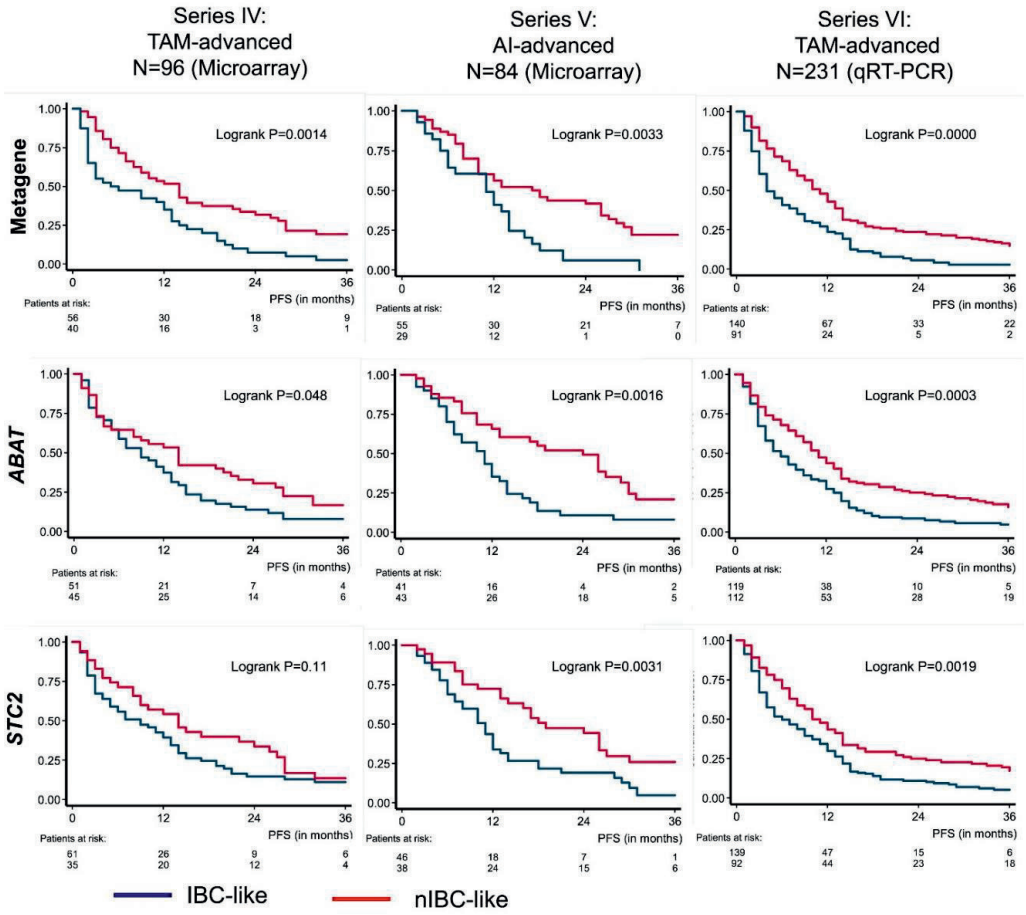


Figure 4 Kaplan–Meier Analyses for outcome after endocrine treatment. The metagene, *ABAT* and *STC2* as IBC/nIBC classifiers and their relation with PFS as measure for treatment outcome in advanced disease after first-line tamoxifen (series IV and VI) and aromatase inhibitors (series V).

Biomarkers and published signatures

The biomarkers were compared for their relation with PFS in advanced disease (series **IV, V, VI**) with published signatures for Recurrence Score and GGI (Table 4). When compared to Recurrence Score and GGI only *ABAT* remained significantly associated with PFS in all series (except for GGI in series V). In contrast, the metagene score and *STC2* were only independent from Recurrence Score and GGI in AI-treated patients (series V). In summary, especially *ABAT* expression levels were independently associated with PFS when compared to published signatures separately and validated with RT-qPCR.

Table 4 Biomarkers and published signatures in advanced disease. The metagene, *ABAT* and *STC2* were compared with the published signatures for Recurrence Score and Genomic Grade Index (GGI) for their relationship with PFS in advanced disease after treatment with tamoxifen (series IV and VI) or aromatase inhibitors (series V)

	Univariate			Bivariate (biomarker together with published signature evaluated)					
				Metagene score		*ABAT*		*STC2*	
Signature	**N**	**HR (95% CI)**	**P**	**HR (95% CI)**	**P**	**HR (95% CI)**	**P**	**HR (95% CI)**	**P**
Series IV. Tam (N = 96)									
Recurrence Score									
low	35	1.00		1.11 (0.99–1.24)	0.085	0.84 (0.71–0.99)	0.036	0.94 (0.82–1.09)	0.430
moderate	12	1.01 (0.49–2.08)	0.977						
high	49	1.61 (1.00–2.60)	0.050						
GGI	96	0.99 (0.90–1.10)	0.899	1.14 (1.03–1.26)	0.010	0.80 (0.69–0.93)	0.005	0.90 (0.78–1.03)	0.113
TAM78	62	2.34 (1.34–4.11)	0.003	1.11 (0.98–1.27)	0.102	0.86 (0.71–1.04)	0.130	0.94 (0.79–1.11)	0.455
ROR-S									
low	33	1.00		1.10 (0.97–1.24)	0.141	0.85 (0.72–1.01)	0.065	0.96 (0.83–1.10)	0.534
moderate	30	1.42 (0.82–2.46)	0.216						
high	33	1.93 (1.14–3.25)	0.014						
ROR-P									
low	23	1.00		1.13 (1.01–1.26)	0.039	0.80 (0.67–0.96)	0.018	0.91 (0.78–1.06)	0.230
moderate	37	0.93 (0.53–1.65)	0.816						
high	36	1.34 (0.77–2.33)	0.307						
Series V. AI (N = 84)									
Recurrence Score									
low	18	1.00		1.23 (1.08–1.42)	0.003	0.77 (0.62–0.95)	0.013	0.79 (0.67–0.93)	0.004
moderate	7	1.85 (0.64–5.36)	0.259						
high	59	2.86 (1.34–6.07)	0.006						
GGI	84	3.64 (1.87–7.08)	<0.001	1.19 (1.03–1.38)	0.019	0.84 (0.67–1.05)	0.124	0.84 (0.71–0.99)	0.043
TAM78	84	1.80 (1.08–3.00)	0.023	1.26 (1.11–1.44)	0.001	0.74 (0.60–0.93)	0.008	0.76 (0.65–0.88)	<0.001
ROR-S									
Low	24	1.00		1.19 (1.03–1.37)	0.016	0.82 (0.66–1.03)	0.087	0.83 (0.71–0.97)	0.018
moderate	31	1.20 (0.61–2.36)	0.603						
high	29	3.20 (1.65–6.21)	0.001						
ROR-P									
low	18	1.00		1.19 (1.03–1.38)	0.017	0.80 (0.64–1.00)	0.050	0.82 (0.71–0.96)	0.015
moderate	38	1.79 (0.84–3.82)	0.132						
high	28	3.94 (1.81–8.59)	0.001						
Series VI. Tam (N = 231), qRT-PCR									
Recurrence Score									
low	65	1.00		1.05 (0.99–1.11)	0.134	0.88 (0.80–0.97)	0.011	0.96 (0.90–1.02)	0.196
moderate	13	1.90 (1.00–3.60)	0.048						
high	131	2.39 (1.70–3.37)	<0.001						
GGI	226	1.53 (1.29–1.81)	<0.001	1.06 (1.01–1.12)	0.032	0.89 (0.81–0.97)	0.009	0.94 (0.89–1.01)	0.057

Biomarkers and clinicopathological predictors

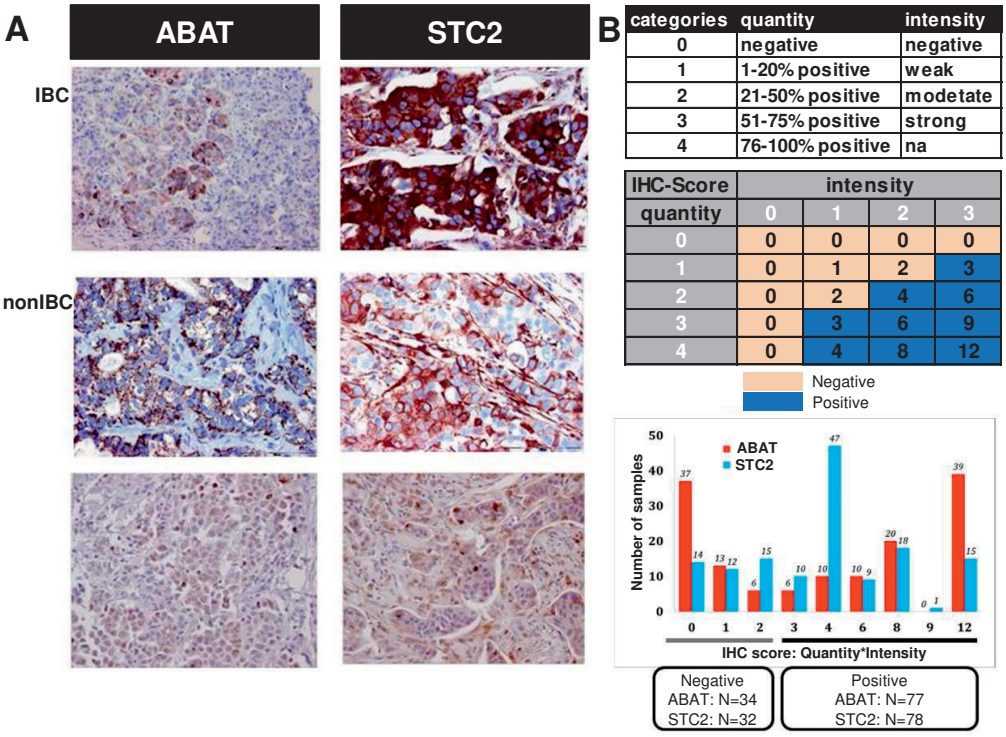
Multivariate analyses were performed by adding the biomarkers separately to a base model of traditional clinicopathological factors for endocrine therapy in advanced disease (Table 5). The model included age, menopausal status, dominant site of relapse, disease-free interval and mRNA expression levels for *ESR1, PGR*, and *ERBB2*. These multivariate analyses showed that low *ABAT* levels were significantly related with poor PFS in the series of 96 patients treated with tamoxifen (series **IV** (HR = 0.78, P = 0.027)), whereas high metagene scores (HR = 1.24, P = 0.005) and low *STC2* levels (HR = 0.79, P = 0.011) were associated with poor PFS in the series of 84 patients treated with AI (series **V**). All three biomarkers were independently related with PFS in the RT-qPCR validation series of 231 patients treated with tamoxifen (series **VI**).

Table 5 Biomarkers and clinico-pathological factors in advanced disease. This table provides the results of the uni- and multivariate Cox regression analyses for PFS performed on the advanced disease patient series (series IV–VI). The biomarkers were separately added to the base model of clinico-pathological factors in multivariate analysis

Clinico-pathological Factors	Series IV. Tam (N=96)					Series V. AI (N=84)					Series VI. Tam (N=231), qRT-PCR				
	N	Univariate (N=96)		Multivariate (N=91)		N	Univariate (N=84)		Multivariate (N=82)		N	Univariate (N=231)		Multivariate (N=231)	
		HR (95% CI)	P	HR (95% CI)	P		HR (95% CI)	P	HR (95% CI)	P		HR (95% CI)	P	HR (95% CI)	P
Age															
<55	34	1.00		1.00		18	1.00		1.00		91	1.00		1.00	
56-70	43	0.75 (0.46-1.22)	0.250	1.09 (0.43-2.79)	0.855	32	1.07 (0.56-2.05)	0.839	0.86 (0.36-2.02)	0.727	81	0.78 (0.57-1.07)	0.123	0.83 (0.53-1.31)	0.427
>70	19	0.90 (0.49-1.65)	0.742	1.21 (0.44-3.38)	0.712	34	0.75 (0.38-1.46)	0.390	0.59 (0.23-1.48)	0.260	59	0.69 (0.48-0.98)	0.038	0.69 (0.43-1.12)	0.134
Menopausal status															
Premenopausal	29	1.00		1.00		7	1.00		1.00		61	1.00		1.00	
Postmenopausal	67	0.81 (0.51-1.30)	0.388	0.99 (0.39-2.54)	0.988	76	0.99 (0.43-2.30)	0.978	1.29 (0.41-4.04)	0.662	170	0.80 (0.59-1.09)	0.155	1.14 (0.72-1.80)	0.586
Dominant site of relapse															
Bone	41	1.00		1.00		46	1.00		1.00		120	1.00		1.00	
LRR or viscera	53	0.81 (0.52-1.26)	0.345	0.78 (0.46-1.32)	0.357	38	0.79 (0.48-1.31)	0.367	0.86 (0.49-1.49)	0.586	111	0.91 (0.69-1.20)	0.518	0.93 (0.70-1.23)	0.600
Disease Free Interval															
<1 year	17	1.00		1.00		14	1.00		1.00		65	1.00		1.00	
2-3 years	24	0.43 (0.22-0.84)	0.013	0.43 (0.21-0.88)	0.021	28	0.64 (0.32-1.27)	0.204	0.84 (0.39-1.79)	0.650	100	0.58 (0.42-0.80)	0.001	0.55 (0.40-0.77)	<0.001
>3 years	52	0.36 (0.20-0.64)	0.001	0.33 (0.18-0.63)	0.001	41	0.38 (0.19-0.75)	0.005	0.44 (0.21-0.91)	0.027	66	0.47 (0.32-0.68)	<0.001	0.46 (0.32-0.67)	<0.001
mRNA levels as continuous variable															
ESR1	96	0.84 (0.73-0.95)	0.007	0.81 (0.68-0.96)	0.016	84	0.67 (0.32-1.40)	0.285	1.03 (0.46-2.35)	0.935	231	0.91 (0.85-0.97)	0.003	0.93 (0.86-1.00)	0.037
PGR	95	0.94 (0.86-1.03)	0.217	0.99 (0.88-1.10)	0.807	84	0.55 (0.37-0.80)	0.002	0.61 (0.40-0.94)	0.026	231	0.90 (0.84-0.97)	0.004	0.92 (0.85-1.00)	0.043
HER2	96	1.05 (0.89-1.25)	0.550	1.13 (0.94-1.37)	0.198	84	1.17 (0.69-1.98)	0.563	1.40 (0.79-2.50)	0.253	231	1.13 (1.00-1.27)	0.044	1.07 (0.95-1.20)	0.279
				Added to the model					Added to the model					Added to the model	
Metagene score	96	1.29 (1.13-1.46)	<0.001	1.09 (0.95-1.24)	0.210	84	1.28 (1.13-1.45)	<0.001	1.24 (1.07-1.44)	0.005	231	1.09 (1.04-1.14)	<0.001	1.08 (1.02-1.14)	0.009
ABAT	96	0.80 (0.69-0.94)	0.005	0.78 (0.63-0.97)	0.027	84	0.74 (0.60-0.91)	0.004	0.83 (0.65-1.05)	0.127	231	0.85 (0.80-0.92)	<0.001	0.87 (0.79-0.96)	0.004
STC2	96	0.90 (0.79-1.03)	0.117	0.99 (0.83-1.18)	0.903	84	0.76 (0.66-0.88)	<0.001	0.79 (0.67-0.95)	0.011	231	0.93 (0.88-0.98)	0.004	0.93 (0.87-0.99)	0.017

Biomarkers and protein expression

In an exploratory study, ABAT and STC2 protein expression were examined in 110 ER-positive primary breast cancer specimens (Figure 5A). Evaluation of quantity and intensity separately showed for both proteins no significant relationships with PFS (Supplementary Figure 1). The quantity and intensity scores were multiplied to generate an IHC-score for the staining and classified 77 specimens (69%) as ABAT-positive and 78 specimens (70%) as STC2-positive (Figure 5B). These dichotomized IHC-scores were not related with PFS, i.e. not for ABAT (HR = 0.79; 95% CI = 0.51–1.23; P = 0.30) and not for STC2 (HR = 0.93; 95% CI = 0.60–1.44; P = 0.74).



categories	quantity	intensity
0	negative	negative
1	1-20% positive	weak
2	21-50% positive	modetate
3	51-75% positive	strong
4	76-100% positive	na

IHC-Score	intensity			
quantity	0	1	2	3
0	0	0	0	0
1	0	1	2	3
2	0	2	4	6
3	0	3	6	9
4	0	4	8	12

Figure 5 ABAT and STC2 protein expression. The expression of ABAT and STC2 protein was evaluated with immunohistochemistry in 110 ER-positive primary tumor specimens of advanced breast cancer patients treated with first-line tamoxifen. In Figure 5**A** representative samples are shown for ABAT and STC2 staining in IBC and nIBC patients. Figure 5**B** demonstrates the staining categories for quantity, intensity, and IHC-scores, and the distribution of IHC-scores for ABAT and STC2. The IHC-scores were dichotomized into positive and negative scores, identifying 77 ABAT-positive and 78 STC2-positive specimens. Both ABAT and STC2 protein expression had no relationship with progression free survival.

DISCUSION

Inflammatory breast cancer is a rare (~5%) but highly aggressive form of locally advanced breast cancer with an elevated invasive and metastatic potential. It is characterized by clinical and pathological characteristics atypical for breast cancer in general, amongst others a low frequency of ER positivity. In the past, we showed that the molecular portrait of IBC indeed contained fingerprints of aggressive tumor cell

behavior in breast cancer in general (Van Laere et al., 2008). Patients with IBC bearing ER expressing tumor cells constitute approximately 30% of all IBC cases and endocrine treatment in these patients is observed to be poorly effective. The molecular profile of samples from patients with ER+ IBC could provide additional hints towards unraveling the molecular biology associated with resistance to endocrine treatment. In this study, we demonstrate that, at least at the molecular level, ER+ IBC is characterized by features associated with endocrine resistance. For instance, the recurrence score and the HOXB13/IL17RB gene expression ratio are both significantly elevated in ER+ IBC compared with ER+ nIBC. Several studies have shown that elevated levels for both parameters are highly predictive of endocrine therapy resistance (Dowsett et al., 2010; Jansen et al., 2007; Ma et al., 2004; Paik et al., 2004). In addition, application of the PAM50-algorithm (Tibshirani et al., 2002) revealed a remarkably low frequency of Luminal A-type samples, which are shown to be more frequently responsive to endocrine treatment compared with their Luminal B-type counterparts. This hypothesis is supported by the observation that samples from tumors with a Luminal B-phenotype frequently exhibit high Recurrence Scores (Fan et al., 2006).

Using repetitive prediction analysis to obtain robust predictors, we identified a metagene of six genes consisting of *ABAT, ADAMDEC1, CLEC7A, ETS1, ITK* and *STC2* to discriminate ER+ IBC from ER+ nIBC samples within the discovery series. These biomarkers were each verified and demonstrated that only the metagene and the genes *ABAT* and *STC2* remained predictive in the test series. The metagene is a slightly better predictor than the single genes, however, its performance in the other series was largely dictated by *ABAT* and *STC2*. It is intriguing that *ABAT* and *STC2*, as 2 genes not yet linked to inflammation, discriminate between ER+ IBC and nIBC. A role in the inflammatory response, however, is less likely to be established since both *ABAT* and *STC2* are down-regulated in IBC compared to nIBC.

Recently, both *ABAT* and *STC2* were described in the 100 rules used in the Absolute Intrinsic Molecular Subtyping (AIMS) (Paquet and Hallett, 2015). AIMS enables subtyping from gene expression profile at mRNA expression levels of an individual sample without the need of large, diverse, and normalized datasets. These findings indicate that both genes are highly relevant in molecular subtyping. Moreover, the molecular subtyping of breast cancer becomes more and more important in the clinical management of patients. The results of our study may therefore contribute with regard to endocrine treatment decision making.

Although our study demonstrated only that both *ABAT* and *STC2* are just biomarkers, literature suggests for both a role in ER signaling. *ABAT* (MIM: 137150) has been identified as a luminal-like gene with an ER-binding site within 20 kb distance from the transcription start site (Krijgsman et al., 2011). This gene is incorporated in Agendia's BluePrint assay, an 80-gene molecular subtyping profile developed in 200 breast cancer specimens and validated in four independent cohorts. *ABAT* encodes for 4-aminobutyrate aminotransferase, an enzyme responsible for the catabolism of gamma-aminobutyric acid (GABA), which might be involved in the hormonal regulation and pathogenesis of breast cancer (Opolski et al., 2000). Moreover, comparative metabolomics demonstrated alterations in glutamine and beta-alanine

metabolism along with low *ABAT* expression with shortened survival in ER+ and ER− breast cancer (Budczies et al., 2013).

Bouras and colleagues have shown that *STC2* (MIM: 603665) as an estrogen responsive gene is co-expressed with ER (Bouras et al., 2002). In fact, independent studies have indeed shown that *STC2* is a dynamic marker of estrogen-driven pathway activation and that constitutive expression after serum withdrawal negatively affects breast cancer cell growth, cell viability and cell migration (Raulic et al., 2008; Urruticoechea et al., 2008)). Therefore, reduced expression of *STC2* in breast cancer cells enables survival and cell growth in the absence of estrogen, thereby contributing to endocrine treatment resistance. Of note, in a recent effort to redefine the molecular portraits of IBC on an extended series of 137 samples, repressed *STC2* expression levels were observed in IBC in a molecular subtype-independent manner (Van Laere et al., 2010). Future studies are needed to provide functional evidence that *ABAT* and *STC2* are mechanistically involved in endocrine therapy response.

Based upon above considerations and findings we evaluated *ABAT* and *STC2* further in different datasets to determine their relationship with prognosis and treatment outcome to adjuvant and first-line endocrine therapy (i.e. tamoxifen and aromatase inhibitors). Both biomarkers were not prognostic, whereas only decreased levels of *ABAT* were associated with shorter MFS after adjuvant tamoxifen. In the advanced disease setting, decreased expression of *ABAT* and *STC2* characterized patients with reduced PFS under either tamoxifen- or AI-based endocrine therapy. Particularly in patients treated with first-line tamoxifen these “ER+ IBC-like” predictors were associated with sensitivity to endocrine treatment in an independent data set profiled with an alternative technology. The latter is important as it proves that determination of *ABAT* and *STC2* expression levels is also applicable with standard PCR technologies, making the “bench-to-bedside” transition more feasible. We also explored whether ABAT and STC2 protein expression might be applicable as predictive biomarkers in immunohistochemical assays, however, our findings showed no relationship with tamoxifen outcome.

Combining these biomarkers with clinico-pathological factors in multivariate analyses demonstrated that *ABAT* remained significantly associated with response to tamoxifen in two independent patient series, whereas the metagene and *STC2* was only independent within the qRT-PCR validation. Moreover, only ABAT remained independent predictive for tamoxifen in both patient series when combined with the published signatures Recurrence Score and GGI. All these multivariate analyses on different patient series summarized indicate *ABAT* as a robust predictor for the response to tamoxifen.

In conclusion, this study has identified an increased metagene score and decreased expression of *ABAT* and *STC2* in IBC, and correlated the metagene and low expression of the genes with poor tamoxifen treatment outcome in the advanced setting as shown with qRT-PCR in an independent validation. ABAT and STC2 protein expression were not informative with regard to treatment outcome. Further studies on the classifier genes are needed to elucidate the mechanism of therapy resistance.

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CHAPTER VI

Cell-free DNA mutations as biomarkers in breast cancer patients receiving tamoxifen

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ABSTRACT

The aim was to identify mutations in serum cell-free DNA (cfDNA) associated with disease progression on tamoxifen treatment in metastatic breast cancer (MBC). Sera available at start of therapy, during therapy and at disease progression were selected from 10 estrogen receptor (ER)-positive breast cancer patients. DNA from primary tumor and normal tissue and cfDNA from minute amounts of sera were analyzed by targeted next generation sequencing (NGS) of 45 genes (1,242 exons). At disease progression, stop-gain single nucleotide variants (SNVs) for *CREBBP* (1 patient) and *SMAD4* (1 patient) and non-synonymous SNVs for *AKAP9* (1 patient), *PIK3CA* (2 patients) and *TP53* (2 patients) were found. Mutations in *CREBBP* and *SMAD4* have only been occasionally reported in breast cancer. All mutations, except for *AKAP9*, were also present in the primary tumor but not detected in all blood specimens preceding progression. More sensitive detection by deeper re-sequencing and digital PCR confirmed the occurrence of circulating tumor DNA (ctDNA) and these biomarkers in blood specimens.

Keywords: breast cancer, tamoxifen therapy, targeted next generation sequencing, cell-free DNA, disease progression

INTRODUCTION

It is increasingly appreciated that the genetic make-up of tumors forms one of the main determinants for outcome to systemic treatments in cancer patients [1]. There is also accumulating evidence that primary tumor characteristics can greatly differ from those of the metastases [2]. This may underlie the relatively poor association of molecular characteristics of primary tumors with outcome in MBC. It is therefore likely that genetic variants important for treatment decision making should be determined in specimens from metastatic tumor rather than from primary tumor tissue. In addition, the genetic constitution of a tumor lesion is not fixed but constantly changes, in particular under treatment pressure. As novel DNA mutations can cause resistance to systemic treatments, longitudinal monitoring of these mutations during treatment is crucial to detect resistance at an early stage and, if possible, to adjust treatment based on the emerging mutations.

DNA from primary and metastatic tumor cells can be detected as cfDNA in the peripheral blood of cancer patients. This cfDNA is therefore a very attractive tool to establish mutational changes occurring in tumor cells in a minimal invasive manner. Its great promise in this respect was recently reviewed [3]. For example, in patients with metastatic *KRAS* wild-type colorectal cancer treated with an anti-EGFR antibody, blood analyses showed that the appearance of *KRAS* mutants, conferring resistance against anti-EGFR antibodies, preceded progressive disease with up to 10 months [4]. Likewise, in breast cancer patients mutations in the estrogen receptor (ESR1) have been hardly detected in primary tumors but are currently frequently reported in plasma from patients with metastatic disease that acquired resistance to aromatase inhibitor therapy [5-8]. In the current study, we aimed to identify tumor-specific mutations in cfDNA that associate with disease progression on tamoxifen in MBC.

RESULTS

Detection of DNA changes

As we were interested in cfDNA mutations that potentially associate with treatment outcome in a particular patient, we characterized DNA changes in serum taken at disease progression in 10 MBC patients who received first-line tamoxifen (Sumpplementary Table S1) . After applying our selection criteria on called variants, 18 cfDNA changes were identified at disease progression which were not detected in normal tissue DNA nor reported by the 1000 Genome database. Of these, 3 variants were only seen in blood specimens and not in the corresponding primary tumor whereas 15 variants were also detectable in the primary tumor. Twelve DNA changes in 6 patients were shown to associate with treatment outcome (Figure 1) Of these, 9 tumor-specific DNA changes were present at disease progression and in the primary tumor but not in all blood specimens preceding progression (Table 1)

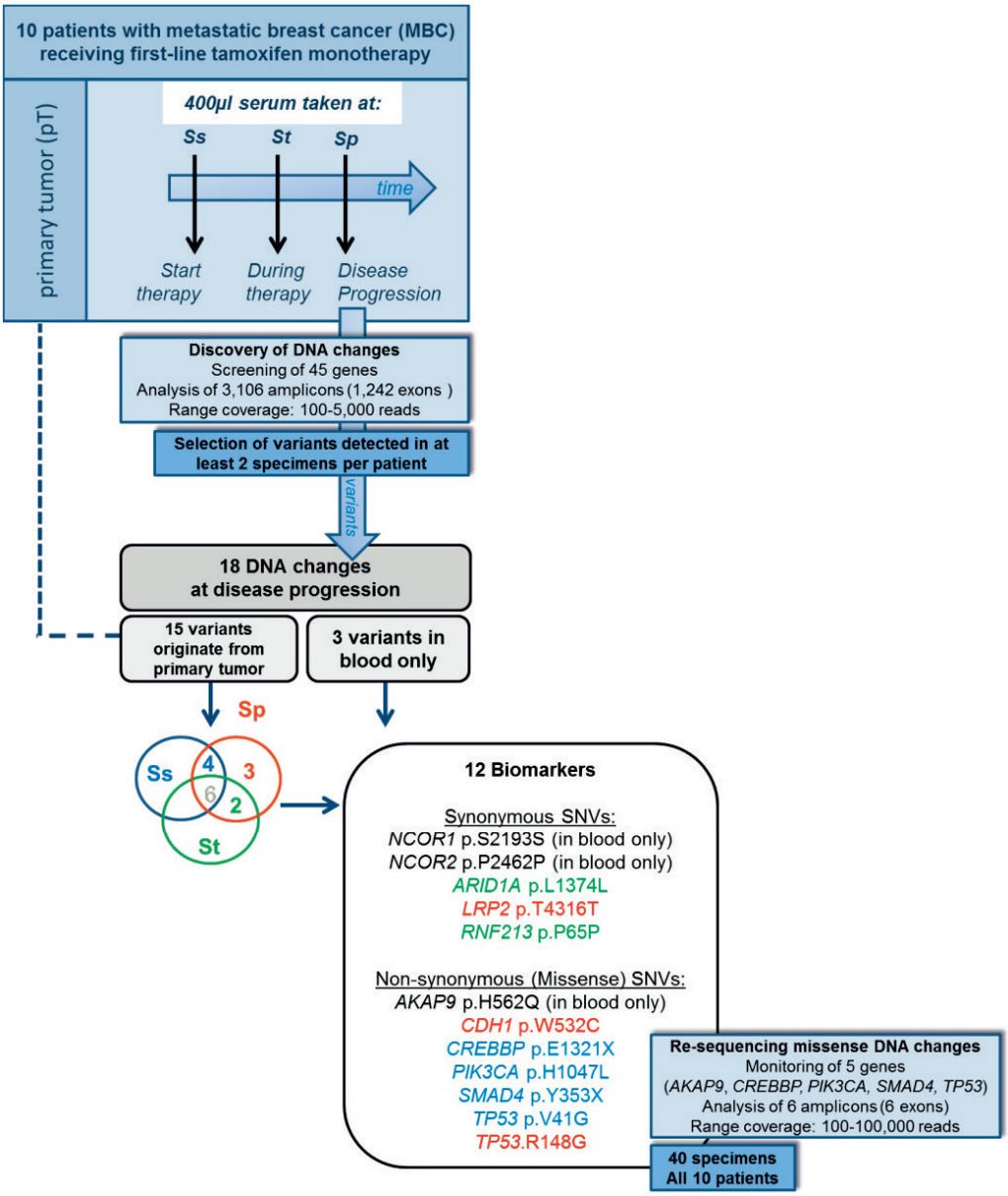


Figure 1 Study design and discovered DNA changes. Targeted ion-PGM (re-)sequencing was performed on DNA isolated from primary tumors and blood specimens from 10 metastatic breast cancer patients who received tamoxifen as first-line therapy. Cell-free DNA (cfDNA) was isolated from 400 µl serum taken at start (Ss), during therapy (St) and at disease progression (Sp). Analysis revealed 12 biomarkers including 9 single nucleotide variants (SNVs) detected at progression and in primary tumor but not in all preceding blood specimens. The SNVs originating from the primary tumor are presented in red when only seen at Sp, in green when seen at Ss and Sp, and in blue when seen at St and Sp.

Table 1 12 cfDNA mutations identified as biomarkers in 6 metastatic breast cancer patients receiving first-line tamoxifen therapy [1]

		Primary Tumor (pT)			Results obtained in cfDNA[2] and calculated per 1ml serum								
					At start therapy (Ss)			During therapy (St)			At disease progression (Sp)		
Gene	Amino Acid change	DNA input (in ng)	variant frequency (in %)	number mutant copies per 1ng DNA	yield cfDNA (in ng)	variant frequency (in %)	number mutant copies per 1ml serum	yield cfDNA (in ng)	variant frequency (in %)	number mutant copies per 1ml serum	yield cfDNA (in ng)	variant frequency (in %)	number mutant copies per 1ml serum
Patient 1 [3]		16.6			30.8			17.0			16.3		
NCOR2	p.P2426P		0	0		0	0		2	103		3	148
AKAP9	p.H562Q		0	0		16	1493		17	876		2	99
Patient 6		15.8			23.0			29.7			24.0		
RNF213	p.P65P		0	0		0	0		0	0		2	145
CDH1	p.W532C		0	0		0	0		0	0		1	73
CREBBP	p.E1321X		23	65		2	139		0	0		4	291
Patient 7		14.8			14.8			7.3			17.5		
PIK3CA	p.H1047L		25	76		2	89		0	0		7	371
SMAD4	p.Y353X		17	52		8	358		0	0		4	212
TP53	p.V41G		35	106		3	134		0	0		2	106
Patient 8		13.0			17.5			24.3			113.8		
NCOR1	p.S2193S		0	0		0	0		4	294		4	1379
LRP2	p.T4316T		10	35		0	0		0	0		2	689
RNF213	p.P65P		2	7		0	0		3	220		3	1034
CDH1	p.W532C		2	7		0	0		0	0		1	345
TP53	p.R148G		35	121		0	0		0	0		3	1034
Patient 9		9.8			34.0			21.0			10.8		
ARID1A	p.L1374L		57	261		50 [4]	5152		60	3818		48	1564
Patient 10		12.2			6.8			10.5			10.3		
CDH1	p.W532C		3	11		0	0		0	0		3	93
PIK3CA	p.H1047L		43	158		0	0		0	0		0	0
TP53	p.R148G		0	0		0	0		2	64		0	0

[1] No DNA changes were identified at disease progression and detected in corresponding primary tumor and/or blood specimens at earlier time-points for patients 2 to 5.
[2] Input was 400 µl serum.
[3] Patient 1 had a *PIK3CA* p.H1047R mutation not called by standard ionPGM settings, but which was confirmed by re-sequencing and digital PCR (See also Figure 2 and additional Table A1).
[4] The *ARID1A* SNV was observed at a mutation frequency of 50% in specimen Ss but not called due to strand bias, i.e. this SNV was detected in 7 forward and 289 reverse strand reads.

Pathogenic somatic single nucleotide substitutions

The 12 DNA changes included 5 synonymous and 7 non-synonymous SNVs. Almost all algorithms predicted the missense SNVs for *CDH1*, *PIK3CA* and *TP53* (p.V41G; p.R148G) as pathogenic and for *AKAP9*, *CREBBP* and *SMAD4* predominantly as not pathogenic (Supplementary Table S2). Moreover, the SNVs in *PIK3CA* and *TP53* have been reported as cancer-specific mutations.

Re-sequencing at 1% detection limit of missense SNVs

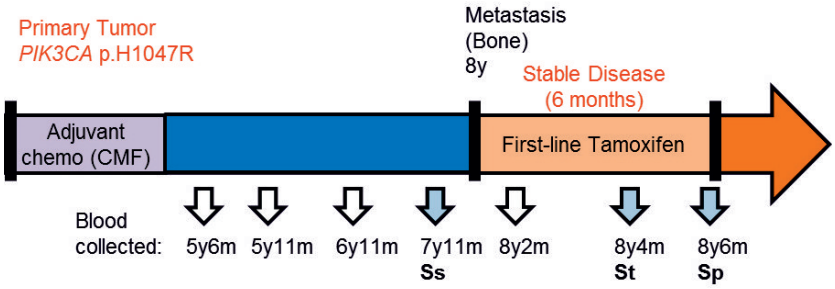
Next the identified non-synonymous SNVs were selected for re-sequencing since only these translate into amino acid changes, which might alter the biological function of the encoded protein and as a result might affect clinical outcome. All specimens were re-sequenced for 1 missense SNV found in blood only and 5 missense SNVs found in primary tumors at diagnostic levels, i.e. higher than 10%. The re-sequencing confirmed the initial results for *AKAP9* p.H562Q in patient 1, *CREBBP* p.E1321X in patient 6, *SMAD4* p.Y353X, *TP53* p.V41G, and *PIK3CA* H1047L in patient 7, and *TP53* p.R148G in patient 8. Moreover, *PIK3CA* exon 20 re-sequencing identified another SNV, i.e. p.H1047R. This SNV was seen at disease progression and all other blood specimens of patient 1 and in the primary tumors of patients 1, 2, and 7 (Supplementary Table S3).

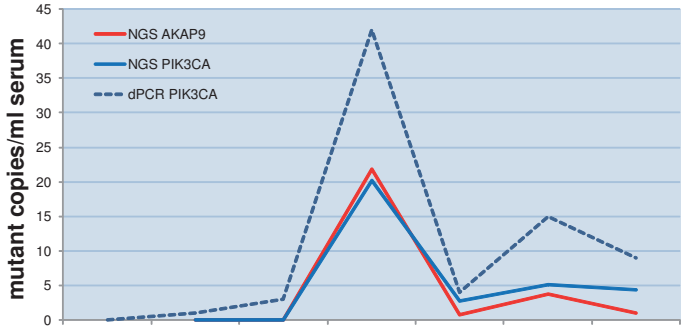
Digital PCR evaluation at 0.05% detection limit of *PIK3CA* mutations

The re-sequencing results for *PIK3CA* were verified by digital PCR using mutation-specific assays (Supplementary Table S3). The p.H1047L mutation was evaluated and confirmed in all specimens of patients 7 and 10, but was additionally seen in serum at start of therapy of patient 10. The p.H1047R mutation was evaluated in all primary tumors as well as in blood specimens of patients 1 and 2. Digital PCR confirmed the occurrence of this mutation in all evaluated blood specimens and primary tumors, except in the primary tumor of patient 7.

cfDNA mutations and disease development

For patient 1 additional blood specimens available between diagnosis of primary tumor and metastatic lesions were evaluated by NGS and digital PCR (Figure 2). Re-sequencing demonstrated *AKAP9* and *PIK3CA* mutations at similar magnitudes in blood specimens taken around the occurrence of metastatic lesions. It also detected PIK3CA mutant reads in blood preceding the metastasis, however, in less than 10 reads. Digital PCR confirmed this *PIK3CA* mutation in blood taken 6 years after diagnosis of primary disease but two years before diagnosis of metastatic lesions. At the time metastatic lesions were recognized, the blood had a large number of copies with this mutation, which dropped after 2 months of first-line tamoxifen therapy, but increased towards therapy resistance after 6 months treatment.





Amount cfDNA (in ng) in 1ml serum			17.6	20.4	6.2	30.8	23.2	17.1	16.1
***AKAP9* p.H562Q**	**ion-PGM**	Proportion (in %)		0.00%	0.00%	23.35%	1.02%	7.22%	1.85%
		mutant/total reads		(0/7,055)	(0/6,285)	(2021/8,653)	(70/6,842)	(534/7,392)	(215/11,604)
		mutant copies/ml serum		0	0	22	1	4	1
***PIK3CA* p.H1047R**	**ion-PGM**	Proportion (in %)		0.00%	0.00%	21.65%	3.79%	9.72%	8.83%
		mutant/total reads		(3/2,564)	(2/3,229)	(1577/7,285)	(177/4,671)	(358/3,682)	(463/5,243)
		mutant copies/ml serum		0	0	20	3	5	4
	Digital PCR	Proportion (in %)	0.00%	1.19%	2.38%	45.15%	5.88%	28.48%	18.62%
		mutant/total copies	(0/125)	(1/134)	(3/115)	(189/424)	(3/60)	(19/73)	(14/76)
		mutant copies/ml serum	0	1	3	42	4	15	9

Figure 2 cfDNA missense mutations and disease development. The *AKAP9* and *PIK3CA* mutations of patient 1 were evaluated in blood specimens during the course of disease. Sera collected five years after clinical diagnosis of breast cancer were evaluated by ion-PGM resequencing and for *PIK3CA* in duplicate by digital PCR (dPCR). The p.H1047R mutation was observed at low magnitude in blood taken five years after diagnosis of primary disease and already two years before radiological diagnosis of metastatic lesions. Although sometimes low numbers of *PIK3CA* mutant copies were detected, all were independently observed after ion-PGM resequencing and in two separate digital PCR reactions. All proportions except for the sample at 5y11m were above the limit of detection.

DISCUSSION

This exploratory study is to our knowledge the first to report on sequential monitoring of serum cfDNA in a homogenous setting of MBC-patients receiving first-line tamoxifen therapy. In total 12 variants for 6 patients were identified in cfDNA at disease progression, including 3 variants that were only found in blood specimens but not in the primary tumor and 9 variants detected in corresponding primary tumor but not in all blood specimens preceding progression. Because of their putative biological relevance, we confirmed the identified missense mutations by re-sequencing and by digital PCR.

Out of these, missense mutations in *PIK3CA*, *TP53, SMAD4* and *CREBBP* were present both at time of progression and in the primary tumor. COSMIC reported

mutations in breast cancer most frequently in *PIK3CA* and *TP53* while occasionally in *CREBBP* and *SMAD4* with our variants for the latter two genes not earlier described. Mutations in all these genes have been found in hormone-resistant breast cancer [9]. The presence of *PIK3CA* and *TP53* mutations in longitudinally collected blood specimens correlated with treatment outcome to PI3K-inhibitors and aromatase inhibitors and was associated with the clinical course of disease [10, 11]. *CREBBP* and *SMAD4* encode proteins that bind to ER as co-activator [12] and co-repressor [13], respectively, suggesting a putative role in endocrine therapy resistance [13, 14]. However, the effect of the *CREBBP* stop-gain mutation revealed in our study remains to be established. The *SMAD4* p.Y353X stop-gain mutation resides within the MH2 domain, a mutational hotspot [15] related to a loss of function, detrimental for TGFb signaling, and poor disease outcome [16]. Importantly, above variants at time of progression likely reflect only tumor load in the blood. The *AKAP9* p.H562Q missense mutation was not seen in the primary tumor and might have been missed due to tumor cell heterogeneity. Alternatively, this mutation might originate from metastatic lesions or acquired due to treatment pressure. Variants in *AKAP9* have been described repeatedly in COSMIC and as SNPs associated with increased breast cancer risk [17], but the particular variant found here has not yet been reported. It is currently unknown whether, and if so, to which extent, all missense mutations actually contribute to resistance against tamoxifen. Many of the mutations are probably bystander mutations due to genomic instability. Therapy resistance, however, may select for tumor cells with specific mutations adapting these cells to the hostile environment, resulting in the survival of fittest and ultimately driving tumor progression [18].

Our study differs from previous studies using ion-PGM targeted NGS on cancer tissue and liquid biopsies to identify tumor-specific DNA changes. Earlier studies [19–21] evaluated 200 amplicons of the commercially available hotspot cancer panel whereas we examined fifteen-fold more amplicons. These studies detected mutations in both plasma and tissue for 27 of 34 cancer patients [20], or like our study, in half of the cancer patients [19, 21] and evaluated plasma specimens collected within a 16 month time-frame after primary tumor tissue was obtained. We instead sequenced and identified mutations in minute cfDNA amounts isolated from serum collected at least 3 years after diagnosis of primary disease. Mutations in low DNA amounts might be missed due to the limited number of genomic equivalents present and corresponding higher limit of detection. Retrospective studies, such as our own, collected mainly limited blood quantities which will often result in minute cfDNA amounts available for analysis. Furthermore, our serum samples have been stored at −80°C for more than 18 years, demonstrating that long-term stored routinely collected sera are suitable for cfDNA isolation and subsequent molecular characterization.

To define ctDNA mutations in blood associated with disease progression on tamoxifen treatment, we screened for biomarkers seen at progression and the corresponding primary tumor but not in all preceding blood specimens in a particular patient. The mutation detection in blood depends on cfDNA quantities, with these quantities changing in time and reflecting tumor load in the course of disease. Our study showed overall no significant differences in blood cfDNA yields at different time-points, however, most mutations were detected in patients with the highest DNA yields at

progression. Deeper re-sequencing confirmed the presence of the 6 missense SNVs in specimens of individual patients in which they were initially reported and absence from those which initially lack them. It also discovered an additional *PIK3CA* mutation, and examination of sequence reads revealed that the p.H1047R mutation was originally present in respective specimens but not called due to stringent settings, indicating that current thresholds are suboptimal for rare variant detection in cfDNA [19, 22]. Digital PCR independently identified both *PIK3CA* mutations in blood specimens at higher frequencies than revealed by NGS and in additional specimens and as proof-of-principle even in blood taken years before diagnosis of the metastatic lesions.

In conclusion, our study demonstrates that targeted ion-PGM sequencing of cfDNA is applicable to discover mutations in archived serum samples. Deeper re-sequencing and digital PCR analyses enables more sensitive detection and monitoring of specific mutations in sequential blood specimens even in samples stored for over 18 years and in minute amounts of cfDNA. Further studies are warranted to investigate whether detection of ctDNA in tamoxifen-treated metastatic breast cancer patients can be used to detect disease progression at an early stage and whether the identified variants play a role in tamoxifen resistance.

MATERIALS AND METHODS

Materials and methods are described briefly below, details are found in the appendix.

Patient and sample collection

This retrospective study investigated fresh frozen primary tumor tissue and sequential sera taken from 10 MBC patients who received tamoxifen as first-line therapy for distant metastatic disease (Figure 1). Blood specimens were selected at start of tamoxifen therapy (Ss), during therapy (St), and at disease progression (Sp). From 6 patients formalin fixed paraffin embedded (FFPE) macro-dissected normal tissue was available and analyzed. The study was approved by the medical ethics committee (MEC 02.953), performed according to the Code of Conduct of Medical Scientific Societies (www.federa.org/codes-conduct) and followed REMARK guidelines where possible [23]. Clinicopathological characteristics are presented in Supplementary Table S4.

DNA isolation, quantification, and sequencing

DNA from tumor and normal tissue specimens was extracted as described previously [24, 25]. The MagnaPure Compact nucleic acid isolation kit (Roche Diagnostics) was applied to isolate cfDNA from 400 µl serum. DNA yields and concentrations were quantified with a Qubit® 2.0 fluorometer (Thermo Scientific). The cfDNA input amounts of each sample were used to establish the genomic equivalents and limits of detection for subsequent molecular analyses (Supplementary Tables S5 and S6). Semiconductor sequencing was performed using the Ion Torrent Personal Genome Machine (Ion-PGM) and consumables, kits, software packages and protocols provided by the manufacturer (Thermo Scientific). Briefly, 10 ng tissue DNA and

minute amounts cfDNA (range: 165-573 pg) were used as input for library preparation and sequenced with a custom-made gene panel. Ion AmpliSeq Library Preparation Kit 2 and Ion PGM Template OT2 200 kit were applied to generate libraries and templates, respectively. Ion Sequencing Kit v2 was used for sequencing on an Ion 318 chip.

Custom gene panel

The 45-gene panel (Supplementary Table S2) included the most frequently mutated genes for breast, colon, prostate and ovarian cancer reported in the catalogue of somatic mutations in cancer (Cosmic Release 67; http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). Thirty-nine genes were sequenced for all exons, 6 oncogenes for hotspot exons only. In total 3,106 amplicons (i.e. 1,242 exons; ~255kb) were sequenced up to a read depth of 5,000x.

Bio-informatics for variant detection and evaluation

Raw data analyses, base calling and alignment were performed using Torrent Suite v4.0. Somatic low stringency filtering was applied in Variant Caller v4.16 (VC) to detect DNA changes when compared to reference genome hg19 (build 37). Variants were annotated by a custom pipeline including ANNOVAR (openbioinformatics.org/annovar) within Galaxy (galaxyproject.org). Only exonic variants with frequencies of 1% or higher and above the cfDNA-specific limit of detection were selected. Uniquely identified variants and those found in sequenced normal DNA or reported within the 1000-Genome database were excluded. These variants had to be sequenced without strand bias at a read depth of 100x or more and showing at least 10 mutant reads. Integrative Genomics Viewer (IGV) (http://www.broadinstitute.org/igv) was used for manual examination. Identified SNVs were evaluated with different *in silico* algorithms to predict the pathogenicity of the SNV on protein function. These tools are embedded in ANNOVAR and included SIFT, PolyPhen2, MutationTaster, FATHMM, GERP++, SiPhy and PhyloP [26].

Re-sequencing and digital PCR analysis

Exons of selected non-synonymous SNVs were re-sequenced for all specimens by ion-PGM after independent library preparation up to 100,000 reads depth, and evaluation was performed similar to the initial analysis. The *PIK3CA* genotype was verified with Taqman p.H1047L- and p.H1047R-specific assays and the QuantStudio™ 3D Digital PCR system (Thermo Scientific). Reaction mixtures, including tumor or serum DNA and QuantStudio™ 3D Digital PCR Master Mix, were loaded on digital PCR chips with 20,000 wells, and cycled under standard conditions for 40 cycles. QuantStudio™ 3D analysisSuite™ determined the proportion mutant and wild-type templates.

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CHAPTER VII

General Discussion and Future Perspectives

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Breast cancer is the most common cancer in women and second most common cancer worldwide, with nearly 1.7 million new cases diagnosed in 2012 (World Cancer Research Fund) [1]. The 5-year survival rate of early stage BC is 80-90%, particularly in many countries with advanced medical care. However it is dropping to 24% when diagnosed at a more advanced stage http://www.wcrf.org/int/cancer-facts-figures/data-specific-cancers/breast-cancer-statistics.

Breast cancer is more common after menopause. Most breast cancer subtypes are hormone-related with approximately 75% of breast cancer cases being ER-positive [2]. Cancer is a disease of the DNA and the most prevalent mutation in (ER-positive) breast cancer is *PIK3CA* (see Introduction). Moreover, genomic studies have also shown that ER-positive breast cancers are of the luminal subtypes. For metastatic breast cancer (MBC) the most common site of metastasis is bone [3].

Anti-hormonal therapy (antiestrogens and/or aromatase inhibitors) used for ER-positive breast cancer patients will eventually fail in almost 50% of the ER-positive breast cancers [4]. In the advanced setting, up to 60% of patients do not respond to anti-hormonal first-line therapy. Moreover, almost all metastatic patients and approximately 40% of patients receiving adjuvant treatment eventually relapse [5,6].

For over 40 years the selective estrogen receptor modulator tamoxifen, which competes with the natural ligand for binding to the estrogen receptor, has been key for premenopausal breast cancer therapy. Moreover, in the adjuvant setting, tamoxifen has been shown to decrease breast cancer recurrence risk and death. Tamoxifen binds to ERα and permits receptor dimerization, but induces conformational changes which differ to those induced by estrogens.

For 20 years, another treatment of ER-positive breast cancer is used: aromatase inhibitors (AI). The mechanism of action for aromatase inhibitors prevents estrogen synthesis, consequently depriving the receptors from their natural ligand. AI are mostly effectively used in postmenopausal women.

In general cancer patients do not die from their primary tumor but they die of the metastases that are no longer responding to therapy. Relevant for this discussion and my thesis aims is that the objective response rate to endocrine therapy for ER-positive MBC is only 20-40% with a median duration of approximately 8-14 months [7]. This shows a clear and urgent need to recognize biomarkers able to identify patients who will or will not benefit from the therapy. As such, for those patients, unnecessarily exposure to undesirable adverse events of (anti-hormonal) therapy can be avoided.

Consequently, this thesis aimed to find potential biomarkers of prediction to anti-hormonal treatment resistance in advanced ER-positive breast cancer patients. Predictive markers, however, are also often prognostic. By definition, the ideal predictive biomarker must have a **sensitivity predictive value** with **no prognostic value** [8].

For the purpose to find potential biomarkers that predict anti-hormonal treatment resistance in advanced ER-positive breast cancer patients different approaches were

followed. Almost all approaches included evaluation of publicly available *in silico* datasets for ER-positive and/or luminal breast cancer. These datasets were mainly used for independent validation of biomarkers, however, we have used the *in silico* datasets for discovery in our analyses of mRNAs and miRs and of protein expression and phosphorylation as described in Chapters III and IV, respectively. The starting point and focus of this thesis is ER-positive/luminal MBC with *PIK3CA* mutations, the most prevalent mutation in breast cancer as mentioned before.

PIK3CA mutations: Its relationship with and relevance for endocrine treatment outcome

In **Chapter II,** *PIK3CA* hotspot mutations were investigated in 1352 primary breast tumors for its relationship with prognosis and endocrine treatment outcome. We were the first to report for MBC, and as shown in this chapter, that ***PIK3CA* mutations** in ER-positive primary tumors associate with **favourable outcome on first-line aromatase inhibitors.** Moreover, ***PIK3CA* mutations** in our cohort were **not prognostic nor predictive for outcome on tamoxifen**.
Regarding endocrine therapy response in the (neo-)aduvant setting, others have shown for adjuvant AI treatment that *PIK3CA* mutations in exon 20 [9] also associates with a favourable outcome, whereas mutations in exon 9 are not related with outcome [10]. With regard to tamoxifen treatment, contradicting findings in the adjuvant setting have been reported. In one study *PIK3CA* mutations and a PI3K-gene expression signature were related with favourable outcome on tamoxifen [11] while in other studies mutations were not related to tamoxifen treatment benefit [12], or were associated with shorter survival [13].

Since preclinical models have identified cross-talks between ER and PI3K/AKT/mTOR pathways as mechanism of endocrine therapy resistance, therapies combining endocrine treatment with PI3K inhibitors have also been evaluated in clinical settings. In these studies, *PIK3CA* mutations might not only relate with response to endocrine therapy but also to PI3K-inhibitor therapy. One of the first reported a neo-adjuvant study which demonstrated significant increased antiproliferative responses when patients received mTOR inhibitor (everolimus) plus AI (letrozole) compared to AI alone. This antiproliferative response for the combination therapy was also seen in a small number of patients with *PIK3CA* exon 9 mutations, but not for patients with exon 20 mutations [14]. Evaluation of this combination therapy in MBC within the BOLERO-2 study, a phase 3 trial, demonstrated a two times longer progression-free survival for the addition of everolimus to AI [15]. Additionally, the BELLE-2 study also suggests that PIK3CA mutations detected in the primary tumor as well as in ctDNA may help select patients who benefit from adding a pan-PI3K inhibitor (buparlisib) to SERD endocrine therapy (fulvestrant). Patients carrying the *PIK3CA* mutation showed to increase from 4.0 to 6.8 months PFS [16]. Unfortunately, buparlisib showed some serious adverse events precluding further development of this drug. [17]. On the opposite, Loi et al. have shown that *PIK3CA* mutations do not correlate with better neoadjuvant mTOR inhibitor (everolimus) treatment outcome when added to AI (letrozole). Instead, they showed their PI3K gene expression signature can predict better treatment outcome after neoadjuvant AI [18]. Schmid et al. (not in refs)also showed in the neoadjuvant setting, that regardless of *PIK3CA* mutation status in luminal B primary BC, the PI3K inhibitor (pictilisib) appears to

suppress tumor cell proliferation significantly when added to AI (anastrozole) [19]. As suggested by both Loi et al. and Schmid et al. and in line with our study design from chapter III onwards, the *PIK3CA* mutation alters genes that are related with tumor aggressiveness. This might explain why treatment with PI3K inhibitors improves the outcomes of BC patients regardless of the *PIK3CA* mutation status. We proposed that genes related to the *PIK3CA* mutation, like the above mentioned PI3K signature, can predict better than *PIK3CA* genotype treatment outcome after adding PI3K inhibitors to AI treatment.

Finally, the *PIK3CA* genotype of primary breast tumors was not prognostic in our cohort, which is in line with the findings by others [18,20,21]. For example, Kim et al. have shown that *PIK3CA* mutations did not affect OS in metastatic BC regardless of ER status [20]. Moreover, unlike *TP53* mutations, *PIK3CA* mutations do not affect its own gene expression level [9,20].

Our observed association of the *PIK3CA* mutation with aromatase inhibitors, but not with tamoxifen might suggest a difference in the mechanism of action for these two types of drugs, which could teach us more about hormonal resistance. The mechanism of action for aromatase inhibitors prevents estrogen synthesis, consequently depriving the receptor from their natural ligand. Tamoxifen is a selective estrogen receptor modulator (SERM), which competes with the natural ligand for binding to the estrogen receptor.

Aromatase inhibitors reduce considerably the presence of estrogens. However, AI does not interact with the ER and does not induce conformational changes. Therefore, it is not expected that AI alters ER functionality. That is why the majority of **aromatase inhibitors resistant tumors still express functional ER** as it has been described by Patani, et al. [22].

Tamoxifen binds to ERα and permits receptor dimerization, but induce conformational changes which differ to those induced by estrogens. Therefore, more **ER functional changes** are **expected to be altered after tamoxifen use** [23,24].

To evaluate *PIK3CA* genotype for its prognostic and predictive value properly, we should have analyzed patient samples with and without treatment in the same clearly defined clinical setting using randomized clinical trials (RCT) as explained by Beelen et al.[8]. However, due to the retrospective nature of collected data from our cohort of patients, we were not able to analyze both prognostic and predictive value in the similar sets of patients for both disease and treatment outcome (in the adjuvant setting). Ideally, we could have done randomized clinical trials (RCT) to ensure that the potential predictive value of *PIK3CA* genotype was correct. Nevertheless, it is currently not standard of care to have RCT including placebo groups without adjuvant systemic treatment [8].

In conclusion, there is an urgent need to verify the relationship for *PIK3CA* genotype with disease and treatment outcome in prospective more clearly defined clinical trials to determine its potential as prognostic and predictive biomarker established in above mentioned retrospective studies[25]. One such randomized phase III trial comparing AI versus tamoxifen followed by AI, however, was inconclusive with regard to *PIK3CA* mutations and treatment outcome (Sabine, Crozier et al., JCO 2014).

Genes, microRNAs and proteins related to PIK3CA mutations and endocrine treatment outcome

In the era of personalized medicine, not only the mutation of *PIK3CA* is of importance but even more downstream affected targets and pathways. This will further aid in the discovery of predictive biomarkers and of novel targets for therapy. To find potential biomarkers for sensitivity to AI in MBC patients based on their relationship with *PIK3CA* genotype, we have studied mRNA (gene) and microRNA expression profiles in **chapter III**. The goal was to identify mRNAs and microRNAs related to AI treatment and to *PIK3CA* genotype. In contrast to the standard procedures, i.e. mostly using one discovery and one validation cohort of patients, and aiming at a lower false rates we have used in this chapter two discovery cohorts. We reasoned that biomarkers identified in two independent cohorts, are certainly more informative at the moment of validation in a third independent cohort.
In each discovery set, expression of several mRNAs and miRs were related with the *PIK3CA* genotype, including *LRG1, PLCL1, FAM81B, CCNO, NEK10, PGR, SERPINA3, SORBS2, VTCN1, hsa-miR-449a, hsa-miR-301a-3p,* and *hsa-miR-205-5p* as overlapping biomarkers. Of these common genes and miRs, only ***LRG1* expression was shown to be related with AI treatment outcome**, and **independent from luminal subtype**. Others have demonstrated that *LRG1* plays a role in protein-protein interaction, signal transduction, and cell adhesion and development [26]. Moreover, *LRG1* is expressed during granulocyte differentiation [26]. Interestingly for liquid biopsies, *LRG1* mRNA is detectable in whole blood using qPCR, whereas LRG1 protein can be measured in plasma by ELISA [27] or by an electrochemical sensor at lower costs. This mLRG1-sensor has been designed for diagnosing adenoma-carcinoma transition in colon cancer [28]. Further studies are needed to explore and apply such a sensor for *LRG1* in plasma of ER positive breast cancer patients and verify our observed relation with aromatase inhibitors response. Our findings in chapter III demonstrated high LRG1 expression levels in primary breast cancer with *PIK3CA* mutations and response on AI treatment.

Importantly to mention is the fact that we always want to show associations with *PIK3CA* genotype after correction for well-known confounders such as molecular subtype. In that way, biomarkers related to the *PIK3CA* mutation status, have a higher likelihood to be related to genotype and not just have been identified by chance or due to confounders.

Based on gene expression related *in silico* data, we aimed in **chapter IV** to identify cancer related proteins of signalling pathways (including the ones of the PI3K/AKT/mTOR pathway). These proteins were investigated for altered expression or phosphorylation in luminal breast cancer with *PIK3CA* mutations. We here showed that an **enhanced MAPK1/3 phosphorylation is related to *PIK3CA* mutated luminal breast cancer** in an exon specific manner. The enhancement was also **associated with favorable metastasis-free survival** especially when located in the nuclei of tumor cells. Others reported previously contrasting findings on the relationship between MAPK1/3 phosporylation and endocrine treatment outcome. Some studies showed no significant findings for adjuvant tamoxifen [29,30] whereas others demonstrated increased MAPK1/3 phosphorylation with better outcome in especially tamoxifen-treated cases [31,32] or resistance to neo-adjuvant AI [33]. Using the

in-silico RPPA data we showed an enhanced MAPK1/3 phosphorylation in luminal *PIK3CA* mutated tumors. In our own clinical data we were not able to confirm this relationship, probably due to a small sample size, since for only 95 out of 721 FFPE samples sufficient DNA could be retrieved. The enhanced MAPK1/3 phoshorylation in luminal *PIK3CA* mutated tumors, however, was also seen by Beelen when analysing TMAs of 563 ER-positive breast tumors for MAPK1/3 phosphorylation and PIK3CA exon 9 and exon 20 mutation status [12]. Moreover Wang et al. have shown already an incremented MAPK1/3 phosphorylation after the knock-in of *PIK3CA* mutations human breast epithelial cells [34]. Nevertheless, this cell line model might not explain completely our found association since breast epithelial cells are not breast cancer cells. New technologies such as for example Crisper/Cas strategies might enable functional studies including the knock-in of *PIK3CA* exon-specific mutations in different luminal *PIK3CA* wild-type cell lines. The insertion of the mutation will confirm whether MAPK1/3 phosphorylation will increase after introducing specific *PIK3CA* genetic alterations in already luminal breast cancer cell lines.

Endocrine therapy resistance and breast cancer phenotype

The next aim was to contextualize ER-positive inflammatory breast cancer with *PIK3CA* mutation. With the era of immunotherapy it is of importance not only to look at the tumor itself but also at the tumor micro-environment. As envisioned every time we make new subgroups, we are trying to find new features, which can give us insights on treatment resistance. Previously, we found that the *PIK3CA* mutation was related to AI-first line sensitivity. Later, we used these insight to go beyond and to find biomarkers based on this initial observation. Our collaborators Van Laere et al. from the translational Cancer Research Unit, GZA Hospitals St-Augustinus, in Antwerp, Belgium observed in their cohort of inflammatory breast cancer (IBC), an aggressive tumor phenotype, that a subgroup of patients (ER+ IBC) exhibited resistance to endocrine therapy compared to ER+ non-IBC patients. Based on this, we used in **chapter V,** this cancer phenotype with worse outcome as alternative approach to identify biomarkers for endocrine therapy resistance.

As for non-IBC, *PIK3CA* mutations have been also described in 28% of IBC cases [35]. Moreover, *PIK3CA* mutations have also been identified (13%) in single circulating tumor cells isolated from blood of IBC patients [36]. The *PIK3CA* mutation frequencies for IBC and for non-IBC, however, were not significant different [37].

In **chapter V**, our analyses revealed that the "ER+ IBC-like" **metagene** *(ABAT, ADAMDEC1, CLEC7A, ETS1, ITK and STC2*), as well as its classifier genes ***ABAT***, and ***STC2*** are **associated with endocrine treatment resistance** but **not with prognosis**.

In line with our findings, down-regulation of STC2 has been identified by Parris et al. as critical gene for breast cancer initiation and progression [38]. Moreover, STC2 gene expression has been associated with shorter disease-free survival [39]. Interestingly, we find another piece of the puzzle, to better understand endocrine response in **chapter III**, we observed that primary tumors of **ER-positive MBC patients treated with first-line AI** have a **longer PFS** and **increased PLCL1**. Thus, ***PLCL1* gene expression** was proposed as **biomarker of AI-first line sensitivity**. *PLCL1* encodes for phospholipase Clike1 (PRIP), a protein that regulates the phosphorylation and

activation of the gamma-aminobutyric acid (GABA) receptor [40], [41]. Therefore, **increased *PLCL1* levels** might result in **reduced GABA receptor activity**. On the other hand, in **chapter V**, **low *ABAT*** gene **expression** was associated with **shorter PFS after anti-hormonal therapy** (tamoxifen and first-line AI) in ER-positive IBC patients when compared to ER-positive non-IBC patients. *ABAT* encodes for the 4-aminobutyrate aminotransferase (ABAT) responsible for catabolism of GABA. Therefore, **low ABAT gene expression levels** might result in **enhanced GABA receptor** downstream **activity** since more GABA is available to interact with the GABA receptor.

In chapter V, we showed that expression of ABAT protein was not informative with regard to treatment outcome. However, Budczies et al., have shown that Low *ABAT* mRNA expression was associated with low ABAT protein expression. Most importantly, they also observed an association between low *ABAT* expression and recurrence-free survival in ER+ and ER- breast cancer patients [42].

This brings me to hypothesise that **resistant tumor cells proliferate not only via estradiol and estrogen receptors but** might also use **GABA and GABA receptors** for this purpose. Moreover, estradiol and GABA might cooperate in this proliferation process since it has been shown by Maggi et al., that estrogens may increase the number of GABA binding sites by direct interaction with the GABA receptor gene or genes involved in the metabolism of GABA receptor [43]. It has also been shown that GABA as estradiol regulates most of the same neural functions [44]. Therefore, GABA receptor and estrogen receptor (the corresponding receptors for GABA and estradiol) might have a huge similarity. Future studies are needed to verify all this and to test above mentioned hypothesis for its validity.

Liquid biopsy biomarkers for endocrine therapy resistance

Nowadays, circulating biomarkers including circulating tumor cells (CTCs) and cell-free DNA are being widely studied since they can be isolated from several body fluids including blood, becoming a useful alternative to get a “liquid biopsy” in an almost non-invasive manner. In breast cancer, these promising tools can be potentially used in diagnosis, prognosis, prediction and monitoring of treatment response. Moreover, the study of these biomarkers will lead researchers to a better understanding of the complexity and dissemination of breast cancer biology as well as treatment resistance [45,46].

Previously in **Chapter II**, we observed no association between *PIK3CA* mutations and first-line tamoxifen treatment outcome, most likely due to differences between primary tumor and metastases as it has already been shown by Dupont Jensen et al. [47]. Therefore, next we aimed to identify *PIK3CA* mutations as well as additional tumor-specific mutations in serum cell-free DNA potentially related to first-line tamoxifen outcome in metastatic breast cancer patients (**Chapter VI**).

Tumor-specific mutations including *PIK3CA* mutations were detected in minute amounts of serum cell-free DNA (**Chapter VI**). Cell-free DNA was extracted using serum samples collected during therapy and stored for over 18 years. To discover specific mutations in cfDNA, we demonstrated that targeted ion-PGM sequencing can be used as shown in chapter VI and by Weerts et al. [48]. However, more sensitive

detection methods such digital PCR are required for mutation detection in blood then in tissue biopsies, since in blood only trace amounts of mutated circulating tumor DNA are often present.

For further liquid biopsy discovery studies more sensitive methods next to digital PCR are needed, including OnTarget assays to enrich for mutant alleles or deep sequencing with unique molecule identifiers used for instance in Oncomine cfDNA assays. These assays enable the simultaneous evaluation of many mutations, whereas digital PCR can analyse in general only one mutation [49,50]. Again the optimal standardization of pre-analytical conditions, i.e. collection of blood samples, is key [50]. Methods must be selected based on its highest detection sensitivity and low cost, which facilitates a real time monitoring on disease progression.

We confirmed with additional techniques (re-sequencing and digital PCR) only the missense identified mutations because of their putative biological relevance. As yet, not studied in detail, the synonymous mutations might also have biological relevance, as it has been reviewed by Gotea V. et al. They presented evidence supporting the direct impact of synonymous mutations on gene function via gene splicing; mRNA stability, folding, and translation; protein folding; and miRNA-based regulation of expression. Their results highlight the functional contribution of synonymous mutations to oncogenesis and the need to further investigate their detection and validation [51].

In chapter II, we showed the association between *PIK3CA* mutations and first-line AI treatment outcome in metastatic breast cancer patients. However, for the retrospective exploratory study in chapter VI, blood samples from patients treated with first-line AI were scarce and unfortunately not available for the study.

Liquid biopsy – Future of Circulating blood biomarkers

CTCs and cell-free DNA as circulating blood biomarkers can complementary provide real-time information. Given the heterogeneity of breast cancer cells, this changes over time and is affected by treatment pressure. Moreover, since metastatic sites are often of restrictive access, liquid biopsies might potentially become surrogates for tissue-based already existing biomarkers. In addition, these are suitable to closely follow the patients over time, which will impact the management of patients and will lead to a more accurate personalized therapy [52].

Moreover, new emerging breast cancer clones can be monitored in real-time for molecular characterization, which will allow a more accurate follow up of the evolution of the tumor and therefore offer the patient an individualized treatment [53]. Breast cancer is well characterized by accumulation of several mutations. Hence, identification of gain or loss of mutations in metastatic tumors not previously identified in the primary tumors is not surprising. A clear example of this has recently been shown by Segal et al., who compiled recent studies comparing the frequency of *ESR1* mutations in metastatic and primary ER-positive breast tumors and concluded that *ESR1* mutations hardly identified in the primary tumors can go up to 37% in metastatic specimens, especially in luminal A and *PIK3CA*-mutated tumors [54]. Thus current anti-estrogen treatments remain partially effective for these tumors harbouring *ESR1*

mutations. Nevertheless, alternative therapies capable to target the remaining resistant clones will be required [55].

Other type of mutations (including *PIK3CA, TP53, ARID1A, PTEN, AKT1, NF1, FBXW7* and *FGFR3*) have been also shown to be discordant between primary and recurrent breast tumors as well as, copy number alterations (as in *MCL1, CCND1, FGFR1, MYC, IGF1R, MDM2, MDM4, AKT3, CDK4, AKT2*) [56]. This evidences the need of developing profiles capable to identify new features of metastatic clones and therefore better predict treatment response. For example a CTC 8-gene signature in MBCP to distinguish between poor and good outcome after first-line AI treatment was recently developed and further validated in an independent set [57].

Certainly, isolation of CTCs compared to cell-free DNA is more expensive and challenging since CTCs are less frequent present in blood [58]. However, unlike plasma cell-free DNA, CTCs can offer wider information not only DNA-related but also at other kind of levels, including proteomics, metabolomics, epigenetics. Additionally, ex-vivo culture of CTCs is a better strategy to identify the right treatment for the right breast cancer patient over the course of the disease. CTCs in the blood can be captured in viable form and used to improve drug screening [59]. This especially of importance for metastatic disease where the characteristics of the primary tumor will differ from the metastasis and will aid in discovery and discrimination between driver and passenger gene mutations/alterations. Future studies will reveal the most cost-effective way to better use these (novel) tools in the clinical practice.

General recommendations and future perspectives

Retrospective studies have the limitation of lack of optimal control groups. Thereby, to better raise firm conclusions, results obtained from these kind of studies must be confirmed in prospective clinical trials including appropriate randomized controls. An accurate study design must be considered based on the current wide knowledge on tumor cell heterogeneity. Patient inclusion criteria should contemplate most comparable group of patients. Since the *PIK3CA* mutation is already known to have a different effect based on subtype, the groups´ comparisons should be based on subtypes in relation to the outcome of the study, for example, in relation to anti-hormonal therapy response.

If we consider that cells are individual entities even though they keep inter-communication with neighbour cells, different tumor clones are present in a tumor tissue. Hence, not only the percentage of tumor cells present in the primary tumor sample but its insight into the temporal and spatial heterogeneity may lead us to adequate conclusions.

In the future, technology advances, including the promising circulating biomarkers such as (profiling of) circulating tumor cells (CTCs) and cell-free (mutant) DNA obtained from liquid biopsies, currently under study in many labs, will lead us to have a closer follow up of the patients considering different possible present clones in the patient. Moreover, timing and frequency of analysis to better prognosticate and predict the outcome of treatment will be also determined more accurately by the study of this biomarkers.

The pathways interaction complexity will be better understood by selecting xenographs and culture of circulating tumor cells, and based on all the variables already known that should be controlled at the moment of study treatment response such as genetic, metabolomic, and proteomic alterations present in the cell, above and beyond the tumor environment, which already has been shown to play a key role in tumor development and its evolution. Moreover, if we consider that pathway analysis has been usually done based on primary tumor samples in which the tumor percentage might drastically differ from one sample to another, the use of circulating biomarkers based on a cell individual approach will help overcoming clonally related issues.

The future scenario for patients´ follow up and treatment will therefore consider a multidisciplinary approach, namely, not only important known features such as subtypes will be taken into account but also new technologies that will let us have access to real time information carried in circulating biomarkers. This will boost the knowledge about the tumor evolution making it easier to find biomarkers capable to **pick the right patient for the right treatment**. Later on, in the era of personalized/precision medicine, once the biomarkers have been validated, the goal will be to **pick the right treatment for the right breast cancer patient**.

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CHAPTER VIII

Summary

Samenvatting

Resumen

Zusammenfassung

SUMMARY

Breast cancer, which is the most common type of cancer in women and second most common cancer worldwide, is a heterogeneous disease. Guidelines to treat this disease are based on prognostic and predictive clinicopathological factors and tumor biomarkers, such as estrogen receptor (ER), progesterone receptor (PR) and HER2 receptor. Most breast cancers are ER-positive (75-80%), for which three types of anti-hormonal therapies are used: selective estrogen receptor modulators (SERMs), -degraders (SERDs) and aromatase inhibitors (AI). Tamoxifen, a SERM, has been a key treatment for (premenopausal) women for 40 years now. Aromatase inhibitors (AI), used for over 20 years, prevent estrogen synthesis. AI have been mostly effectively used in postmenopausal women. Finally, SERDs like Fulvestrant are nowadays more often used as therapy for patients with acquired resistance to AI treatment due to gained mutations in the ligand binding domain of ER.

Important for the aims of this thesis is the fact that the objective response rate to anti-hormonal therapy for ER-positive metastatic breast cancer (MBC) is only 20-40% with a median duration of approximately 8-14 months. This shows a clear and urgent need for biomarkers which are able to identify patients who will or will not benefit from the therapy. As such, for those patients, unnecessary exposure to undesirable adverse events of (anti-hormonal) therapy can be avoided.

The aim of this thesis was to find potential biomarkers which are able to predict anti-hormonal treatment responsiveness and/or resistance in mainly advanced ER-positive breast cancer patients. To reach this goal different research approaches were followed. Since mutations in *PIK3CA* are the most prevalent mutations in luminal tumors and occur in maximum 45% (see introduction) of the ER-positive breast cancers, the thesis was mainly focused on the relationship between *PIK3CA* genotype and PI3K pathway with treatment outcome.

First, in **chapter II**, we studied the effect of the *PIK3CA* mutation in relation to progression-free survival (PFS) in primary tumors of 531 MBC patients after first-line AI or tamoxifen treatment. We found that patients with a *PIK3CA* mutation have significantly longer PFS after first-line AI therapy when compared to wild-type tumors. Therefore, we proposed the *PIK3CA* mutation as a potential predictive biomarker for first-line AI sensitivity. Moreover, no association with treatment outcome was found after first-line tamoxifen therapy. Thus, the ***PIK3CA* mutation has no predictive value for first-line tamoxifen sensitivity nor resistance**. We also studied the mutation in relation to metastasis-free survival (MFS) in 342 LNN (neo)adjuvant systemic therapy naïve patients and demonstrated that the ***PIK3CA* mutation has no value as a prognostic biomarker**.

In **chapter III**, we investigated mRNAs and miRs expression profiles as potential predictive biomarkers correlated with *PIK3CA* genotype and AI therapy outcome. First, expression of mRNAs and miRs from primary breast tumors was investigated for the relation with *PIK3CA* genotype in two independent discovery sets. One set included 286 luminal samples from *in silico* data of the TCGA consortium. The second dataset included 84 ER-positive samples of MBC patients treated with first-line AI. Expression of 9 mRNAs (*CCNO, FAM81B, LRG1, NEK10, PLCL1, PGR, SERPINA3,*

SORBS2, VTCN1) and 3 miRs (miR-449a, miR-205-5p, miR-301a-3p) was related to the *PIK3CA* mutation status in both sets. Further analyses revealed that ***LRG1* and *PLCL1* expression related to longer PFS after first-line AI therapy**, independent of luminal subtype and traditional predictive factors. Next, these 9 mRNAs and 3 miRs were evaluated in a publicly available *in silico* dataset of 25 breast cancer patients who received neo-adjuvant AI. Expression levels of 3 genes (*LRG1, PGR, SERPINA3*) were shown to decrease significantly after neo-adjuvant AI treatment in patients with clinical response. Based on all these findings, **we proposed *LRG1* as potential luminal subtype independent biomarker related to the *PIK3CA* genotype and AI therapy outcome.**

We next, in **chapter IV,** aimed to identify cancer related proteins of signalling pathways with altered expression or phosphorylation in luminal breast cancer harbouring *PIK3CA* mutations. For that purpose, *in silico* data from 361 primary tumors of breast cancer patients of the TCGA consortium were used. Here we showed that enhanced phosphorylation of MAPK1/3, p38 and YAP and decreased expression of p70S6K and 4E-BP1 in luminal breast cancer related to *PIK3CA* mutations. Phosphorylated MAPK1/3, as most significant upregulated protein for *PIK3CA* exon specific mutations, was further explored in tissue microarrays containing 721 primary breast cancer specimens including a subset of 290 luminal tumors from systemic untreated LNN patients, to evaluate its relationship with metastasis free survival. We showed that especially nuclear MAPK1/3 phosphorylation has a potential value as a prognostic biomarker. Our findings indicate that **phosphorylation of MAPK1/3 and its subcellular localization associate with a favourable disease outcome.**

Until this chapter, *PIK3CA* mutation had been our starting point to find biomarkers with potential prognostic or predictive value after anti-hormonal treatment. In the epoch of immune therapy, in **chapter V**, we evaluated a subgroup of patients with inflammatory breast cancer (ER-positive IBC) who are more often resistant to anti-hormonal therapy compared to ER-positive non-IBC patients. *PIK3CA* mutations are also present in IBC, however, no significant differences in prevalence have been observed between IBC and non-IBC. Based on this and since anti-hormonal therapy resistance might depend also on cancer phenotype we now compared IBC with non-IBC samples. **We identified a metagene of six genes (*ABAT, ADAMDEC1, CLEC7A, ETS1, ITK and STC2*) that distinguished IBC from non-IBC.** This metagene and its individual genes were further evaluated in 6 independent *in silico* datasets and validated by qRT-PCR in a cohort of MBC patients receiving first-line tamoxifen. It was shown that **the metagene, ABAT and STC2 were not prognostic**, that **decreased expression of ABAT and STC2 was associated with poor anti-hormonal therapy outcome in MBC** and that only ABAT expression was related to outcome in patients receiving adjuvant tamoxifen.

Finally in **chapter VI**, in the current era of liquid biopsies and personalized/precision medicine, we aimed at the characterization of tumor-specific mutations in cell-free DNA (cfDNA) from sequential sera taken from MBC patients while on tamoxifen treatment. As a proof of principle, we demonstrated that mutations can be detected in minute amounts of serum cell-free DNA by next generation sequencing (NGS) using a targeted ion-PGM gene panel. Moreover, cfDNA can be analyzed in blood samples stored for almost two decades. In this exploratory study**, we identified mutations in**

***PIK3 P53, AKAP9, CREBBP* and *SMAD4* which, (except *AKAP9*) were present in both primary tumor as well as in serum cfDNA taken at disease progression.** In most cases, however, these mutations were not detected in sera preceding progression, not even using deeper re-sequencing. Interestingly, one case had a *PIK3CA* mutation which was traced back in cfDNA of serum taken two years before diagnosis of metastatic lesions. It was detected in only a few reads by NGS, but confirmed by digital PCR.

In conclusion, in this thesis we showed that *PIK3CA* mutations detected in primary breast tumors have a predictive value for AI response in the advanced disease setting, but not for tamoxifen nor for prognosis. Related to this *PIK3CA* genotype we demonstrated in primary breast tumors that expression of *LRG1* can be used as potential biomarker for AI therapy outcome, which upon neo-adjuvant AI treatment showed decreased levels in patients with clinical response. At the proteomic level, MAPK1/3 phosphorylation levels in luminal breast cancer was shown to be related with *PIK3CA* exon specific mutations. This MAPK1/3 phosphorylation, especially when localized in the nuclei, has also prognostic value in breast cancer. Next, using an alternative approach to discover biomarkers for endocrine therapy resistance, we constructed a metagene to identify ER-positive breast cancers with an IBC phenotype. This metagene and its individual genes *ABAT* and *STC2* were shown to be predictive for tamoxifen and AI therapy resistance in the advanced disease setting. In the adjuvant setting only *ABAT* was related to tamoxifen resistance. Finally, we explored the potential of tumor-specific mutations in serum cfDNA as biomarkers for tamoxifen resistance in MBC patients. Mutations in *PIK3CA, TP53, AKAP9, CREBBP and SMAD4* were shown in serum cfDNA taken at disease progression and all mutations except for AKAP9 also in the primary tumor.

All above potential biomarkers described in this thesis hopefully will improve the identification of patients who will or will not benefit from anti-hormonal therapy. For these patients, undesirable adverse effects due to unnecessary exposure to therapy might be avoided. Thus, today with these biomarkers we are able to better **pick the right patient for the right endocrine treatment**. Promisingly in the near future, when the biomarkers are more extensively validated by others, we will finally be able **to pick the right treatment for the right breast cancer patient.**

SAMENVATTING

Borstkanker, de meest voorkomende vorm van kanker bij vrouwen en de tweede meest voorkomende kanker wereldwijd, is een heterogene ziekte. Richtlijnen voor de behandeling van deze ziekte zijn gebaseerd op klinische pathologische factoren en tumorbiomarkers zoals oestrogeen receptor (ER), progesteron receptor (PR) en HER2 receptor. De meeste borstkankers zijn ER-positief (75-80%), waarvoor drie soorten anti-hormonale therapieën bekend zijn: selectieve oestrogeenreceptor modulatoren (SERM's) en -degraders (SERD's) en aromatase-remmers (AI). Tamoxifen, een SERM, is al 40 jaar een belangrijk medicijn voor de behandeling van (premenopauzale) vrouwen. Aromatase remmers (AI), nu al meer dan 20 jaar in gebruik, voorkomen oestrogeen synthese. AI worden voornamelijk effectief gebruikt bij postmenopauzale vrouwen. Tenslotte worden SERD's, zoals Fulvestrant, vaker gebruikt als therapie voor patiënten met verworven resistentie tegen AI-behandeling als gevolg van mutaties in het ligand bindende domein van de ER.

Belangrijk voor de doelstellingen van dit proefschrift is het feit dat de objectieve response voor anti-hormonale therapie bij ER-positieve uitgezaaide borstkanker (MBC) slechts 20-40% bedraagt, met een mediane duur van ongeveer 8-14 maanden. Dit laat een duidelijke en dringende behoefte aan biomarkers zien die in staat zijn om die patiënten te identificeren die wel of niet op de therapie zullen reageren. Als zodanig kan voor deze patiënten onnodige blootstelling aan ongewenste bijwerkingen van (anti-hormonale) therapie worden voorkomen.

Het doel van dit proefschrift was het vinden van potentiële biomarkers die in staat zijn het effect van anti-hormonale therapie, responsiviteit dan wel resistentie, in voornamelijk uitgezaaide ER-positieve borstkankerpatiënten te voorspellen. Om in ons onderzoek dit doel te bereiken, werden verschillende benaderingen gevolgd. Aangezien mutaties in *PIK3CA* de meest voorkomende mutatie in deze luminale tumoren zijn, in maximaal 45% van de ER-positieve borstkanker, was het proefschrift vooral gericht op de relatie tussen *PIK3CA* genotype en PI3K signaalpaden en de behandelingsuitkomst.

In het eerste **hoofdstuk II**, bestudeerden we het effect van *PIK3CA* mutaties in relatie tot progressie-vrije overleving (PFS) in primaire tumoren van 531 MBC patiënten na eerste lijn AI- of tamoxifen behandeling. We hebben vastgesteld dat patiënten met een *PIK3CA* mutatie een significant langere PFS hebben na de eerste lijns AI therapie in vergelijking met wild-type tumoren. Daarom hebben we de *PIK3CA* mutatie aangedragen als een potentiële predictieve biomarker voor de eerste lijn AI gevoeligheid. Bovendien werd geen associatie met de behandelingsuitkomst gevonden na eerste lijns tamoxifen therapie. Dus heeft de ***PIK3CA* mutatie geen voorspellende waarde voor de eerste lijns tamoxifen gevoeligheid of resistentie**. We hebben ook de mutatie bestudeerd in relatie tot metastase-vrij overleving (MFS) bij 342 LNN (neo) adjuvante systemische therapie naïeve patiënten en aangetoond dat de ***PIK3CA* mutatie geen waarde heeft als prognostische biomarker**.

In **hoofdstuk III** onderzochten we mRNAs en miRs expressieprofielen, die gecorreleerd waren met *PIK3CA* genotype en AI therapie uitkomst, als potentiële predictieve biomarkers. Ten eerste werd de expressie van mRNA's en miR's in primaire borsttumoren onderzocht voor de relatie met *PIK3CA*-genotype in twee onafhankelijke data sets. Een set omvatte 286 luminale borstkanker weefsels uit de in silico data set van het TCGA consortium. De tweede dataset omvatte 84 ER-positieve weefsels van MBC patiënten die waren behandeld met eerste lijn AI. Expressie van 9 mRNA's (*CCNO, FAM81B, LRG1, NEK10, PLCL1, PGR, SERPINA3, SORBS2, VTCN1*) en 3 miRs (miR-449a, miR-205-5p, miR-301a-3p) toonden een verband met de *PIK3CA* mutatie status in beide data sets. Uit verdere analyses bleek ook dat ***LRG1* en *PLCL1* expressie gerelateerd was met een langer PFS na eerste lijn AI therapie**, en dit was onafhankelijk van het luminale subtype en de traditionele predictieve factoren. Vervolgens werden deze 9 mRNAs en 3 miRs geëvalueerd in een publiek beschikbare in silico data set van 25 borstkankerpatiënten die neo-adjuvante AI hadden kregen. Expressie niveaus van 3 genen (*LRG1, PGR, SERPINA3*) bleken significant af te nemen na neo-adjuvante AI behandeling bij patiënten met klinische respons. Op basis van al deze bevindingen **hebben we LRG1 voorgesteld als potentiële luminale subtype onafhankelijke biomarker die verband houdt met het *PIK3CA* genotype en AI therapie uitkomst**.

Vervolgens hebben we in **hoofdstuk IV** geprobeerd om kanker gerelateerde eiwitten met gewijzigde expressie dan wel fosforylering of signaal routes te identificeren bij luminale borstkankers die *PIK3CA* mutaties bevatten. Daartoe werden in silico data, van het TCGA consortium, gebruikt van 361 primaire tumoren van patiënten met borstkanker. Hieruit bleek dat verhoogde fosforylering van MAPK1/3, p38 en YAP en verlaagde expressie van p70S6K en 4E-BP1 bij luminale borstkanker in verband kon worden gebracht met *PIK3CA* mutaties. Het gefosforyleerde MAPK1/3, het meest significant verhoogde eiwit voor *PIK3CA* exon specifieke mutaties, werd verder bestudeerd in weefselmicroarrays van 721 primaire borstkanker weefsels. In een subset van 290 luminale tumoren met systemisch onbehandelde LNN patiënten is de relatie met metastase vrij overleving geevalueerd. We laten zien dat vooral nucleaire localisatie van MAPK1/3 fosforylering een potentiële waarde heeft als een prognostische biomarker. Onze bevindingen wijzen erop dat **fosforylering van MAPK1/3 en de subcellulaire lokalisatie ervan associeert met een gunstige ziekte-uitkomst**.

Tot aan dit hoofdstuk was *PIK3CA* mutatie ons uitgangspunt om biomarkers te vinden met potentiële prognostische of voorspelling op anti-hormonale behandeling. In het tijdperk van immuuntherapie, hebben we in **hoofdstuk V** een subgroep patiënten met inflammatoire borstkanker geevalueerd (ER-positieve IBC) die juist vaker resistent zijn tegen anti-hormonale therapie in vergelijking met ER-positieve niet-IBC-patiënten. Ook hier zijn *PIK3CA* mutaties aanwezig in IBC, en er zijn geen significante verschillen in prevalentie waargenomen tussen IBC en niet-IBC. Gebaseerd op dit en daar anti-hormonale therapie ook afhankelijk kan zijn van kankerfenotype, hebben we deze IBC met niet-IBC-weefsels vergeleken. **We hebben een metagen van zes genen (*ABAT, ADAMDEC1, CLEC7A, ETS1, ITK* en *STC2*) geïdentificeerd die IBC kan onderscheiden van niet-IBC**. Dit metagen en de individuele genen werden verder bestudeerd in 6 onafhankelijke in silico datasets en gevalideerd met behulp

van qRT-PCR in een cohort van MBC patiënten die eerste lijns tamoxifen kregen. Er werd aangetoond dat **het metagen, en de afzonderlijke genen *ABAT* en *STC2* niet prognostisch waren**. **De verlaagde expressie van *ABAT* en *STC2* was geassocieerd met een slechte anti-hormonale therapie-uitkomst in MBC** en dat alleen *ABAT*-expressie was gerelateerd aan de uitkomst bij patiënten die adjuvante tamoxifen kregen.

In het huidige tijdperk van *liquid biopsies* en persoonsgerichte/precisie geneeskunde, richtten wij ons in **hoofdstuk VI** op de karakterisatie van tumor-specifieke mutaties in celvrij DNA (cfDNA) die uit sequentiële sera die van MBC-patiënten werden verkregen tijdens de behandeling met tamoxifen. We hebben aangetoond dat mutaties kunnen worden gedetecteerd in kleine hoeveelheden serum waaruit het celvrij DNA werd geisoleerd. Dit werd middels *next generation sequencing* (NGS) met behulp van een gerichte ion-PGM genpaneel geanalyseerd. Bovendien is cfDNA stabiel en kan worden geanalyseerd in bloedmonsters die bijna twee decennia zijn opgeslagen. In deze verkennende studie **hebben we mutaties geïdentificeerd in *PIK3CA, TP53, AKAP9, CREBBP* en *SMAD4*, die (behalve *AKAP9*) aanwezig waren in zowel de primaire tumor als in het serum cfDNA verkregen bij ziekteprogressie**. In de meeste gevallen werden deze mutaties echter niet gedetecteerd in de sera voorafgaand aan progressie van de ziekte, ook niet na meer gevoeligere re-sequentie bepalingen. Interessant was dat één casus een *PIK3CA*-mutatie had, die werd teruggevonden in cfDNA van serum dat twee jaar voor de diagnose uitzaaiing werd genomen. Het werd in lage frequentie gedetecteerd door NGS, maar bevestigd door digitale PCR.

Concluderend, in dit proefschrift laat ik zien dat *PIK3CA* mutaties gedetecteerd in primaire borsttumoren wel een voorspellende waarde hebben voor AI respons van uitgezaaide ziekte, maar niet voor tamoxifen behandeling noch voor prognose. Gerelateerd aan dit *PIK3CA* genotype hebben we in primaire borsttumoren aangetoond dat de expressie van *LRG1* kan worden gebruikt als potentiële biomarker voor AI therapie uitkomst, en ook dat *LGR1* verlaagd was bij neo-adjuvante AI behandelde patiënten met klinische respons. Op het proteoom niveau liet ik zien dat de MAPK1/3 fosforyleringsniveaus bij luminale borstkanker gerelateerd zijn met *PIK3CA* exon specifieke mutaties. Deze MAPK1/3 fosforylering, met name gelokaliseerd in de kernen, heeft ook een prognostische waarde in borstkanker. Daarnaast is met behulp van een alternatieve benadering om biomarkers te ontdekken voor resistentie tegen endocriene therapie, een metagen geconstrueerd om ER-positieve borstkanker te identificeren met een IBC-fenotype. Dit metagen en de individuele genen *ABAT* en *STC2* bleken voorspellend te zijn voor de resistentie tegen tamoxifen en AI in de uitgezaaide ziekte. Bij de adjuvante behandeling was alleen ABAT gerelateerd aan tamoxifen resistentie. Tenslotte hebben we de waarde van tumor-specifieke mutaties in serum cfDNA verkend als mogelijke biomarkers voor tamoxifen resistentie bij uitgezaaide (MBC) patiënten. Mutaties in *PIK3CA, TP53, AKAP9, CREBBP* en *SMAD4* werden in de primaire tumor getoond en in de meeste gevallen alleen in serum cfDNA afgenomen bij progressie van de ziekte.

Alle bovenstaande potentiële biomarkers die in dit proefschrift worden beschreven, zullen hopelijk de selectie van patiënten verbeteren die al dan niet van anti-hormonale

therapie profijt mogen hebben. Bij deze patiënten kunnen ongewenste bijwerkingen als gevolg van onnodige blootstelling aan therapie vermeden worden. Zo kunnen we met deze biomarkers **beter de juiste patiënt kiezen voor de juiste endocriene behandeling.** Hoopvol voor de nabije toekomst, wanneer deze biomarkers meer uitgebreid worden gevalideerd door anderen, zullen we uiteindelijk de **juiste behandeling voor de juiste borstkankerpatiënt kunnen kiezen.**

RESUMEN

El cáncer de mama, una enfermedad heterogénea, es el tipo de cáncer más común en mujeres y el segundo más común en todo el mundo. Guías de tratamiento para esta enfermedad se basan en factores clínico-patológicos pronósticos y predictivos al igual que en biomarcadores tumorales tales como receptor de estrógenos (ER), receptor de progesterona (PR) y receptor HER2. La mayoría de los cánceres de mama son ER-positivo (75-80%). Para su tratamiento se usan tres tipos de terapia anti-hormonal: Moduladores selectivos del receptor de estrógenos (SERMs), degradadores selectivos del receptor de estrógenos (SERDs) e inhibidores de aromatasa (AI). El tamoxifeno, un SERM, ha sido por más de 40 años el tratamiento clave para mujeres pre-menopáusicas. Por otro lado, los AI que previenen la síntesis de estrógenos, han sido usados por algo más de 20 años y han sido principalmente efectivos para tratar a mujeres posmenopáusicas. Finalmente, los SERDs como el fulvestran, son usados hoy en día como terapia para pacientes con resistencia adquirida a los AI debido a mutaciones adquiridas en el dominio de unión al ER.

Es importante para los objetivos de esta tesis, que las tasas de respuesta objetiva a la terapia anti-hormonal para pacientes de cáncer de mama metastásico es solo 20-40%, con una duración media de aproximadamente 8-14 meses. Esto muestra una necesidad clara y urgente de encontrar biomarcadores capaces de identificar pacientes que se beneficiarán o no de la terapia; de este modo se podrá evitar la exposición innecesaria a efectos adversos no deseados en los pacientes en quienes la terapia anti-hormonal no surtirá efecto.

El objetivo de la presente tesis fue encontrar biomarcadores con potencial, capaces de predecir respuesta y/o resistencia al tratamiento anti-hormonal, principalmente en pacientes de cáncer de mama ER avanzado. Para lograr este objetivo, se usaron diferentes estrategias, teniendo en cuenta que las mutaciones *PIK3CA* son las más prevalentes en tumores luminales y ocurren hasta en un 45% de los cánceres de mama ER positivo (ver introducción), el enfoque se centró básicamente en la relación entre el genotipo *PIK3CA* y la ruta PI3K con el resultado del tratamiento.

Primero, en el **capítulo II**, se estudió el efecto de la mutación *PIK3CA* en relación con la supervivencia libre de progresión (PFS) en tumores primarios de 531 pacientes MBC luego de seguir la primera línea de tratamiento con AI o tamoxifeno. Se encontró que pacientes con la mutación *PIK3CA* comparados con los silvestres tienen una PFS significativamente más larga luego de seguir la terapia de primera línea con AI. Por lo que la mutación *PIK3CA* fue propuesta como biomarcador potencial de predicción para la primera línea de tratamiento con AI. Adicionalmente, no se encontró dicha asociación tras la primera línea de tratamiento con tamoxifeno. Por lo tanto, **la mutación *PIK3CA* no tiene valor predictivo de respuesta ni resistencia para la primera línea de tratamiento con tamoxifeno**. Dicha mutación también se estudió en relación con la supervivencia sin metástasis (MFS) en 342 pacientes con ganglio linfático negativo sin terapia sistémica neo-adyuvante y se demostró que la **mutación *PIK3CA* no tiene valor como biomarcador pronóstico**.

En el **capítulo III**, se investigó el perfil de expresión de ARN mensajeros (mRNAs) y micro ARNs (miRs) como biomarcadores potenciales de predicción correlacionados

con el genotipo *PIK3CA* y con el resultado tras la terapia. En primer lugar, la expresión de mRNAs y miRs de tumores primarios de mama fue investigada en relación con el genotipo *PIK3CA* en dos grupos de descubrimiento independientes. Un grupo incluyó 286 muestras luminales de datos *in silico* del consorcio TCGA, mientras que el segundo grupo de datos incluyó 84 muestras ER-positivas de pacientes MBC tratados con la primera línea de AI. La expresión de 9 mRNAs (*CCNO, FAM81B, LRG1, NEK10, PLCL1, PGR, SERPINA3, SORBS2, VTCN1*) y 3 miRs (miR-449a, miR-205-5p, miR-301a-3p) se encontró relacionada con el estatus de la mutación *PIK3CA* en ambos grupos. Análisis posteriores revelaron que **la expresión de *LRG1* y *PLCL1* está relacionada con una PFS más larga tras la terapia de primera línea con AI**, independientemente del subtipo luminal y de factores tradicionales de predicción. Posteriormente, estos 9 mRNAs y 3 miRs fueron evaluados en una base de datos *in silico* pública de 25 pacientes de cáncer de mama quienes recibieron AI neo-adyuvante. Se observó que en pacientes con respuesta clínica, los niveles de expresión de 3 genes (*LRG1, PGR, SERPINA3*) mostraron disminuir significativamente después del tratamiento neo-adyuvante con AI. Con base en estos hallazgos, ***LRG1* fue propuesto como biomarcador potencial independiente del subtipo luminal relacionado con el genotipo *PIK3CA* y el resultado tras la terapia con AI**.

A continuación, en el **capítulo IV**, el objetivo fue identificar proteínas de vías de señalización asociadas con cáncer cuya expresión o fosforilación estuviese alterada en cáncer de mama luminal con mutaciones *PIK3CA*. Para ello, se utilizaron datos *in silico* de 361 tumores primarios de pacientes de cáncer de mama tomados del consorcio TCGA. Aquí se mostró que una fosforilación aumentada de MAPK1/3, p38 y YAP y una expresión disminuida de p70S6K y 4E-BP1 en cáncer de mama luminal, se relaciona con la mutación *PIK3CA*. La proteína MAPK1/3 fosforilada, la cual fue encontrada sobre regulada en tumores con mutaciones *PIK3CA* exón específicas, fue estudiada con mayor detalle haciendo uso de microarreglos de tejidos de 721 especímenes de cáncer de mama, incluyendo un subgrupo de 290 tumores tipo luminal de pacientes con ganglio linfático negativo no tratados sistémicamente, para evaluar su relación con la supervivencia sin metástasis. Se mostró que especialmente la fosforilación MAPK1/3 nuclear tiene un valor potencial como biomarcador pronóstico. Estos resultados indican que **la fosforilación de MAPK1/3 y su localización subcelular están asociadas con un desenlace favorable de la enfermedad**.

Hasta este capítulo, la mutación *PIK3CA* había sido el punto de partida para encontrar biomarcadores con valor potencial pronóstico y predictivo luego del tratamiento anti-hormonal. En la época de la inmunoterapia, en el **capítulo V**, se evaluó un subgrupo de pacientes con cáncer de mama inflamatorio (IBC ER-positivo), quienes son resistentes con mayor frecuencia a la terapia anti-hormonal comparados con los pacientes ER-positivo no-IBC. Las mutaciones *PIK3CA* están presentes también en IBC; sin embargo, no se han observado diferencias significativas entre IBC y no-IBC. Con base en esto y teniendo en cuenta que la resistencia a la terapia anti-hormonal dependerá también del fenotipo del cáncer, se compararon especímenes de IBC con no-IBC. **Se identificó un metagen de seis genes (*ABAT, ADAMDEC1, CLEC7A, ETS1, ITK* y *STC2*), el cual es capaz de distinguir IBC de no-IBC**. Este metagen, así como sus genes individuales fueron posteriormente evaluados en seis bases de

datos *in silico* independientes y validados usando qRT-PCR en una cohorte de pacientes MBC, quienes habían recibido tamoxifeno como primera línea de tratamiento. Se mostró, que **el metagen**, al igual que los genes individuales ***ABAT*** **y** ***STC2*** **no tienen valor pronóstico**, además que **la expresión disminuida de** ***ABAT*** **y** ***STC2*** **se asocia con un desenlace desfavorable luego de la terapia anti-hormonal en pacientes MBC**. Se mostró también que solo la expresión de *ABAT* se relacionó con el resultado en pacientes que recibieron tamoxifeno como terapia adyuvante.

Finalmente en el **capítulo VI**, en la era de las biopsias líquidas y medicina personalizada, el objetivo fue caracterizar mutaciones tumor-específicas en ADN libre de células (cfDNA) a partir de suero tomado secuencialmente de pacientes MBC mientras estaban bajo tratamiento con tamoxifeno. Como prueba de concepto, se demostró que diferentes mutaciones pueden ser detectadas en cantidades ínfimas de AND en suero libre de células usando secuenciación de nueva generación (NGS) con un panel gen dirigido mediante el uso de la tecnología ion-PGM. Adicionalmente, el cfDNA puede ser analizado en muestras de sangre almacenadas por casi dos décadas. En este estudio exploratorio, **se identificaron mutaciones en** ***PIK3CA, TP53, AKAP9, CREBBP*** **y** ***SMAD4*****, las cuales (excepto** ***AKAP9*****) estuvieron presentes tanto en el tumor primario como en el suero cfDNA tomado durante la progresión de la enfermedad**. Sin embargo, en la mayoría de los casos, estas mutaciones no fueron detectadas en sueros precedentes a la progresión, ni siquiera tras una re-secuenciación más profunda. Cabe notar que en un caso con presencia de la mutación *PIK3CA*, se rastreó el cfDNA en suero tomado dos años antes del diagnóstico de lesiones metastásicas y se logró detectar la mutación en unas pocas lecturas usando NGS, aunque no fue confirmada con PCR digital.

En conclusión, en esta tesis se mostró que las mutaciones *PIK3CA* detectadas en tumores primarios de mama tienen un valor predictivo de respuesta a AI en estado avanzo de la enfermedad. Por el contrario, no tienen valor predictivo de respuesta a tamoxifeno, ni tampoco valor pronóstico. En relación con el genotipo *PIK3CA*, se demostró que en tumores primarios de mama, la expresión del gen *LRG1* puede ser usada como un biomarcador potencial de respuesta a la terapia con AI. Dicho biomarcador mostró disminuir sus niveles en pacientes con respuesta clínica bajo tratamiento neo-adyuvante con AI. A nivel proteómico, el grado de fosforilación de MAPK1/3 en cáncer de mama de tipo luminal mostró estar relacionado con mutaciones *PIK3CA* exón específicas. La MAPK1/3 fosforilada, especialmente cuando se localiza en el núcleo, también tiene un valor pronóstico en cáncer de mama. El siguiente paso fue descubrir biomarcadores de resistencia a la terapia endocrina mediante una estrategia alternativa, para lo cual fue construido un metagen capaz de identificar cáncer de mama ER-positivo con un fenotipo IBC. Este metagen y sus genes individuales *ABAT* y *STC2* mostraron ser capaces de predecir resistencia a las terapias con AI y tamoxifeno en pacientes con enfermedad avanzada. Mientras que en el contexto adyuvante, solo *ABAT,* se encontró relacionado con resistencia a tamoxifeno. Finalmente, se exploraron mutaciones tumor-específicas en suero cfDNA como biomarcadores potenciales de resistencia a tamoxifeno en pacientes MBC. Mutaciones en *PIK3CA, TP53, AKAP9, CREBBP* y *SMAD4*, fueron identificadas en suero cfDNA tomado durante la progresión de la enfermedad. Todas las mutaciones (excepto *AKAP9*) fueron halladas también en el tumor primario.

Cabe esperar que todos los biomarcadores potenciales descritos anteriormente en esta tesis, mejoren la identificación de pacientes quienes se beneficiarán o no de la terapia anti-hormonal. Para estos últimos pacientes quienes no se beneficiarán del tratamiento, los efectos adversos no deseados debidos a exposición innecesaria a la terapia podrán evitarse. De este modo, con estos biomarcadores hoy podemos **seleccionar el paciente adecuado para el tratamiento endocrino adecuado**, con la promesa que en un futuro no muy lejano, una vez los biomarcadores estén más extensamente validados por otros, sea posible finalmente **seleccionar el tratamiento adecuado para el paciente de cáncer de mama adecuado**.

ZUSAMMENFASSUNG

Brustkrebs, die am häufigsten auftretende Krebsart bei Frauen und die zweithäufigste weltweit, ist eine sehr heterogene Krankheit. Richtlinien zur Behandlung basieren auf prognostischen und prädiktiven Faktoren, sowie Tumor-Biomarkern wie zum Beispiel Östrogenrezeptor (ER), Progesteronrezeptor (PR) und HER2-Rezeptor. Die meisten Mammakarzinome sind ER-positiv (75-80%), für die es drei verschiedene Arten der antihormonellen Therapie gibt: selektive Östrogenrezeptormodulatoren (SERMs), selektive Östrogenrezeptorabbauer (SERDs) und Aromataseinhibitoren (AIs). Tamoxifen, ein SERM, ist seit 40 Jahren das Schlüsselelement der Therapie bei prämenopausalen Frauen. Aromataseinhibitoren, welche seit 20 Jahren genutzt werden, verhindern die Östrogensynthese und wurden am effektivsten bei postmenopausalen Frauen zur Therapie genutzt. SERDs wie Fulvestrant werden heutzutage vor allem bei Patienten genutzt, die eine Resistenz auf AIs, aufgrund von erworbenen Mutationen in der ER-Bindungsdomäne entwickelt haben.

Für die Ziele dieser Dissertation war die Tatsache wichtig, dass ER-positive, metastasierende Mammakarzinome (MBC) nur in 20-40% der Fälle auf eine antihormonelle Therapie ansprechen und dann auch nur mit einer medianen Dauer von 8-14 Monaten. Dies zeigt die Notwendigkeit Biomarker zu finden, welche es ermöglichen, Patienten die von der Therapie profitieren oder nicht profitieren würden, zu identifizieren. Somit könnten die Patienten, die nicht von einer Behandlung profitieren, vor den negativen Folgen einer unnötigen (antihormonellen) Therapie und ihren unerwünschten Nebenwirkungen geschützt werden.

Das Ziel dieser Dissertation war es, potenzielle Biomarker vor allem für fortgeschrittene ER-positive Mammakarzinome zu finden, die das Ansprechen oder die Resistenz auf antihormonelle Therapien besser vorhersagen können. Zu diesem Zweck wurden mehrere verschiedene Forschungsansätze verfolgt. Da Mutationen des *PIK3CA*-Gens die vorherrschenden Mutationen bei luminalen Tumoren sind und in bis zu 45% der ER-positiven Tumoren vorkommen (siehe Einleitung), konzentriert sich diese Dissertation auf die Beziehung zwischen dem *PIK3CA*-Genotyp und dem PI3K-Signalweg in Bezug auf das Therapieergebnis.

Im **zweiten Kapitel** untersuchten wir den Effekt der *PIK3CA*-Mutation in Primärtumoren auf das progressionsfreie Überleben von 531 MBC-Patienten, nach einer Erstlinientherapie von AIs oder Tamoxifen. Es zeigte sich, dass Patienten mit einer *PIK3CA*-Mutation ein deutlich längeres progressionsfreies Überleben hatten, wenn sie mit AIs therapiert wurden als bei Wildtyptumoren. Daher schlugen wir die *PIK3CA*-Mutation als potenziellen prädiktiven Biomarker für die Sensitivität der AI-Erstlinientherapie vor.
Im Gegensatz dazu wurde kein Zusammenhang mit dem Ergebnis bei einer Erstlinientherapie mit Tamoxifen gefunden. Dementsprechend hat **die *PIK3CA*-Mutation keinen prädiktiven Wert für die Resistenz oder Sensitivität auf die Tamoxifen-Erstlinientherapie**. Wir untersuchten die Mutation auch in Bezug auf das metastasenfreie Überleben (MFS) in 342 systemischen (neo-) adjuvanten-Therapie-

naiven LNN-Patienten und zeigten, dass die ***PIK3CA*-Mutation keinen Nutzen als prognostischer Biomarker** darstellt**.**

Im **dritten Kapitel** untersuchten wir ob mRNA- und miR-Expressionsprofile als potenzielle prädiktive Biomarker mit dem *PIK3CA*-Genotyp und dem AI-Therapieergebnis korrelieren. Zuerst wurde die Expression von mRNAs und miRs in primären Mammakarzinomen, im Zusammenhang mit dem *PIK3CA*-Genotyp, in zwei unabhängigen Versuchskohorten untersucht. Die erste Kohorte enthielt 286 luminale Proben aus *in silico* Datensätzen des TCGA-Konsortiums. Die zweite enthielt 84 ER-positive Proben von MBC-Patienten, die mit einer AI-Erstlinientherapie behandelt wurden. In beiden Kohorten konnte ein Zusammenhang zwischen dem *PIK3CA*-Mutationsstatus und der Expression von neun mRNAs (*CCNO*, *FAM81B*, *LRG1*, *NEK10*, *PLCL1*, *PGR*, *SERPINA3*, *SORBS2*, *VTCN1*) sowie drei miRs (miR-449a, miR-205-5p, miR-301a-3p) nachgewiesen werden. Weitere Analysen zeigten**, dass die Expression von *LRG1* und *PLCL1* mit einem längeren progressionsfreien Überleben nach AI-Erstlinientherapie assoziierten**, unabhängig vom luminalen Subtyp und traditionellen prädiktiven Parametern. Als nächstes wurden diese neun mRNAs und drei miRs in einem öffentlich zugänglichen *in silico* Datenset von 25 Mammakarzinompatienten, die mit neoadjuvanter AI-Therapie behandelt wurden, geprüft. Hier zeigte sich, dass die Expression von drei Genen (*LRG1*, *PGR*, *SERPINA3*) nach neoadjuvanter AI-Therapie signifikant abgenommen hatte in Patienten, die auf die Therapie ansprachen. Basierend auf diesen Ergebnissen schlugen wir ***LRG1* als potenziellen, luminal-subtypunabhängigen, Biomarker für den *PIK3CA*-Genotyp und das AI-Therapieergebnis vor.**

Im **vierten Kapitel** wollten wir tumorassoziierte Proteine der Signalkaskaden mit veränderter Expression oder Phosphorylierung in luminalem Brustkrebs mit *PIK3CA*-Mutationen detektieren. Hierfür nutzten wir Proben eines *in silico* Datensatzes von 361 Primärtumoren von Brustkrebspatienten des TCGA-Konsortiums. Eine verstärkte Phosphorylierung von MAPK1/3, p38 und YAP, sowie eine verringerte Expression von p70S6K und 4E-BP1 in luminalen Brustkrebsproben im Zusammenhang mit *PIK3CA*-Mutationen konnte nachgewiesen werden. Phosphoryliertes MAPK1/3, das am signifikantesten hochregulierte Protein assoziiert mit *PIK3CA*-Exonmutationen, wurde in einer weiteren Testreihe mit Gewebemikroarrays aus 721 primären, luminalen Mammakarzinomen, einschließlich einer Subgruppe von 290 luminalen Tumoren von systemisch-unbehandelten LNN-Patienten, untersucht, um den Bezug zum metastasenfreien Überleben zu evaluieren. Wir konnten zeigen, dass vor allem die nukleare MAPK1/3-Phosphorylierung einen potenziellen Wert als prognostischer Biomarker hat. **Unsere Ergebnisse zeigen, dass die Phosphorylierung und subzelluläre Lokalisierung von MAPK1/3 mit einer besseren Prognose assoziieren.**

In den bisherigen Kapiteln stand immer die Mutation des *PIK3CA*-Gens als Startpunkt im Fokus, um Biomarker mit potenziellem prognostischem oder prädiktivem Wert nach Antihormontherapie zu finden. In den Zeiten der Immuntherapie, untersuchten wir in **Kapitel fünf** eine Untergruppe von Patienten mit inflammatorischem Mammakarzinom (ER-positiver IBC), welche deutlich häufiger therapieresistent auf

antihormonelle Therapien reagieren, im Vergleich zu ER-positiven non-IBC-Patienten.
PIK3CA-Mutationen kommen auch bei diesem Karzinomtyp vor, ein signifikanter Unterschied in der Prävalenz zwischen IBC und Non-IBC konnte allerdings nicht gefunden werden. Aufgrund dieser Beobachtung und da die antihormonelle Therapieresistenz auch vom Krebsphänotyp abhängig sein könnte, verglichen wir Proben von IBC-Patienten mit Non-IBC-Patienten. Wir identifizierten ein **Metagen aus sechs Genen (*ABAT*, *ADAMDEC1*, *CLEC7A*, *ETS1*, *ITK* und *STC2*), welches sich zwischen IBC und Non-IBC unterschied**. Dieses Metagen und seine individuellen Gene wurden in 6 unabhängigen *in silico* Datensätzen evaluiert und durch qRT-PCR in einer Kohorte von MBC-Patienten mit Tamoxifen als Erstlinientherapie validiert. Hierbei konnte gezeigt werden, dass **das Metagen, *ABAT* und *STC2* keinen prognostischen Wert hatten**, eine **reduzierte Expression von *ABAT* und *STC2* mit einem schlechteren antihormonellen Therapieergebnis in MBC assoziierte** und dass nur die Expression von *ABAT* bei Patienten mit adjuvanter Tamoxifentherapie relevant in Bezug auf das Therapieergebnis war.

In der heutigen Ära der Liquid-Biopsy-Analytik und der personalisierten Medizin/Präzisionsmedizin charakterisierten wir im **sechsten Kapitel**, tumorspezifische Mutationen in zellfreier DNA (cfDNA) aus den konsekutiv entnommenen Seren von MBC-Patienten, die unter Tamoxifentherapie standen. In einer Pilotstudie, zeigten wir, dass es möglich ist, Mutationen in geringen Mengen zellfreier, aus Serum gewonnener, DNA unter Verwendung eines Targeted-Ion PGM Genpanels mittels "Next generation sequencing" (NGS) nachzuweisen. Des Weiteren kann cfDNA auch in fast zwanzig Jahre alten Blutproben untersucht werden. In dieser explorativen Studie identifizierten wir **Mutationen in *PIK3CA*, *TP53*, *AKAP9*, *CREBBP* und *SMAD4*, die (mit Ausnahme von *AKAP9*) sowohl im Primärtumor als auch in der, beim Fortschreiten der Erkrankung gewonnenen, Serum-cfDNA auftraten**. In den meisten Fällen konnten die Mutationen allerdings nicht in den Seren vor der Tumorprogression nachgewiesen werden, dies war auch nicht der Fall bei tiefergehender Re-Sequenzierung. Interessanterweise wurde allerdings in einem Fall eine *PIK3CA*-Mutation, in cfDNA aus Serum, das 2 Jahre vor dem Nachweis von Metastasen abgenommen wurde gefunden. Diese Mutation wurde nur in wenigen NGS-Reads detektiert, aber konnte mit digitaler PCR bestätigt werden.

Zusammenfassend wurde in dieser Dissertation gezeigt, dass *PIK3CA*-Mutationen in primären Mammakarzinomen einen prädiktiven Nutzen für die Aromataseinhibitortherapie bei fortgeschrittener Erkrankung haben, aber jedoch keine Aussagekraft in Bezug auf Tamoxifentherapie oder Prognose besitzen. Im Zusammenhang mit dem *PIK3CA*-Genotyp konnte auch in primären Mammakarzinomen gezeigt werden, dass die Expression von *LRG1* als potenzieller Biomarker für den AI-Therapieerfolg genutzt werden kann, da *LRG1* im Rahmen einer neoadjuvanten Therapie geringere Expression zeigte, wenn die Therapie auch klinisch anschlug. Auf Proteinebene konnten wir für luminalen Brustkrebs nachweisen, dass der MAPK1/3-Phosphorylierungsgrad in Zusammenhang mit *PIK3CA*-spezifischen Exonmutationen steht. Diese Phosphorylierung von MAPK1/3 hat auch einen prognostischen Wert für Brustkrebs, vor allem, wenn sie in den Zellkernen auftritt.

Im Rahmen einer alternativen Herangehensweise um Biomarker für eine Resistenz bei der endokrinen Therapie zu finden, konstruierten wir ein Metagen um ER-positive Mammakarzinome mit IBC-Phänotyp zu identifizieren.
Dieses Metagen und seine Einzelgene *ABAT* und *STC2* waren prädiktiv für Therapieresistenz gegen Tamoxifen oder AIs im fortgeschrittenen Krankheitsstadium. Im Fall der adjuvanten Therapie war nur *ABAT* mit Tamoxifenresistenz assoziiert. Zuletzt untersuchten wir das Potenzial von tumorspezifischen Mutationen in Serum-cfDNA als Biomarker für Tamoxifenresistenz bei MBC-Patienten. Mutationen in *PIK3CA*, *TP53*, *AKAP9*, *CREBBP* und *SMAD4* konnten sowohl im Serum bei Tumorprogression als auch, mit Ausnahme von *AKAP9*, im Primärtumor nachgewiesen werden.

Alle in dieser Dissertation beschriebenen potenziellen Biomarker können hoffentlich die Identifikation von Patienten verbessern, die von einer antihormonellen Therapie profitieren oder nicht profitieren. Für Patienten, die nicht von einer Behandlung profitieren würden, könnten dann unerwünschte Nebenwirkungen, die durch eine unnötige Therapie entstehen, vermieden werden.

So können wir heute mithilfe dieser Biomarker **den richtigen Patienten für die richtige endokrine Therapie auswählen**, und hoffentlich in naher Zukunft, wenn diese Biomarker auch von Anderen ausführlich validiert worden sind, das richtige **Therapiekonzept für jeden Brustkrebspatienten individuell bestimmen.**

APPENDICES

Acknowledgements

List of Publications

PhD portfolio

About the author

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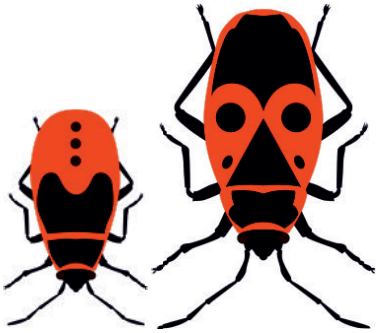
Natürlich kann ich das Lob für die tolle Cover- und Layoutgestaltung nicht überspringen. Er hat all meine Ideen miteinbezogen, verbessert und daraus ein gelungenes Cover und Layout erstellt.



LIST OF PUBLICATIONS

Ramirez-Ardila D, Timmermans AM, Helmijr JA, Martens JWM, Berns E and Jansen M. Increased MAPK1/3 Phosphorylation in Luminal Breast Cancer Related with PIK3CA Hotspot Mutations and Prognosis. Transl Oncol 2017; 10: 854-866.

Ramirez-Ardila DE, Ruigrok-Ritstier K, Helmijr JC, Look MP, van Laere S, Dirix L, Berns EM and Jansen MP. LRG1 mRNA expression in breast cancer associates with PIK3CA genotype and with aromatase inhibitor therapy outcome. Mol Oncol 2016; 10: 1363-1373.

Jansen MP, Martens JW, Helmijr JC, Beaufort CM, van Marion R, Krol NM, Monkhorst K, Trapman-Jansen AM, Meijer-van Gelder ME, Weerts MJ, **Ramirez-Ardila DE**, Dubbink HJ, Foekens JA, Sleijfer S and Berns EM. Cell-free DNA mutations as biomarkers in breast cancer patients receiving tamoxifen. Oncotarget 2016; 7: 43412-43418.

Jansen MP, Sas L, Sieuwerts AM, Van Cauwenberghe C, **Ramirez-Ardila D**, Look M, Ruigrok-Ritstier K, Finetti P, Bertucci F, Timmermans MM, van Deurzen CH, Martens JW, Simon I, Roepman P, Linn SC, van Dam P, Kok M, Lardon F, Vermeulen PB, Foekens JA, Dirix L, Berns EM and Van Laere S. Decreased expression of ABAT and STC2 hallmarks ER-positive inflammatory breast cancer and endocrine therapy resistance in advanced disease. Mol Oncol 2015; 9: 1218-1233.

Ramirez-Ardila DE, Helmijr JC, Look MP, Lurkin I, Ruigrok-Ritstier K, van Laere S, Dirix L, Sweep FC, Span PN, Linn SC, Foekens JA, Sleijfer S, Berns EM and Jansen MP. Hotspot mutations in PIK3CA associate with first-line treatment outcome for aromatase inhibitors but not for tamoxifen. Breast Cancer Res Treat 2013; 139: 39-49.

PHD PORTFOLIO

A summary of PhD training and teaching activities

Name PhD student:	Diana Esperanza Ramirez Ardila
Erasmus MC department:	Medical Oncology
Research school:	Postgraduate school of Molecular Medicine
Promotor:	Prof. dr. P.M.J.J. Berns
Supervisor:	Dr.Ir M.P.H.M. Jansen

PhD training

Courses	**Year**	**ECTS**
General courses		
Study Design	2010	4.3
Classical Methods for Data-analysis	2010	5.7
Clinical Epidemiology	2010	5.7
Methodologic Topics in Epidemiologic Research	2010	1.4
Modern Statistical Methods	2010	4.3
Specific courses		
Principles of Research Medicine	2010	0.7
Clinical Decision Analysis	2010	0.7
Methods of Public Health Research	2010	0.7
Clinical Trials	2010	0.7
Topics in Meta-analysis	2010	0.7
Pharmaco-epidemiology	2010	0.7
Genome Wide Association Analysis	2011	1.4
Principles of Genetic Epidemiology	2011	0.7
Genomics in Molecular Medicine	2011	1.4
Markers and Prognostic Research	2011	0.7
Molecular Diagnostics	2011	1.0
The Get Out of Your Lab Days 2011	2011	0.6
Technology Facilities	2011	0.9
Signal Transduction Pathways Regulating Aging and Disease	2011	1.2
The Course on Molecular Medicine	2011	0.7
The Ensemble Workshop	2011	0.5
The workshop Basic data analysis on gene expression arrays	2011	0.7
The EBI: Bioinformatics Roadshow	2012	0.9
The course on the Analysis of microarray gene expression data using R/BioC and web tools	2012	2.0
Course on R	2015	1.4

Skills Courses		
Summer Course English	2010	1.4
Introduction to Medical Writing	2011	1.1
Working with SPSS for Windows	2011	0.15
The workshop on Photoshop and Illustrator CS5	2012	0.3
Dutch A1 Beginners	2012	2.2
Dutch A2.1 Pre-Intermediate 1	2013	2.2
Dutch A2.2 Pre-Intermediate 2	2013	2.2
Presentations		
Oral Presentations		
Annual Scientific Meeting of the	2011	0.3
Journal Club	2011 - 2013	3.0
Annual JNI Scientific Lab meeting	2012	1.0
TI Pharma Consortium at Agendia meeting	2012	0.3
TI Pharma 501 kick off meeting	2013	0.3
Half yearly "Antwerpen meets Rotterdam" Scientific meeting	2013	0.3
REDH Colombia, Academic Expo 2013	2013	0.3
TI Pharma consortium at NKI meeting	2014	0.3
Poster Presentations		
Poster presentation at the annual Molecular Medicine Day	2012	1.0
Poster presentation at the annual San Antonio Breast Cancer Symposium	2012	1.0
Seminars and workshops		
Annual Molecular Medicine Day in Rotterdam	2011-2013	0.9
Sixth EORTC PathoBiology Group Annual Meeting Rotterdam	2011	0.3
Annual Cancer Genomics Centre Scientific Meeting Amsterdam	2011	0.3
Montly JNI Oncology Lectures	2011-2014	0.9
Weekly JNI Scientific Lab Meetings	2011-2014	2.2
Teaching activities		
Lecture on genomic techniques JMS	2011	0.2
Supervision intership HBO student	2012	2.0
Supervision intership Bachelor student	2012	2.0
Virtual class	2016	2.0
Total ECTs		**67.85**

ABOUT THE AUTHOR

Diana Esperanza Ramirez Ardila was born on March 14th 1983 in Bogota, capital of Colombia, located in South America. In September 2005, she obtained her Bachelor of Sciences (BSc) degree in Pharmaceutical Chemistry - Pharmacy from the National University of Colombia, Bogota. One month before her graduation she already started working as a Pharmacy manager at *Medicamentos del dos mil,* located in the same city. She implemented essential standard operating procedures (SOPs) improving the overall quality service of the pharmacy. Being there, she got involved in phase III double blinded clinical trials. In November 2006, she started working as a Clinical trial Pharmacist at the Rheumatology clinic *Riesgo de Fractura*. There, she initiated different clinical trials ensuring the site to meet all regulatory requirements. In July 2007, she started a simultaneous job in the National Institute of Legal Medicine and Forensic Sciences in the toxicology department. Passionate by the toxicology field, she decided to start an International Magister in Toxicology - distance learning program with the Ilustre Colegio de Químicos de Sevilla – Spain. She graduated in January 2010. In August 2009 she was awarded with the Award of Excellence in Scientific Research by the National Institute of Legal Medicine and Forensic Sciences. With this award, she presented her poster on an analytical methodology that she had developed for the determination of anticonvulsants in whole blood and urine using liquid-liquid extraction and GC/MS in the 47th International Meeting TIAFT in Geneva –Switzerland.

In December 2009, for three months, she did an internship in cellular and molecular biology. She worked on the translocation of connective tissue growth factor (CTGF) during apoptosis of endothelial cells at the University of Montréal – Canada under the supervision of Prof. dr. Marie Joseé Herbert, director of the University of Montréal Transplant Program. Inspired by the research surroundings she discovered being there, she decided to continue her research education. Granted by the ERACOL program in August 2010 Diana started her MSc/PhD program in the Integrated genomics of therapy resistance group under the supervision of Prof.dr. Els Berns and mentored by dr. ir. Maurice Jansen. In September 2011 she obtained her Master of Science (MSc) in Health Sciences, with specialisation Clinical Epidemiology, degree at Erasmus University of Rotterdam.

Motivated by the project she was working on during her Master program (Picking the right patient for aromatase inhibitors treatment in breast cancer) she decided to continue her PhD on the same research topic. During her PhD research, Diana got two awards: The first one, on the Molecular Medicine Day in February 2009 for her work entitled “Picking the right patient for Aromatase and PI3K inhibitors treatment in breast cancer”. In December 2012 she got a second (travel) award to attend the 2012 CTRC-AACR San Antonio Breast Cancer Symposium (SABCS) to present her poster

entitled “Hotspot mutations in PIK3CA predict treatment outcome on aromatase Inhibitors but are not predictive for tamoxifen”. Her PhD research has led to five published scientific articles, three of them as a first-author.

Since she always has looked for balance in life, in April 2013, she started in the striking world of Circus arts. In June 2014, she decided to do volunteering work in the Social Innovation Academy -SINA in Mpigi, Uganda. She was driven by need to have a higher and direct impact on the society. There, she worked on empowering facilitators and scholars in health and self-awareness topics. In Uganda she met Martin and in 2016, their baby, Luna Sofia was born, who brought her back to life.