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TESIS DOCTORAL

# MOLECULAR ALTERATIONS IN METASTATIC BREAST CANCER AND EFFICACY OF PI3K/AKT/MTOR INHIBITORS IN EARLY PHASE CLINICAL TRIALS

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UAB
Universitat Autònoma de Barcelona

UAB - Facultad de Medicina. Programa Doctorado en Medicina I 2017

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*A mis padres y a mi hermano*
*A José Miguel, Julián, y a mis amigas de Casa*

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*Y a todas las pacientes, que con entereza, coraje y dignidad lucháis cada día para vencer el cáncer. Sois la inspiración y el motor de nuestro trabajo.*

*“The most beautiful experience we can have is the mysterious.*
*It is the fundamental emotion that stands at the cradle of true art and true science.*
*Whoever does not know it and can no longer wonder, no longer marvel,*
*is as good as dead, and his eyes are dimmed”*

*Albert Einstein,* "The World As I See It," *1930*

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# 1
# Introduction and Background

# 1. Introduction and Background

## 1.1. Precision medicine in oncology

Precision medicine is an evolving field where detailed information on the molecular characteristics of the disease and its host are used to determine which therapies will work best for each individual patient. The aspiration of delivering the patient the most personalized treatment possible has existed since the very birth of medicine itself. Hippocrates combined an assessment of the four humors – blood, phlegm, yellow bile, and black bile – to determine the cause and the best treatment for each patient[1]. While the first connection between genetic inheritance and susceptibility to a disease (alkaptonuria) was described in 1902 by Sir Archibald Garrod[2], real momentum toward personalized medicine was marked in 2003 with the complete sequencing of the human genome. Back in the early days, sequencing was both time consuming and expensive but now, thanks to tremendous technical progress, massive sequencing has rapidly evolved; today complete sequencing of a human genome takes between 24-48h with a price tag of less than $5,000. We are also now moving beyond the genome into the entire spectrum of molecular medicine, including the proteome, metabolome, and epigenome. All these data must be interpreted and integrated within the context of precision medicine.

When applied to cancer, precision medicine involves the use of molecular characteristics of the tumor and its microenvironment, integrated with patient's characteristics, in order to match therapies to the specificities of individual patients, and treat cancer more effectively and with less toxicity[3].

The ultimate goal in identifying biologically important genes and pathways disrupted in cancer is the generation of clinically relevant diagnostic, prognostic, and therapeutic data that can serve as the basis of delivering precision medicine. The aberrations that meet these criteria are often referred to as "actionable"[4], and if they are also potentially targets for therapeutic development, they are known as "druggable"[5].

Success stories of matching treatment with targeted drugs and recurrent molecular alterations in different tumor types include endocrine therapy for hormonal receptor positive breast cancer[6], anti-HER2 drugs (trastuzumab, pertuzumab, T-DM1) in *ERBB2* amplified breast cancer[7-9], imatinib for chronic myeloid leukemia with BCR-ABL fusion gene[10] and in KIT-mutant gastrointestinal stromal tumors[11], gefitinib and erlotinib in patients with EGFR-mutant lung cancer[12,13], vemurafenib[14] and double BRAF and MEK inhibition[15] for *BRAF* V600E mutant melanoma, and crizotinib[16] and ceritinib[17] for non-small cell lung cancer (NSCLC) with EML4-ALK translocation.

### 1.2. Metastatic breast cancer: epidemiology and treatment goals

Breast cancer is the most frequently diagnosed cancer worldwide and the second leading cause of cancer mortality among females. In the United States (US), it is estimated that 246.660 women were diagnosed in 2016, accounting for 29% of all cancer cases (Figure 1)[1].

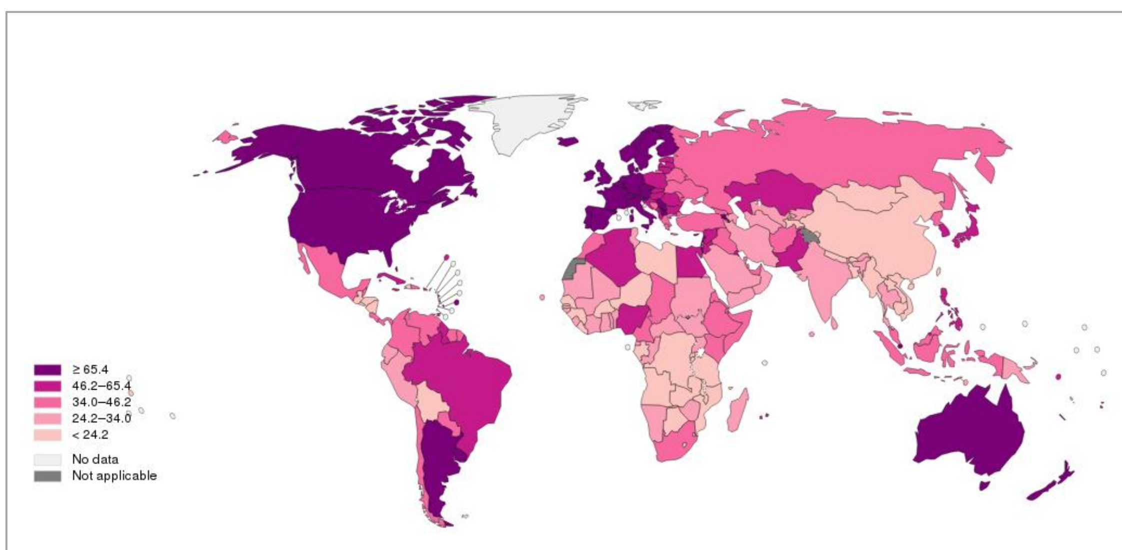


**Figure 1**: Estimated age-standardized incidence cases of breast cancer (women) worldwide (2012). Data source: GLOBOCAN 2012. Map production: IARC (http://gco.iarc.fr/today), World Health Organization.

Despite being the most frequent cancer in women, it follows lung cancer as the most frequent cause of death from cancer in females, accounting for 14% of total cancer deaths[18]. In Europe, the 5-year relative survival rate for women diagnosed

with breast cancer from 2000-2007 was 81.8%, reaching 82.8% in Spain (Figure 2)[19].

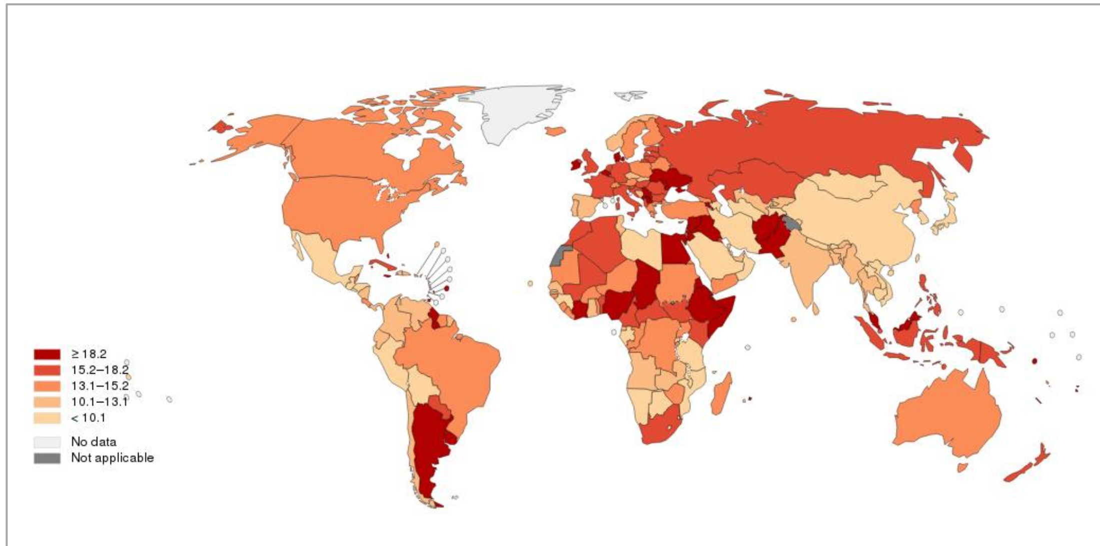


**Figure 2:** Estimated age-standardized rates of deaths from breast cancer (women) worldwide (2012). Data source: GLOBOCAN 2012. Map production: IARC (http://gco.iarc.fr/today), World Health Organization.

These numbers suggest that most breast cancers, especially in developed regions, are nowadays diagnosed in early, curable stages. Classically, the decrease in mortality from breast cancer has been attributed to the implementation of large scale population-based screening programs[20], although this concept has been questioned over recent years[21].

Despite the decrease in mortality from breast cancer, up to 25-40% of patients will eventually develop recurrence or metastases during their lives[22]. Additionally, 5-6% of patients will have overt metastatic disease at the time of breast cancer diagnosis[23].

Metastatic breast cancer (MBC) is a treatable yet virtually incurable condition[24]. In the last few decades, a constant improvement in overall survival (OS) of women affected with MBC has been observed, but median OS is still only 2-3 years, although this range may in some cases be wider[25,26]. As patients live longer, it is essential to preserve their quality of life throughout the course of their disease and therapy.

The two main goals of care in MBC are to extend survival and optimize quality of life[26]. The management of MBC is complex, and therefore crucially involves all related specialties working together as multidisciplinary teams (including medical oncologists, radiation oncologists, surgical oncologists, imaging experts, pathologists, psychologists, social workers, nurses, palliative care experts, among others)[27].

Benefits in OS in MBC have a multifactorial origin, but key factors behind improved OS are increased insights into the mechanism that drives the disease, and a better management of both targeted therapies and cytotoxic agents. Data of HER2+ MBC patients treated with trastuzumab suggest that survival may be higher for patients treated in specialized institutions[28]. For this reason, if at all possible, management of MBC patients by multidisciplinary teams in specialized institutions should be encouraged[26].

Research remains a priority in this setting. Several international guidelines recommend participation in well-designed, independent, prospective trials as a treatment option to all eligible patients, whenever possible[26,27].

### 1.3. Classification of breast cancer

Breast cancer is a heterogeneous disease. The discovery of the estrogen receptor (ER)[29] and the human epidermal growth factor receptor-2 (HER2)[30] as drivers of breast carcinogenesis has shed important light on different breast cancer subtypes and led to the development and use of targeted agents against these receptors. In 2000, Perou and Sørlie described the messenger RNA (mRNA)-based intrinsic subtypes of breast cancer and this classification has changed the way we understand the disease[31]. In recent years, an increasing body of information regarding the mutational and copy number variation (CNV) landscape of breast cancer has also emerged.

We will now briefly discuss each of these tremendous advances in our understanding of breast cancer biology.

### *1.3.1. Targeting ER and HER2*

ER is a nuclear protein that acts as a transcription factor. Upon physiological conditions, and after activation by its ligand, ER binds DNA and promotes gene transcription. It can also assume additional functions that are independent of DNA-binding[32]. The two most common isoforms of ER are ERα and ERβ, codified by the genes *ESR1* (6q25.1) and *ESR2* (14q23.2), respectively. Although both ERα and ERβ are widely expressed in different tissues, ERα is preferentially expressed in endometrium, breast, ovarian stromal cells, and hypothalamus, while ERβ is found in ovarian granulosa cells, kidney, brain, bone, heart, lungs, intestinal mucosa, prostate, and endothelial cells[33].

Binding of estrogen to the ER stimulates proliferation of mammary cells, with the resulting increase in cell division and DNA replication, which may lead to the development of mutations that can cause disruption of cell cycle, apoptosis and DNA repair and, eventually, tumor formation[34]. ER is overexpressed in around 70% of breast cancer cases, referred to as ER-positive (ER+) tumors[35].

Tamoxifen, a selective ER modulator with antagonist properties in the breast tissue, was the first widely used targeted therapy in breast cancer[6]. Tamoxifen improves the outcomes of ER+ breast cancer in both the early and metastatic settings. The meta-analysis reported by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) analyzed data from 10.645 women with ER+ breast cancer treated with tamoxifen or with placebo[36]. In this study, 5 years of adjuvant tamoxifen reduced the 15-year risk of breast cancer recurrence (relative risk [RR] 0.53 during years 0-4 and RR 0.68 during years 5-10, both $2P<0.00001$) and death (RR 0.71 during years 0-4 and RR 0.66 during years 5-9, and 0.68 during years 10-14; $P<0.0001$ for extra mortality reduction during each separate time period), irrespective of the use of chemotherapy, age, progesterone receptor (PgR) status, or other tumor characteristics.

Aromatase inhibitors (AI), such as letrozole, anastrozole, or exemestane, further improve these results, resulting in a proportional reduction of recurrence rates and mortality by about 30% and 15%, respectively, when compared to tamoxifen in the adjuvant treatment of post-menopausal breast cancer patients[37].

HER2 is a transmembrane protein encoded by the *ERBB2* gene, located in chromosome 17q21. HER2 is a member of the human epidermal growth factor receptor (EGFR/ERBB) family and a well-recognized oncogene in breast cancer[38]. Approximately 20-25% of all breast cancers have amplification or overexpression of HER2, which is a marker of worse prognosis[30].

Targeting the HER2 receptor in HER2-positive (HER2+) breast cancer has also dramatically changed the natural history of this subtype of disease. The pivotal trial that led to the approval of the first anti-HER2 drug tested the addition of trastuzumab (a humanized IgG1 monoclonal antibody targeting HER2) to conventional chemotherapy in 469 women with HER2+ MBC[39]. In this study, treatment with trastuzumab associated with a longer time to disease progression (median, 7.4 vs. 4.6 months; P<0.001), a higher rate of objective response (50% vs. 32%; P<0.001), and an improved survival (median survival, 25.1 vs. 20.3 months; P=0.01) when compared to placebo.

A further step in improving the prognosis of HER2+ MBC patients came with the addition of pertuzumab, a humanized monoclonal antibody that binds the subdomain II of the HER2 extracellular domain, to standard therapy[8]. The CLEOPATRA trial enrolled 808 patients to receive pertuzumab plus trastuzumab plus docetaxel or placebo plus trastuzumab plus docetaxel in the first line treatment of HER2+ MBC. In the final pre-specified OS results, pertuzumab significantly improved the median OS to 56.5 months as compared with 40.8 months in the group receiving the placebo combination (hazard ratio [HR] 0.68, 95% confidence interval [CI] 0.56-0.84; P<0.001). Median progression-free survival (PFS) as assessed by investigators also improved by 6.3 months in the

pertuzumab group (HR 0.68, 95%CI 0.58-0.80). These outstanding results led to the approval of pertuzumab in the first-line treatment of HER2+ MBC.

T-DM1 is another anti-HER2 drug that has improved outcomes of HER2+ MBC patients. With a different mechanism of action with respect to trastuzumab and pertuzumab, it is an antibody-drug conjugate that incorporates the HER2-targeted antitumor properties of trastuzumab with the cytotoxic activity of the microtubule-inhibitory agent DM1 (a derivative of maytansine); the antibody and the cytotoxic agent are conjugated by means of a stable linker[40]. In the EMILIA trial, 991 patients that previously progressed to anthracyclines, taxanes, and trastuzumab, were randomized to receive T-DM1 or lapatinib plus capecitabine[9]. Treatment with T-DM1 significantly reduced the risk of progression or death in this setting: HR for PFS 0.65 (95%CI 0.55-0.77, P<0.001; 9.6 vs. 6.4 months) and HR for OS 0.68 (95%CI 0.55-0.85, P<0.001; 30.9 vs. 25.1 months). Similar benefits were observed in the TH3RESA trial that randomly assigned 602 patients who had previously progressed to two or more HER2-directed regimens in the advanced setting and taxanes in any setting to receive T-DM1 or treatment of physician's choice (TPC)[41]. PFS significantly improved with T-DM1 compared with TPC (median 6.2 months [95%CI 5.59-6.87] vs. 3.3 months [2.89-4.14]; stratified HR 0.53, 95%CI 0.42-0.66; P<0.0001). The trial also showed positive results in terms of OS in the final results presented at the San Antonio Breast Cancer Symposium in 2015 (median OS 22.7 vs. 15.8 months; HR 0.68, 95%CI 0.54-0.85; P=0.0007)[42]. These results led to the approval of T-DM1 in patients with HER2+ MBC who previously received trastuzumab and a taxane, separately or in combination.

Regarding early stage disease, trastuzumab is routinely used in the adjuvant setting since the publication of several trials consistently showing an improvement of PFS and OS with the addition of trastuzumab to standard adjuvant chemotherapy[7,43-45]. Pertuzumab and T-DM1 are also being tested in the adjuvant setting, but results from these clinical trials are not yet available.

### *1.3.2. The intrinsic subtypes of breast cancer*

In their seminal work published initially in *Nature* in 2000, Perou and Sørlie analyzed 65 surgical samples from 42 patients using cDNA microarrays that represented 8102 human genes, and identified four different subtypes of breast cancer, based on their gene expression pattern: luminal, normal-like, basal, and HER2-enriched (HER2-E)[31]. They further refined this classification by analyzing an additional 78 samples, as well as three mammary fibroadenomas and four samples of normal breast tissue. This new analysis subdivided the luminal type (characterized by the high expression of genes associated with luminal cells from normal breast tissue) into at least two additional subtypes – Luminal A and Luminal B – according to the low or high expression of proliferation-associated genes[46].

This classification provides important prognostic information: basal and HER2-E subtypes have consistently shown to have a worse prognosis than luminal B (intermediate prognosis) and the good-prognosis luminal A tumors[46,47]. A commercially available test (PAM50 Prosigna®, Nanostring) was developed to assess the intrinsic subtypes of breast cancer, and is used together with other gene platforms such as OncotypeDX® (Genomic Health / Palex), Mammaprint® (Ferrer in Code) or Endopredict® (Myriad Genetics) to evaluate prognosis of early stage breast cancer and the potential benefit of adjuvant chemotherapy.

Surrogate immunohistochemistry (IHC) profiles from the intrinsic subtypes of breast cancer are usually applied in clinical practice to classify breast cancer. Different cut-offs for Ki67, as well as different classification of tumors according to grade or PgR status, have been used in the literature. To harmonize such classification, the St. Gallen consensus in 2013 issued various recommendations that are now widely adopted (Table 1)[48]. Although the correlation with the intrinsic subtypes is not perfect, this classification is a useful tool to routinely assess prognosis and better tailor therapy to the individual patients.

**Table 1:** Surrogate definitions of intrinsic breast cancer subtypes (St. Gallen Consensus).

| Intrinsic Subtype | Clinic-pathologic surrogate definition |
|---|---|
| **Luminal A** | **Luminal A-like:**<br>- ER and PgR positive[a] *and*<br>- HER2 negative *and*<br>- Ki67 low[b] *and*<br>- Recurrence risk "low" based on multi-gene-expression assay (if available)[c] |
| **Luminal B** | **Luminal B-like (HER2 negative)**<br>- ER positive<br>- HER2 negative<br>*and* at least one of:<br>- Ki67 high[b]<br>- PgR negative or low[a]<br>- Recurrence risk "high" based on multi-gene-expression assay (if available)[c]<br>**Luminal B-like (HER2 positive)**<br>- ER positive<br>- HER2 over-expressed or amplified<br>- Any Ki67<br>- Any PgR |
| **HER2-E** | **HER2 positive (non-luminal)**<br>- HER2 over-expressed or amplified *and*<br>- ER and PgR absent |
| **Basal-like** | **Triple negative (ductal)**<br>- ER and PgR absent<br>- HER2 negative |

[a] The added value of PgR in distinguishing between Luminal A-like and Luminal B-like subtypes derives from a work using a PgR cut-point of ≥20% to best correspond to Luminal A subtype[49].
[b] The cut-point between "high" and "low" values for Ki67 is not consensual. Cut-off of <14%[50], 20%, or local laboratory specific value may be used.
[c] Based on Reference Prat A. et al[51].
ER: estrogen receptor. HER2-E: HER2 enriched. PgR: Progesterone Receptor.

### *1.3.3. Genomic landscape of breast cancer*

Undoubtedly, the introduction of anti-ER and anti-HER2 therapies, together with a better understanding of the biology of primary breast cancers, has improved patients' outcomes. However, there is still need for improvement. Of note, no specific targeted agents have been approved whatsoever for triple negative MBC, and overall survival has remained stable since the early nineties in ER+ MBC[25],

suggesting that a deeper understanding of breast cancer biology is needed in order to optimize therapy.

Cancer genomics refers to the study of tumor genomes at different levels, including changes in the DNA sequence (copy number alterations, mutations, and rearrangements), epigenome (DNA methylation and histone modification patterns), and transcriptome (gene or microRNA expression)[52]. Tumor development may occur as a consequence of several genomic dysregulations, such as gene inactivation (by mutations, deletions, or gene promoter silencing), changes in gene expression (by methylation or copy number changes), and mutations or gene rearrangements that result in gene activation[52,53].

Recent advances in massively parallel sequencing technologies allow faster, more sensitive, and more precise analyses of cancer genomes, including breast cancer[4]. Several international initiatives including The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC) have helped to characterize the genomic alterations and the mutational processes that occur in early breast cancer (EBC)[54-56].

Using DNA copy number arrays, DNA methylation, whole-exome sequencing, mRNA arrays, microRNA sequencing, and reverse-phase protein arrays (RPPA), these studies have shown that the most frequent alterations overall in breast cancer (independently of intrinsic subtype) are *TP53* and *PIK3CA* mutations (around 28% for both genes), amplifications in *ERBB2*, *FGFR1*, and *CCND1* (10-20%), phosphatase and tensin homolog deleted on chromosome 10 (PTEN) mutations and deletions (leading to PTEN loss of expression), and *AKT1*, *RB1*, *BRCA1* and *BRCA2* mutations (Figure 3). Other less commonly mutated genes are *KRAS, APC, NF1, NF2, SKT11, MAP2K4, MAP3K1, and AKT2*[55,56]. A high proportion of mutations in noncoding regions of the genome, such as *PLEKHS1*, *TBC1D12*, and *WDR74* promoters, as well as in the *MALAT1* and *NEAT1* long non-coding RNAs have also been identified, but the clinical significance of this finding has yet to be established.

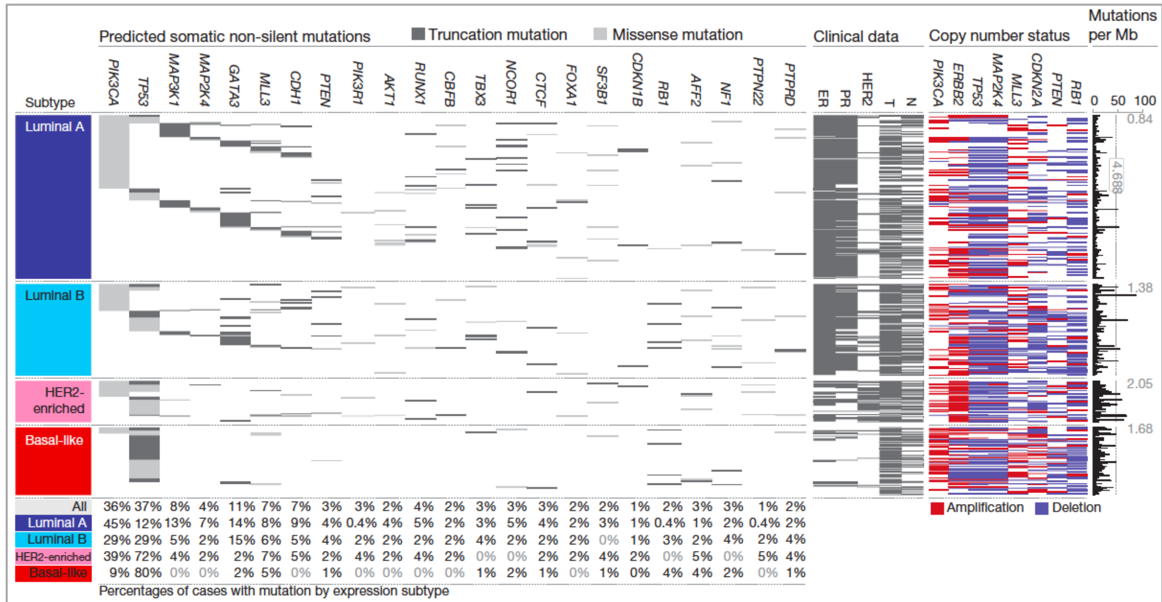


**Figure 3:** Significantly mutated genes and correlations with genomic and clinical features in TCGA. From Reference TCGA Nature 2012[56].

Transcript fusions as a result of chromosomal rearrangements are driver and druggable events in some tumor types, such as NSCLC[57]. In a study including 1019 breast cancer samples, Yoshihara and colleagues identified 94 cases of protein kinase fusion (9.2%). However, only 4 of these (0.4%) were potentially targetable alterations (fusions involving *RET*, *NTRK3*, *FGFR1*, and *FGFR2*)[58].

The relative proportion of mutations in different genes varies according to breast cancer IHC and PAM50 intrinsic subtype, as reported in the 2012 study from TCGA (Figure 3)[56]. In this analysis, *PIK3CA* mutations were by far the most common alterations in Luminal A tumors and were present in up to 49% of primary breast cancer samples. In Luminal B tumors the most frequent alteration was *CCDN1* amplification (58%), although *PIK3CA* mutations were also common (32%). In Basal-like tumors, *TP53* mutations were present in up to 84% of the samples, followed by *PTEN* mutation or PTEN loss (35%) and Inositol polyphosphate 4-phosphatase type II (INPP4B) loss (30%). Finally, in HER2-E tumors, *TP53* (75%) and *PIK3CA* (42%) mutations were also frequent, on top of *ERBB2* amplification (71% of samples).

Interestingly, the genetic landscape of breast tumors also varies according to histological subtype. The comprehensive profiling of 817 breast tumors, including 127 invasive lobular cancers (ILC), 490 invasive ductal cancers (IDC), and 88 mixed IDC/ILC, showed that besides E-cadherin loss (that defines ILC), ILC is enriched in *PTEN* mutations (leading to increased AKT phosphorylation when compared to IDC samples), *TBX3* mutations, and *FOXA1* mutations. Conversely, *GATA3* mutations and GATA3 high expression characterized luminal A IDC[54].

In a second study that also assessed the specific genetic alterations in ILC, it was reported that, besides the expected high mutation frequency of *CDH1* (65%), half of the tumors were mutated in at least one of the three key genes of the PI3K/AKT/mTOR (PAM) pathway, namely *PIK3CA* (43.3%), *PTEN* (3.9%), or *AKT1* (4.1%)[59]. Other relevant gene alterations were mutations in *ERBB2* (5.1%), *ERBB3* (3.6%), and in several transcriptional regulators such as *TBX3* (13.3%), *FOXA1* (9%), *KMT2C* (8%), *GATA3* (7.3%), and *ARID1A* (6.3%).

### 1.4. Molecular screening initiatives in breast cancer

To keep abreast of all the aforementioned developments as well as translate genomic data into improved patient care, several Institutions across the globe have strategically planned to integrate precision medicine in daily clinical practice[60-62]. These so-called “pre-screening programs” aim at sequencing tumor samples of patients that are potential candidates for early-phase clinical trials. Genomic information can then help guide treatment decisions and the inclusion of patients who can potentially derive most benefit from targeted treatments. There are some challenges in establishing these programs, including: (1) selection of the most suitable screening platforms; (2) the somatic variant calling strategy; (3) the availability of a clinically adequate clinical trials portfolio within a given Institution; and (4) the availability of patients that make such a strategy cost-effective. Comprehensive reviews on these issues can be found elsewhere[52,60].

In recent years, multicenter and/or international prospective trials have been launched to better understand the molecular alterations associated with MBC. We

can divide these into first generation studies aimed at a better understanding of cancer biology, and prospective randomized clinical trials that test the hypothesis that the matching of molecular alterations to specific targeted drugs improves MBC outcomes.

One of the first reported initiatives was the French SAFIR-01 trial (Figure 4)[63]. This multicenter molecular screening study was designed to identify molecular abnormalities in individual patients in order to provide targeted therapy matched to the individuals' genomic alterations. Comparative genomic hybridization (CGH) array and Sanger sequencing on *PIK3CA* (exon 9 and 20) and *AKT1* (exon 4) were performed in 407 fresh metastatic samples obtained from 423 patients with MBC. A targetable genomic alteration was identified in 195 (46%) patients, most frequently *PIK3CA* mutation (25%), *CCND1* amplification (19%), and *FGFR1* amplification (13%). Of note, 39% of patients had rare genomic alterations (defined as occurring in less than 5% of the general population), including *AKT1* mutations, and *EGFR*, *MDM2*, *FGFR2*, *AKT2*, *IGF1R*, and *MET* high-level amplifications. Therapy could be personalized in only 13% of the patients, either due to issues related with the sample (low percentage of tumor cells to perform the test, no confirmation of metastatic tumor, failure of CGH or sequencing), or to the lack of access to the matched drug once the genotyping result was available. Notably, response rate among those patients that received matched targeted therapies (85% as single agent) was generally higher than 30%. While the authors concluded that performing such a molecular screening in MBC is feasible, the low yield of such an effort should be considered.

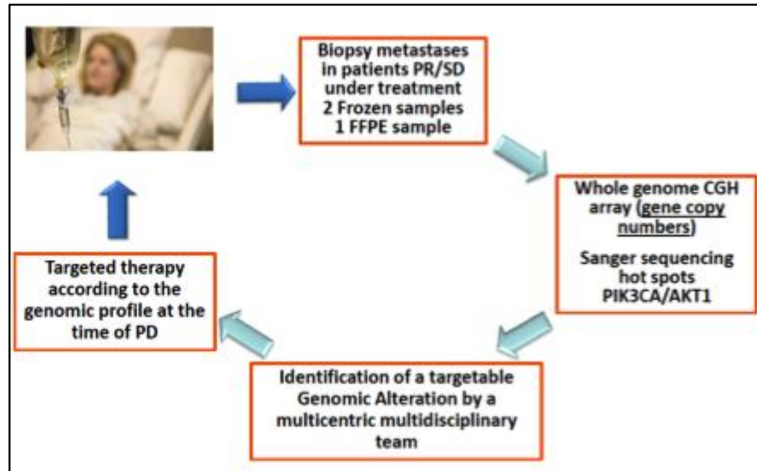


**Figure 4**: SAFIR-01 Study design.

The Breast International Group (BIG) AURORA study (NCT02102165) is a large longitudinal cohort study with downstream biologically driven clinical trials focusing on patients with MBC[64]. One thousand and three hundred patients treated with no more than one line of systemic treatment in the metastatic setting will be enrolled in several European countries, including Spain. Next-generation sequencing (NGS) with an extensive targeted panel of cancer-related genes and RNA sequencing will be performed in tumor tissues from metastatic site(s) [formalin-fixed paraffin embedded (FFPE) and frozen tissue] and archived FFPE tissue from the primary tumor. Whole blood samples, plasma samples, and serum samples will also be collected. Patients will then receive treatment as per the respective physician's discretion, and they will be followed with systematic collection of clinical data and plasma and serum samples every 6 months to determine response (locally assessed) and clinical outcome endpoints for a period of 10 years. The main objective of the AURORA program is to improve the biological understanding of MBC, delineate its molecular evolution life cycle, and explore intratumor heterogeneity.

The SOLTI-sponsored AGATA trial (NCT02445482) is the first genomic screening platform ever attempted in Spain. This study is an observational and prospective trial that aims to determine the effectiveness of molecular testing to include patients in clinical trials with targeted agents based on the tumor molecular

profiling. It will enroll 260 patients across eight Spanish sites, and it is expected to conclude in 2017.

The SAFIR-02 Breast trial (NCT02299999, sponsored by the French UNICANCER Group) compares a targeted treatment administered according to the identified molecular anomalies of the tumor with standard therapy (Figure 5). In this study, molecular screening will be performed prior to treatment with standard chemotherapy for the advanced setting. For those patients who remain stable or respond to standard chemotherapy and for whom a potentially targetable molecular alteration is identified, there will be a randomization between standard maintenance treatment with chemotherapy or targeted therapy according to the results of NGS and CGH. The primary endpoint is PFS in the targeted drug arm compared to standard maintenance therapy arm.

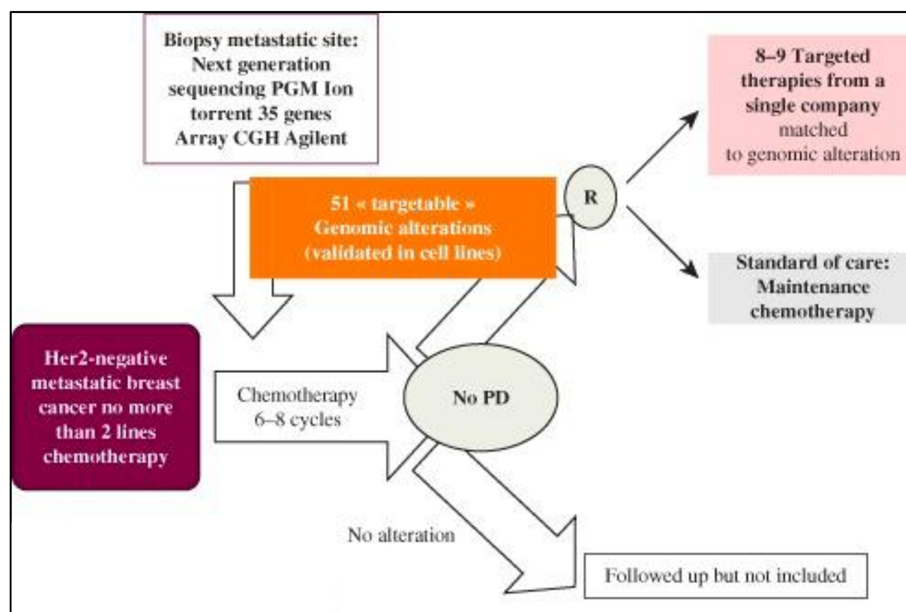


**Figure 5:** SAFIR-02 Study design.

## 1.5. The PAM pathway in human cancer

The phosphatidylinositol 3-kinases (PI3Ks) are a family of lipid kinases that mediate a cascade of intracellular signal transduction that ultimately regulates several key cellular functions, such as protein synthesis, cell cycle control, and control of cellular proliferation, growth and survival[65]. There are four classes of PI3Ks grouped according to their structure and function: IA, IB, II, and III (Figure 6)[66]. Class IA PI3K is the one most clearly implicated in human cancer[67] and consists of a catalytic subunit linked to a regulatory subunit[68]. Human cells contain three genes (*PIK3CA*, *PIK3CB* and *PIK3CD*) that encode the catalytic subunits of class IA PI3K enzymes, termed PI3Kα, PI3Kβ and PI3Kδ, respectively. The major polypeptides produced by these three genes are p110α, p110β and p110δ, collectively termed p110. p110α and p110β are ubiquitously expressed, whereas p110δ is expressed primarily in immune and hematopoietic cells[69-71]. The regulatory subunit is codified by three mammalian genes – *PIK3R1*, *PIK3R2*, and *PIK3R3* – that encode proteins collectively known as p85[65,70]. Class IB consists of *PIK3CG*, which encodes p110γ. Class II PI3Ks (PI3K-C2α, PI3K-C2β and PI3K-C2γ) do not constitutively associate with regulatory subunits and are characterized by a C-terminal C2 domain. The sole class III PI3K is the phosphatidyl inositol phosphate (PIP)-specific vacuolar protein sorting-associated protein 34 (VPS34).

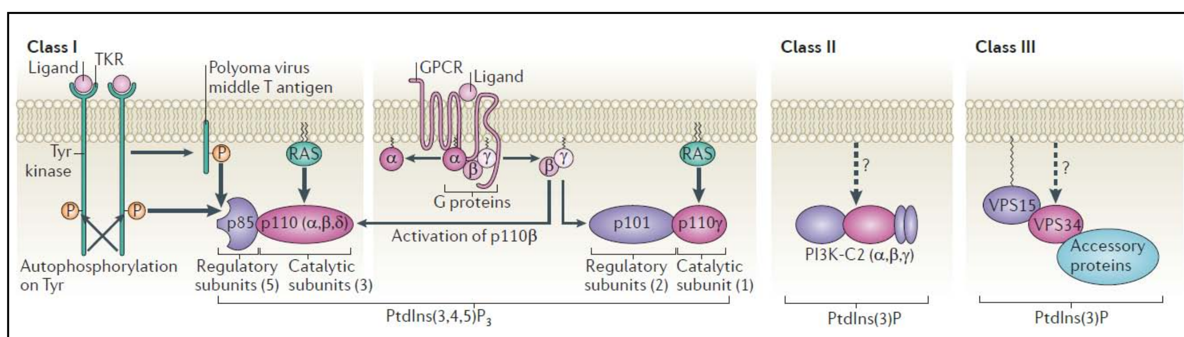


**Figure 6**: The distinct classes of mammalian PI3Ks.
From Reference Vanhaesebroeck B. et al[66].

The activation of the PAM pathway may be initiated by receptor tyrosine kinases (RTK) or G-protein-coupled receptors located at the cell surface, as well as by some oncogenic proteins, such as RAS[68]. Kinase interactions downstream of PI3K are complex; several different feedback loops exist, and the pathway is known to

interact with other signaling cascades. In short, upon growth factor stimulation, RTKs localized in the membrane activate Class IA PI3Ks. The binding of p85 to phosphotyrosine residues on RTKs and/or adaptors relieves the intramolecular inhibition of the p110 catalytic subunit by p85. This leads to the localization of PI3K in the plasma membrane where it phosphorylates phosphatidylinositol 4,5-bisphosphate (PI[4,5]P2) to phosphatidylinositol 3,4,5-triphosphate (PI[3,4,5]P3). The tumor suppressor PTEN acts as a shutdown mechanism of this pathway, dephosphorylating PIP3 to PIP2. INPP4B, in turn, dephosphorylates PIP2 to PIP1, also contributing to the regulation of the pathway[72]. PIP3 directly binds pleckstrin homology domains of various signaling proteins, namely phosphoinositide-dependent kinase 1 (PDK1) and AKT. PDK1 activates AKT by phosphorylating it at its threonine 308 (T308) residue. AKT then phosphorylates several downstream proteins that ultimately lead to cell proliferation, growth, survival, and resistance to apoptosis. AKT also phosphorylates TSC2, thereby inhibiting the rheb GTPase activity of the TSC1/TSC2 dimer. Activated rheb stimulates the mammalian target of rapamycin (mTOR)-containing protein complex mTORC1, leading to increased p70S6 kinase activity, with the consequent increase in protein synthesis. A second mTOR complex, mTORC2, contributes to complete AKT activation by phosphorylating AKT on its serine 473 (S473) residue. Upon activation of S6 kinase, there is a negative feedback loop via insulin receptor substrate 1 (IRS1) that diminishes PI3K activation, providing a mechanism of regulation for pathway activation. Figure 7 depicts the components of the PAM pathway and summarizes their interaction.

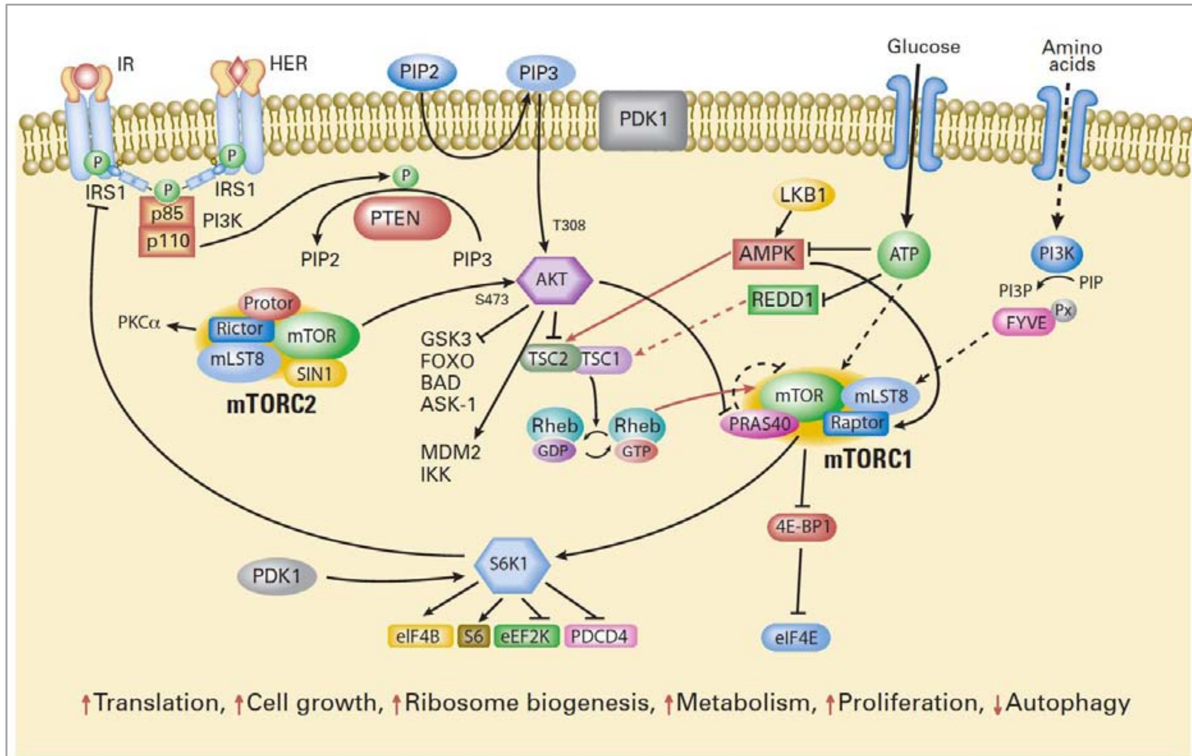


**Figure 7:** The PAM pathway signaling network.
Arrows represent activation and bars represent inhibition.
From Reference Meric-Berstam F. & González-Angulo A.M.[73]

The disruption of this tightly regulated system may occur by a variety of mechanisms. These include mutation and/or amplification of genes encoding for RTKs (*EGFR*, *ERBB2*), or subunits of PI3K (*PIK3CA*, *PIK3CB*, *PIK3R1* and *PIK3R2*), AKT (*AKT1*), or activating isoforms of RAS. Loss of function or expression of PTEN (through mutations, deletions, or epigenetic silencing) is also common[74,75]. These alterations lead to an increase in enzymatic function, enhance downstream signaling elements (including AKT) and, importantly, promote oncogenic transformation in pre-clinical models[75-77].

### 1.6. Prevalence of PAM pathway alterations in breast cancer

Up to 70% of breast cancers have some form of molecular alteration of the PAM pathway[78]. The most common alterations of this pathway are mutations of *PIK3CA* (10-45% depending on breast cancer subtype) and PTEN low expression (~50%)[79,80]. Additionally, *AKT1* mutation can occur in about 3-4% of ER+ breast cancers[54-56,81].

There are many studies describing the prevalence and prognostic implications of PAM pathway alterations in breast cancer. In the following paragraphs, we will briefly discuss the most important findings from three seminal analyses: the TCGA data, the ICGC study, and the recently published data from the METABRIC Consortium.

### *1.6.1. TCGA data*

Alterations in the PAM pathway were analyzed in detail in 357 primary breast cancer samples from the TCGA consortium[56]. This analysis was further extended to 817 samples, with a special focus on ILC (N=127)[54].

#### *1.6.1.1. ER+/HER2-negative breast cancer*

Within ER+/HER2-negative breast tumors, the proportion of PAM pathway alterations varies according to the different intrinsic subtypes[56]. In ER+/HER2-negative Luminal A tumors, *PIK3CA* mutations occurred in 49%, PTEN mutations or loss in 13%, and INPP4B loss in 9% of the samples; in ER+/HER2-negative Luminal B tumors, the proportions were 32%, 24%, and 16%, respectively. *AKT1* mutations were also present in 4% of Luminal A and 2% of Luminal B tumors. Interestingly, RPPA data did not show a correlation between *PIK3CA* mutations in Luminal A samples and downstream markers of PAM pathway activation, such as increased pAKT, pS6 and p4EBP1, in contrast to what was observed in basal-like and HER2-E subtypes (the latter having frequent *PIK3CA* mutations). This had been previously reported[82], and may explain the lack of correlation between *PIK3CA* mutations and worse prognosis in ER+/HER2-negative breast cancer[83].

#### *1.6.1.2. Basal like breast cancer*

In basal-like tumors, *PIK3CA* mutations were less frequent (7%) than in luminal or HER2-E tumors. Interestingly, basal-like breast tumors showed the highest levels

of PAM pathway activation, as measured by protein readouts. This activation was secondary to either mutation/loss of PTEN (35%) and INPP4B (30%), and/or amplification of *PIK3CA* (49%).

#### *1.6.1.3. HER2-E breast cancer*

In the TCGA dataset, not all clinically HER2+ tumors were of the HER2-E subtype. Conversely, not all HER2-E tumors were clinically HER2+. These observations are similar to other published studies[84-86]. The most frequent alterations in the PAM pathway in HER2-E subtype were *PIK3CA* mutations (42%), PTEN mutation or loss (19%), and INPP4B loss (30%).

#### *1.6.1.4. ILC and expanded TCGA data*

The updated TCGA analysis published in 2015 included 817 samples, with a focus on 127 ILC samples[54]. Regarding PAM pathway alterations, *PIK3CA* mutations were more frequent in ILC than in IDC (48% vs. 33%). Luminal A ILC (81% of ILC cases) had the highest proportion of *PIK3CA* mutations (51%), followed by Luminal A IDC (47%) and Luminal B IDC (35%). In this analysis, *PIK3CA* mutation frequency in HER2-E and Basal-like tumors was 37.3% and 6.5%, respectively.

Although the percentage of *PIK3CA* mutations did not significantly differ between Luminal A ILC and Luminal A IDC, PTEN inactivation did emerge as a discriminant feature between these two subtypes. Collectively, PTEN inactivating alterations were identified in 14% of Luminal A ILC versus 3% of Luminal A IDC ($P=10^{9E-4}$). PTEN genetic alterations across all ILC cases included homozygous deletions (6%) and somatic mutations (7%), and were largely mutually exclusive with *PIK3CA* mutations (48%). PTEN mutations in HER2-E and Basal-like tumors represented 7.8% and 5.6% of the cases, respectively.

Interestingly, ILC tumors showed significantly increased AKT phosphorylation at both S473 (P=0.004) and T308 ($P=10^{7E-5}$) when compared to Luminal A IDC,

which is consistent with the higher proportion of PTEN inactivation in ILC. In fact, ILC pAKT levels were comparable with those typically observed in the more aggressive HER2+ and ER-negative/basal-like breast tumors. *AKT1* mutations were infrequent events, accounting for 2% of ILC (3% in Luminal A ILC) and 3% of IDC (5% in Luminal A IDC). The percentage of *AKT1* mutations in Luminal B IDC, and HER2-E IDC was 2.6% and 2%, respectively. No AKT1 mutations were detected in Basal-like tumors.

### *1.6.2. The International Cancer Genome Consortium*

The ICGC is a global initiative aimed at comprehensively elucidating the genomic changes – somatic mutations, abnormal expression of genes, epigenetic modifications – present in many forms of cancers. In a recent study, Nik-Zeinal et al. reported data from whole-genome sequencing of 560 primary breast cancer samples[55]. Compared to targeted gene and exome sequencing, whole-genome sequencing yields information about the mutational landscape of untranslated, intronic and intergenic regions in a cancer genome. This may provide important insights into activating driver rearrangements[87] forming chimeric (fusion) genes/proteins or relocating genes adjacent to new regulatory regions, or the role of driver substitutions and indels in non-coding regions of the genome, originating for instance long non-coding RNAs that may influence gene transcription (epigenetic modulation)[88].

Analysis of the whole genomes of 560 breast cancers and non-neoplastic tissue from each individual (556 female and 4 male) detected 3,479,652 somatic base substitutions, 371,993 small indels and 77,695 rearrangements, with substantial variation in the number of each between individual samples. Prevalence of mutations in PAM pathway-associated genes in ER+ and ER-negative samples, respectively, were as follows: *PIK3CA* 38% and 20%, *AKT1* 4% and 1%, and *PTEN* 8% and 30%.

### *1.6.3. METABRIC Consortium*

In this study published in 2016, the authors provided information on 2433 primary breast tumors regarding sequencing of 173 genes, copy number aberration (CNA), and gene expression, together with long-term clinical follow-up data[81]. In this dataset, *PIK3CA* was the most frequently mutated gene (40.1%). Other PAM pathway genes frequently mutated were *AKT1* (4%) and *PTEN* (4%). Again, the rate of *PIK3CA* mutations differed according to intrinsic subtype: Luminal A 61%, Luminal B 38%, HER2-E 37%, Basal-like 10%.

Interestingly, the authors found that *PIK3CA* mutations have distinct prognostic associations in ER+ tumors stratified into the previously described Integrative Clusters (IntClust)[89*]. Significant interactions between the presence of *PIK3CA* mutations and a worse breast cancer specific survival were identified in IntClust 1+, 2+ and 9+, but not in IntClust 3+, 4+, 7+ and 8+. These results may suggest that integration of IntClust information to *PIK3CA* mutational status could be important to establish prognostic and also potentially predictive information beyond the presence of *PIK3CA* mutation alone.

### *1.6.4. Online resources*

Several online resources are available to easily check the frequency of genomic alterations within a particular subset of breast cancer. They constitute user-friendly tools for data mining and biology learning, and can be consulted at any time. cBioportal, for instance, integrates updated information from the TCGA, as well as other databases, such as METABRIC[79,80].

Figure 8 summarizes the output data from querying the cBioportal database with relevant PAM pathway genes in breast cancer (*PIK3CA*, *AKT1*, and *PTEN*).

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[*] In brief, this study performed a joint clustering of copy number and gene expression data from over 2000 primary breast cancers, and classified breast tumors in 10 different groups (IntClust) with distinct clinical outcomes. See Reference Curtis C. et al[87] for more information.

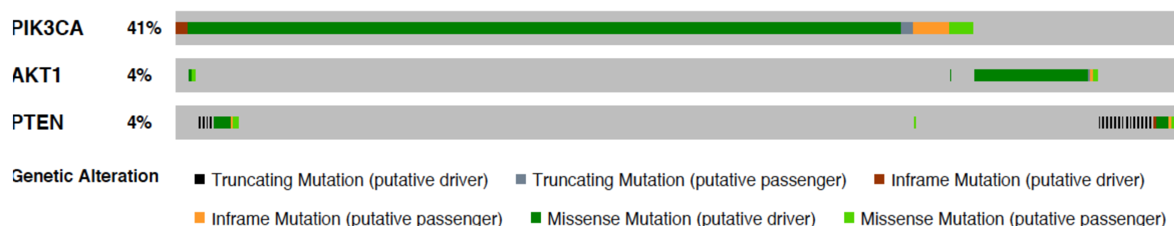


**Figure 8:** Oncoprint from relevant PAM pathway genes (PIK3CA, AKT1, and PTEN). Assessed from cBioportal in 4/23/2017.

## 1.7. Drug modulation of the PAM pathway in breast cancer

Several agents targeting the PAM pathway at different levels are currently in clinical development. They include PI3K inhibitors (either pan-isoform PI3K or isoform-specific PI3K inhibitors: PI3Kα, β, and γ), dual PI3K/mTORC1/2 inhibitors, mTOR inhibitors (rapalogs and mTORC1/2), or AKT inhibitors[90,91].

In the next few paragraphs, we will provide a brief overview of these drugs and their current development phase in breast cancer.

### *1.7.1. mTOR Inhibitors*

#### *1.7.1.1. Rapalogs*

Rapalogs were the first PAM pathway inhibitors to enter the clinic. These compounds, such as temsirolimus and everolimus, are allosteric inhibitors of the mTORC1 complex, and are effective as single agents in renal and neuroendocrine tumors[92,93]. In breast cancer, they have limited activity as single agents.

HORIZON was a Phase III randomized, placebo-controlled study that enrolled 1112 AI-naïve, ER+ and/or PgR+ advanced or MBC patients to receive letrozole plus temsirolimus 30 mg daily (5 days every 2 weeks) or letrozole plus placebo in the first line setting[94]. In this study, the addition of temsirolimus to letrozole resulted in more G3 and G4 adverse events (AEs; 37% vs. 24%), with no benefit in PFS (HR 0.90, 95%CI 0.76-1.07; P=0.25).

BOLERO-4 tested the efficacy and safety of first line everolimus plus letrozole in 202 postmenopausal patients with ER+/HER2-negative MBC[95]. After progression, patients were allowed to continue with everolimus and switch endocrine therapy to exemestane until further progression or unacceptable toxicity. Data that were presented at the ESMO Congress in 2016 were not mature. With a median follow-up of 17.5 months, PFS was not reached at data cutoff, and the estimated PFS at 12 months was 71.4% (95%CI 64.0%-77.5%). Overall response rate (ORR) and clinical benefit rate (CBR) were 42.6% (95%CI 35.7%-49.7%) and 74.3% (67.7%-80.1%), respectively. The most common AEs with the combination therapy were stomatitis (67.8%), weight loss (42.6%), and diarrhea (36.1%)[95].

The BOLERO-2 trial included patients with ER+/HER2-negative advanced or MBC that had progressed to prior non-steroidal AI to receive exemestane in combination with everolimus or placebo. Treatment with everolimus reduced the risk of progression or death from any cause in 57% when compared to placebo (HR 0.43, 95%CI 0.35-0.54; P<0.0001; median PFS 6.9 vs. 2.8 months)[96]. The final PFS analysis reported later on confirmed these results (HR 0.45, 95%CI 0.38-0.54; P<0.0001; median PFS 7.8 vs. 3.2 months)[97]. This PFS advantage did not translate to OS advantage: median OS in patients receiving everolimus plus exemestane was 31.0 months (95%CI 28.0-34.6 months) compared with 26.6 months (95%CI 22.6-33.1 months) in patients receiving placebo (HR 0.89, 95%CI 0.73-1.10; P= 0.14)[98]. However, the study was not powered to show OS superiority with its sample size, and for that reason these results must be interpreted with caution. The PFS results of the BOLERO-2 trial led to the approval of everolimus in ER+/HER2-negative, AI-resistant, advanced or MBC, both in the US and in Europe.

TAMRAD is a Phase II study that randomized 111 postmenopausal women with ER+/HER2-negative, AI-resistant, MBC to receive tamoxifen plus everolimus or tamoxifen alone[99]. Randomization was stratified by type of hormone resistance (primary versus secondary). Primary resistance was defined as relapse during or within 6 months of stopping adjuvant AI treatment or progressing within 6 months of starting AI treatment in the metastatic setting. Secondary resistance was

defined as relapse after 6 months of stopping adjuvant AIs or responding for more than 6 months to AIs in the metastatic setting. CBR (defined as the presence of complete response [CR], partial response [PR], or stable disease [SD] at 6 months) was 61% (95%CI 47-74) with tamoxifen plus everolimus and 42% (95%CI 29-56) with tamoxifen alone. Time to progression (TTP) was 8.6 months with tamoxifen plus everolimus and 4.5 months with tamoxifen alone (HR 0.54, 95%CI 0.36-0.81). The toxicity profile was expected and manageable, with fatigue (72%), stomatitis (56%), rash (44%), anorexia (43%), and diarrhea (39%) as most common AEs. Interestingly, the benefit of everolimus in terms of TTP was larger in the group of patients with secondary endocrine resistance (median TTP 14.8 vs. 5.5 months; HR 0.46, 95%CI 0.26-0.83, exploratory log-rank P=0.009) than in those with primary endocrine resistance (5.4 vs. 3.8 months; HR 0.70, 95%CI 0.40-1.21, exploratory log-rank P=NS). The authors concluded that treatment with everolimus plus tamoxifen might revert endocrine resistance in ER+/HER2-negative MBC, especially in the presence of secondary resistance[99].

The BOLERO-1 trial tested the combination of paclitaxel, trastuzumab, and everolimus in the first line treatment of patients with advanced HER2+ breast cancer[100]. In this trial, the addition of everolimus did not improve PFS when compared to trastuzumab plus paclitaxel. Median PFS in the paclitaxel plus trastuzumab plus everolimus arm was 15 months and in the placebo arm 14.5 months (HR 0.89, 95%CI 0.73-1.08; P=0.117).

The BOLERO-3 trial tested the combination of everolimus with trastuzumab and vinorelbine in patients with advanced HER2+ breast cancer after progression to trastuzumab and a taxane. The triple combination statistically improved PFS over trastuzumab and vinorelbine alone (HR 0.78, 95%CI 0.65-0.95; P=0.0067)[101]. However, the small absolute PFS improvement of 1.2 months (7 vs. 5.8 months) was not enough to establish this treatment as a new standard of care.

Collectively, these differences according to the setting in which rapalogs are used, either in combination with endocrine therapy (HORIZON and BOLERO-4 vs. BOLERO-2 and TAMRAD) and in combination with chemotherapy and HER2-

blockade (BOLERO-1 vs. BOLERO-3), may suggest that targeting mTOR in breast cancer has a role in reverting acquired, but not primary, resistance to standard therapies.

#### *1.7.1.2. mTORC1/2 inhibitors*

ATP-competitive mTOR inhibitors or mTORC1/2 inhibitors, inhibit both mTORC1 and mTORC2. By also blocking mTORC2, these compounds prevent the feedback activation of AKT seen with rapalogs[102]. Some examples are AZD2014 (AstraZeneca) and MLN0128 (Millennium Pharmaceuticals, Inc.). Both drugs are being tested in early-phase clinical trials in breast cancer, in combination to standard endocrine and chemotherapy. Currently, we have only limited data from phase I trials.

The Phase I trial of combination of AZD2014 with fulvestrant was presented at ASCO 2016[103]. This trial enrolled 99 ER+ MBC patients in several dose escalating cohorts of AZD2014 given twice a day (BID) either in a continuous schedule (N=43) or in an intermittent schedule (N=56), in combination with fulvestrant. The recommended Phase II dose (RP2D) was AZD2014 50mg BID continuous and 125mg BID on days 1 and 2 weekly. Dose limiting toxicities (DLTs) in the continuous schedule included stomatitis, rash, and hyperglycemia. No DLTs were observed in the intermittent schedule. Most common G3/4 AEs in the continuous and intermittent schedule were hyperglycemia (12% and 14%, respectively), rash (12% and 0%), fatigue (9% and 7%), stomatitis (5% and 4%), diarrhea (2% and 5%), and vomiting (2% and 5%). ORR was 17% in the continuous schedule and 13% in the intermittent schedule. CBR at 6 months was 33% and 25%, respectively.

The MANTA trial (NCT02216786) is an open-label, multicentric, 4-arm randomized Phase II trial of fulvestrant + AZD2014 (continuous and intermittent schedule) versus fulvestrant + everolimus versus fulvestrant alone in patients with AI-resistant, ER+/HER2-negative advanced or MBC. Approximately 300 patients are randomized (2:3:3:2) to one of the four treatment arms: (1) fulvestrant; (2)

fulvestrant + AZD2014 (continuous daily schedule); (3) fulvestrant + AZD2014 (intermittent schedule 2 days on, 5 days off); (4) fulvestrant + everolimus. The primary endpoint is PFS.

AZD2014 has also been tested in combination with paclitaxel in a Phase I trial, results of which were presented at the ASCO Annual Meeting in 2014[104]. In this study, patients with advanced solid tumors where treatment with paclitaxel was appropriate were enrolled in several dose escalating cohorts of AZD2014 given BID either in a 3/7 (3 days on, 4 days off) schedule (N=12) or in a 2/7 (2 days on, 5 days off) schedule (N=8), in combination with weekly paclitaxel 80mg/m$^2$. In the 3/7 schedule, three patients had DLTs of G3 fatigue and/or mucositis at 75 mg of AZD2014. G3 diarrhea was seen at 50mg BID, but this did not require treatment discontinuation, and was short-lived when managed with antidiarrheals. In the 2/7 schedule two patients had DLT of G3 rash at 100 mg of AZD2014. Maximum tolerated dose (MTD) for the 3/7 schedule is 80 mg/m$^2$/week of paclitaxel and 50 mg BID of AZD2014. DLTs were G3 fatigue and mucositis. The 2/7 schedule of AZD2014 at 100 mg BID in combination with weekly paclitaxel was declared as non-tolerable due to two patients with G3 skin rash. At MTD of the 3/7 schedule, the combination was well-tolerated with rates of neutropenia not exceeding single agent paclitaxel in a heavily pre-treated population. Regarding efficacy, 3/5 patients with ovarian cancer had PR by Gynecologic Cancer Intergroup (GCIG) and Response Evaluation Criteria In Solid Tumors (RECIST) criteria. Additionally, 2/2 patients with squamous cell lung cancer, and 1/3 patient with lung adenocarcinoma showed tumor necrosis and RECIST radiological response.

MLN128 is being tested in a randomized Phase II trial in ER+/HER2-negative MBC (NCT02756364). This study evaluates the efficacy and safety of the combination of fulvestrant + daily MLN0128 and fulvestrant + weekly MLN0128 compared with fulvestrant alone in approximately 153 patients. The primary endpoint is PFS.

### *1.7.2. Dual PI3K/mTOR inhibitors*

Dual inhibitors of PI3K and mTOR target the active sites of both holoenzymes, resulting in pathway inhibition both upstream and downstream AKT. Although the rationale to use such drugs is compelling – especially in tumors with alterations downstream of PI3K but upstream of mTOR (e.g., PTEN or TSC1/2) – their use in the clinical setting has been limited by their challenging toxicity profile[90]. Examples of these compounds that are or have been investigated in breast cancer are GDC-0980 (Genentech)[105,106], BEZ-235 (Novartis), XL-765 (Sanofi/Exelixis)[107], PF-05212384 (Pfizer), and PQR309 (PIQUR Pharmaceuticals). The development of the first three has been discontinued. The Pfizer compound is being tested in combination with chemotherapy (NCT01920061 and NCT02069158) and with endocrine therapy plus palbociclib (NCT03065062 and NCT02626507). The PIQUR compound is being tested as a single agent in all solid tumors (NCT02483858) and in combination with eribulin, a chemotherapeutic compound, in HER2-negative MBC (NCT02723877).

### *1.7.3. PI3K inhibitors*

There are mainly two classes of PI3K inhibitors: pan-PIK3K inhibitors, and isoform-specific PI3K inhibitors. The first ones inhibit at a similar half maximal inhibitory concentration (IC50) the 4 isoforms of PI3K, while compounds in the second group have more affinity for one of the isoforms (α, β, γ, or δ) compared to the others.

#### *1.7.3.1. Pan-PI3K Inhibitors*

Examples of pan-PI3K inhibitors tested in breast cancer are BAY80-6946 (Bayer, NCT02705859), buparlisib (BKM120, Novartis)[108-112], Pictilisib (GDC-0941, Genentech)[106,113,114], and SAR245408 (XL-147; Sanofi/Exelixis)[115]. For their relevance in breast cancer, we will briefly discuss the results of the trials with buparlisib and pictilisib.

#### 1.7.3.1.1. *Buparlisib (BKM120)*

Buparlisib (BKM120, Novartis) was one of the first pan-PI3K inhibitors entering clinical trials, and was tested in multiple tumors types. The MTD of buparlisib in the Phase I escalation trial was 100 mg once a day (QD)[112]. Frequent treatment-related AEs included rash, hyperglycemia, diarrhea, anorexia, mood alteration (37% each), nausea (31%), fatigue (26%), pruritus (23%), and stomatitis (23%). In terms of efficacy, a partial response was observed in a triple negative breast cancer patient with a *KRAS* mutation. Following these results, several trials of buparlisib were launched in breast cancer. The most relevant results presented thus far are summarized in the next paragraphs.

The BELLE-2 trial (NCT01610284) enrolled 1147 postmenopausal women with ER+/HER2-negative locally advanced or MBC that progressed on or after AI therapy to receive either buparlisib or placebo in combination with fulvestrant[116]. Randomization was stratified by PI3K pathway status (as per central assessment) and presence of visceral metastasis. PI3K pathway activation was assessed in archival tumor tissue provided at screening. It was defined as *activated* in the presence of a *PIK3CA* mutation by Sanger sequencing (any mutations in exons 1, 7, 9, or 20) and/or loss of PTEN expression by IHC (1+ expression in <10% of cells). PFS according to circulating tumor DNA (ctDNA) *PIK3CA* status (assessed by BEAMing) was an exploratory endpoint, and could be determined in 587 patients. The trial met its primary endpoint of increasing PFS in the buparlisib-treated group in the full population (6.9 vs. 5.0 months; HR 0.78, 95%CI 0.67-0.89; P<0.001). However, in the PI3K-activated population, the difference in PFS between arms did not reach statistical significance: 6.8 vs. 4.0 months; HR 0.76, 95%CI 0.60-0.97; P=0.014 (one-sided α=0.01 level of significance). Interestingly enough, detection of a *PIK3CA* mutation in ctDNA just prior to the randomization predicted for better outcome, with larger differences in PFS in the buparlisib-treated patients than in the full population (7.0 vs. 3.2 months; HR 0.56, 95%CI 0.39-0.80; P<0.001). Toxicity led to dose reductions and discontinuation of treatment in 46.4% and 13.2% of patients in the buparlisib and placebo arms, respectively. Most common toxicities in the buparlisib arm were hyperglycemia

(43.1%, G3-4 15.4%), ALT elevation (40.1%, G3-4 25.5%), AST elevation (37.3%, G3-4 18%), rash (32.1%, G3-4 7.9%), fatigue (31.9%, G3-4 4.9%), anxiety (22.3%, G3-4 5.4%), and depression (26.2%, G3-4 4.4%). Authors concluded that the trial met its primary endpoint of improving PFS when combining buparlisib and fulvestrant in postmenopausal women with ER+/HER2-negative advanced breast cancer that had progressed after prior AI therapy. However, frequent dose reductions and discontinuations due to AEs reduced treatment duration in the buparlisib arm, potentially limiting the efficacy of combination therapy. Additionally, the PFS results of patients with a *PIK3CA* mutation detected in ctDNA suggest that assessment of *PIK3CA* mutations in ctDNA may help select patients who would most likely benefit from adding a PI3K inhibitor to endocrine therapy.

The BELLE-3 trial (NCT01633060) randomly assigned (2:1) 432 MBC patients previously treated with an AI and who had progressed to endocrine therapy plus everolimus to receive the combination of daily buparlisib plus fulvestrant or placebo plus fulvestrant[117]. The rationale behind this trial was that a pan-PI3K inhibitor, such as buparlisib, could revert resistance to an mTOR inhibitor, like everolimus, as suggested by some pre-clinical data[118]. The trial met its primary endpoint of increasing PFS in the buparlisib arm, with a median PFS of 3.9 months, versus 1.8 months in the placebo arm (HR 0.67, 95%CI 0.53-0.84; P<0.001). The 6-month PFS rates were 30.6% and 20.1%, respectively. Among patients with *PIK3CA* mutations detected in ctDNA (39% of the trial population), PFS was 4.2 months in the buparlisib arm, versus 1.6 months in the placebo arm (HR 0.46, 95%CI 0.29-0.73; P<0.001). Similarly to BELLE-2, patients receiving buparlisib in BELLE-3 had a higher incidence of G3/4 AEs (62% vs. 34%), dose reductions (31% vs. 18%) and dose discontinuations (21% vs. 8%) related to treatment or patient/physician decision. Most common AEs in the buparlisib arm were ALT elevation (39%, G3/4 22%), AST elevation (37%, G3/4 18%), hyperglycemia (36%, G3/4 12%), nausea (36%, G3/4 1%), diarrhea (26%, G3/4 3%), fatigue (23%, G3/4 4%), depression (21%, G3/4 1%), and anxiety (18%, G3/4 1%). Despite the positive results, the PFS benefit is modest, and toxicity may be an important limitation to introduce this treatment as a standard option to ER+/HER2-negative MBC patients who progress after an AI and everolimus.

The BELLE-4 trial (NCT01572727) was a randomized, double-blind, Phase II/III study of buparlisib plus paclitaxel in women treated in the first line setting for HER2-negative locally advanced or MBC[119]. Three hundred thirty-eight patients (73% with ER+ tumors) were randomized (1:1) to receive buparlisib (100 mg QD) or placebo with weekly paclitaxel (80 mg/m$^2$). The trial failed to meet its primary endpoint of improved PFS in the buparlisib-treated group (HR 1.18, 95%CI 0.82-1.68; median PFS in the buparlisib arm 8.0 months vs. 9.2 months in the placebo arm) and was stopped due to futility. The most frequent AEs (≥35% of patients) in the buparlisib arm were diarrhea (55% vs. 34% in the placebo arm), alopecia (49% vs. 52%), nausea (45% vs. 27%), hyperglycemia (45% vs. 11%), rash (40% vs. 24%), fatigue (37% vs. 34%), and neutropenia (35% vs. 30%). The authors concluded that no PFS benefit was conferred by the addition of buparlisib to paclitaxel, and that the PI3K pathway may not drive paclitaxel resistance in untreated HER2-negative MBC.

Buparlisib has also been tested in HER2+ tumors in combination with trastuzumab and/or capecitabine[111] and in triple negative MBC either as single agent (NCT01629615) or in combination with olaparib[120]. However, mainly due to its unfavorable side effects profile, Novartis decided to discontinue its development, and all the trials testing buparlisib will be stopped during 2017.

##### *1.7.3.1.2. Pictilisib (GDC-0941)*

Pictilisib (GDC-0941, Genentech) is a pan-PI3K inhibitor that has been tested as a single agent and in combination with anti-HER2, endocrine and chemotherapy in breast cancer. In the Phase I dose-finding trial, 60 patients with solid tumors received pictilisib at 14 dose levels from 15 to 450 mg QD, initially on days 1 to 21 every 28 days, and later using continuous dosing for selected dose levels[113]. The most common toxicities were G1-2 nausea, rash, and fatigue. The DLT was G3 maculopapular rash at 450mg. A patient with V600E BRAF–mutant melanoma and another with platinum-refractory epithelial ovarian cancer exhibiting PTEN loss and

*PIK3CA* amplification demonstrated partial response by RECIST and GCIG-CA125 criteria, respectively. The RP2D was continuous dosing at 330 mg QD.

FERGI was a two-part, randomized, double-blind, placebo-controlled, Phase II study, that enrolled postmenopausal women with ER+/HER2-negative breast cancer resistant to treatment with an AI in the adjuvant or metastatic setting, to receive pictilisib (340mg QD in part 1 and 260mg QD in part 2) plus fulvestrant vs. placebo plus fulvestrant[106]. Part 1 included 168 patients and randomized them 1:1 according to the presence of *PIK3CA* mutation; part 2 included 61 patients with known *PIK3CA* mutations, and randomized them 2:1 to receive pictilisib or placebo. In part 1, there was no difference in median PFS between the pictilisib and the placebo arms (HR 0.74, 95%CI 0.52-1.06; P=0.096). Median PFS for the pictilisib and the placebo arms were 6.6 months (95%CI 3.9-9.8) and 5.1 months (95%CI 3.6-7.3) respectively. No differences were observed according to the presence of *PIK3CA* mutation. In part 2, there was also no difference in PFS between groups (5.4 vs. 10.0 months; HR 1.07, 95%CI 0.53-2.18; P=0.84). Most common G3-4 AEs were rash (9%), diarrhea (8%), fatigue (8%), ALT elevation (5%), and hyperglycemia (7%).

Pictilisib was also tested in combination with chemotherapy, in the PEGGY trial (NCT01740336)[121]. In this randomized Phase II, placebo-controlled study, 183 ER+/HER2-negative patients were assigned (1:1) to receive paclitaxel (90 mg/m2 weekly for 3 weeks in every 28-day cycle) with either 260mg pictilisib or placebo (QD on days 1-5 every week). The primary endpoint was PFS in the intention-to-treat (ITT) population and in patients with *PIK3CA*-mutant tumors. In the ITT population, median PFS was 8.2 months with pictilisib (N=91) versus 7.8 months with placebo (N=92); HR 0.95, 95%CI 0.62-1.46; P=0.83. In patients with *PIK3CA*-mutant tumors, median PFS was 7.3 months for pictilisib (N=32) versus 5.8 months with placebo (N=30); HR 1.06, 95% CI 0.52-2.12; P=0.88. In the pictilisib arm, there were more AEs leading to dose interruption (58.2% vs. 48.9% in the placebo arm), dose reduction (49.5% vs. 22.8%), and dose discontinuation (25.3% v. 15.2%). The authors suggested that this compromise in drug exposure might explain the lack of efficacy of the combination respect to paclitaxel alone. Rash

was the main AE observed in the pictilisib arm (48.4%, vs. 33.7% in placebo arm). Grade 3 rash was observed in 5.5% of the patients. Other common G3 or higher AEs related to pictilisib were neutropenia (15.4%), hyperglycemia (5.5%), and diarrhea (6.6%). The authors concluded that PEGGY did not meet its primary endpoint, revealing no significant benefit from adding pictilisib to paclitaxel for patients with ER+/HER2-negative locally recurrent or MBC.

Due to the observed toxicities and the modest efficacy results across trials, Genentech has stopped the clinical development of pictilisib.

#### *1.7.3.2. p110α-specific PI3K inhibitors*

Isoform-specific PI3K inhibitors have been developed with the aim of selectively targeting specific alterations in the PI3K pathway (*PIK3CA* mutations, PTEN alterations), while avoiding the cumulative toxicity of inhibiting multiple isoforms of PI3K. There are three groups of isoform-specific inhibitors in clinical development: p110α, p110β, and p110δ inhibitors. p110α inhibitors target preferentially tumors with *PIK3CA* mutations; p110β inhibitors target those with PTEN alterations; and p110δ inhibitors are mainly used in hematological malignancies, since leukocytes and lymphocytes are particularly enriched in this isoform of PI3K. p110β inhibitors, such as AZD8186, are just beginning to be explored in triple negative breast cancer (TNBC) with or without PTEN deficiencies, and no clinical data are available as of yet (NCT01884285).

Selective p110α-specific inhibitors were designed to target tumors harboring *PIK3CA* mutations[122]. Examples of p110α-specific inhibitors that are being developed in breast cancer are alpelisib (BYL719, Novartis), taselisib (GDC-0032, Genentech), and MLN1117 (Millennium / Takeda). To be more accurate, taselisib is actually a PI3K β-sparing inhibitor (meaning that it inhibits the β isoform 30x less than the α isoform) that also displays differential activity in tumors with *PIK3CA* mutations. It will be therefore discussed in this section. Genentech is developing a new p110α selective inhibitor in breast cancer, GDC-0077, but the Phase I trial has just started, and no clinical data are presently available (NCT03006172).

We will now focus in the discussion of two promising compounds in breast cancer, alpelisib and taselisib.

##### *1.7.3.2.1. Alpelisib (BYL719)*

Alpelisib (BYL719) was tested in a Phase I trial that enrolled 132 patients with *PIK3CA*-altered advanced solid tumors, or *PIK3CA*-altered or wild type (WT) ER+ MBC, to receive different doses and schedules of treatment[123]. DLTs were reported in: 4 patients at 450mg QD (hyperglycemia N=2, nausea N=2), 4 patients at 200mg BID (hyperglycemia N=4), and one patient at 150mg BID (hyperglycemia and hypophosphatemia). BYL719 QD and BID MTDs were declared at 400mg (used for dose expansion) and 150mg, respectively. Most common AEs at 400mg were hyperglycemia (51%), nausea (48%), diarrhea (41%), decreased appetite (38%), fatigue (32%), vomiting (30%), and rash (20%). Most common AEs at 150mg BID were hyperglycemia and nausea (both 53%), diarrhea, decreased appetite, fatigue, and stomatitis (33% each). Overall response rate for alpelisib as a single agent was 11%.

A Phase Ib trial combined alpelisib with letrozole in 26 patients with ER+/HER2-negative MBC[124]. In this study, MTD of alpelisib in combination with letrozole was 300 mg QD. Common drug-related AEs at the MTD included hyperglycemia (55%, G3 10%), nausea (60%, all G1-2), fatigue (45%, all G1-2), diarrhea (80%, G3 10%), and rash (45%, all G1-2). The CBR, defined as lack of progression ≥6 months, was 35% (44% in patients with *PIK3CA* mutant and 20% in *PIK3CA* WT tumors), including five objective responses. The authors concluded that the combination of letrozole and alpelisib was safe, with reversible toxicities, and active. Clinical activity was observed independently of *PIK3CA* mutation status, although clinical benefit was higher in patients with *PIK3CA*-mutant tumors.

Phase II and III trials of alpelisib and endocrine therapy or chemotherapy in patients with ER+ breast cancer, either in the metastatic (NCT02437318,

NCT02379247, NCT01872260) or neoadjuvant settings (NCT01923168) are ongoing.

#### *1.7.3.2.2. Taselisib (GDC-0032)*

Taselisib (GDC-0032, Genentech) is an orally bioavailable, potent, and selective inhibitor of Class I PI3Kα, δ, and γ isoforms, with 30-fold less inhibition of the PI3K β isoform relative to the PI3Kα isoform[125]. As a single agent, taselisib RP2D was established at 9mg QD in capsule formulation, equivalent to 6mg QD in its tablet formulation[126]. Most common Grade ≥3 AEs that occurred at a frequency greater than 5% included hyperglycemia (15%), rash (12%), diarrhea (6%), fatigue (6%), and pruritus (6%). Responses were observed in *PIK3CA* mutant breast cancer patients.

Within the Phase I trial (NCT01296555), several expansion cohorts combining taselisib with endocrine treatment have been enrolled and/or are completed. Twenty-eight patients were enrolled in the dose escalation part of taselisib plus letrozole[127]. Most common G3-4 AEs were diarrhea (14%), hyperglycemia (7%), and mucosal inflammation (7%). Other relevant AEs included nausea, fatigue, rash, and muscle spasms. Overall response rate was 38% in patients with *PIK3CA* mutant breast cancer and 9% in patients with *PIK3CA* WT breast cancer.

Results from a Phase II trial combining taselisib with fulvestrant were presented at the ASCO 2016 Annual Meeting[128]. In this trial, 60 post-menopausal ER+/HER2-negative locally advanced or MBC patients who had progression or no response to ≥ 1 prior endocrine therapy in adjuvant or MBC settings were enrolled. Seventeen of them had *PIK3CA* mutations detected in archival tumor tissue, 27 had WT *PIK3CA* and 16 had unknown *PIK3CA* mutation status. Among patients with baseline measurable disease, confirmed response rates were higher in patients with a *PIK3CA* mutation (41.7% vs. 14.3% in WT). Common G≥3 AEs were colitis (13.3%), diarrhea (11.7%), hyperglycemia (6.7%), and pneumonia (5%). These results have prompted a registration randomized Phase III study of taselisib or

placebo plus fulvestrant in *PIK3CA* mutant and WT ER+/HER2-negative MBC patients (SANDPIPER, NCT02340221), which is currently enrolling.

Taselisib is also being tested in the following trials: a Phase Ib/randomized Phase II trial in combination with tamoxifen (POSEIDON, NCT02285179), a Phase I trial in combination with palbociclib (NCT02389842), a Phase I trial in combination with anti-HER2 therapies (NCT02390427), and in a neoadjuvant trial in combination with letrozole (LORELEI, NCT02273973).

### *1.7.4. AKT inhibitors*

The PAM pathway can also be inhibited at the AKT level. There are 2 main classes of AKT inhibitors: allosteric (such as MK-2206) and ATP-competitive (such as GDC-0068 and AZD5363). Allosteric inhibitors lock AKT in a closed conformation, with its phospholipid binding site blocked by the kinase domain[129]. In contrast, cells treated with ATP-competitive inhibitors display increased binding of AKT to PIP2 and PIP3 and increased localization of AKT at the plasma membrane, resulting in its hyperphosphorylation at both T308 (PDK1 site) and S473 (mTORC2 site)[130]. For this reason, phospho-AKT levels are increased upon treatment with ATP-competitive AKT inhibitors.

Importantly, some data suggest that the presence of AKT1 mutations (such as E17K), frequent in cancer, associate with reduced sensitivity to allosteric inhibitors compared with ATP-competitive inhibitors[131].

#### *1.7.4.1. MK-2206*

MK-2206 (Merck) is an allosteric AKT-inhibitor that has been tested in breast cancer in combination with endocrine therapy[132], chemotherapy[133,134], and anti-HER2 therapy[135,136].

In a Phase Ib trial that enrolled 31 ER+/HER2-negative breast cancer patients to receive MK-2206 with anastrozole or fulvestrant, main toxicity was rash (33.3%,

G3 in 23.3%)[132]. Rash was dose limiting and required the amendment of the protocol after the first three patients were treated to include prophylaxis with prednisone. The RP2D was then defined as MK-2206 150 mg orally weekly with prednisone prophylaxis for each endocrine combination. In addition to rash, the most common AEs were hyperglycemia (20%), hypophosphatemia (16.7%), and fatigue (10%). The CBR was 36.7% (95%CI 20%-56%), including 2 patients with partial response and 9 patients with SD for more than 6 months. In this study, *PIK3CA* mutation did not associate with response to MK-2206. The combination of MK-2206 and anastrozole is being further evaluated in a Phase II neoadjuvant trial for newly diagnosed ER+/HER2-negative breast cancer (NCT01776008).

MK-2206 was also evaluated as part of the I-SPY 2 trial, a multicenter, adaptive Phase II trial of neoadjuvant therapy for high-risk clinical stage II or III breast cancer[137]. The results of this part of I-SPY2 were presented at the ASCO 2015 Annual Meeting[138]. Ninety-three patients received MK-2206 135 mg orally QD with weekly paclitaxel (and trastuzumab if HER2+), followed by AC. In the I-SPY2 trial, a given drug graduates to further study if the probability of success (calculated by a Bayesian predictive probability model) in a 2-arm, N=300, Phase III randomized 1:1 trial with pathological complete response as an endpoint, is ≥85%. MK-2206 graduated in the signatures of ER-negative/HER2+, ER-negative, and HER2+. Most common AEs included mucositis (55%), rash (88%, G3/4 24%), and neutropenia (G3/4 21%).

#### *1.7.4.2. Ipatasertib (GDC-0068)*

Ipatasertib (GDC-0068, Genentech) is a highly selective, orally bioavailable AKT kinase inhibitor that shows pharmacodynamic inhibition of AKT signaling and antitumor activity in human cancer cells *in vitro* and *in vivo*[139] and in tumor biopsies from patients[140]. The combination of ipatasertib with chemotherapy (including paclitaxel) shows increased efficacy when compared to ipatasertib alone in breast cancer cell lines and xenografts[141].

In the Phase I study as single agent (NCT01090960), 52 patients were enrolled in three stages: 30 patients in dose-escalation (Stage 1), and 22 patients in the two dose-expansion cohorts of tumor-specific indications (Stage 2: MBC n=11, metastatic castration-resistant prostate cancer n=5) or all solid tumors (Stage 3, n=6)[142]. Overall, 16 patients with MBC were enrolled. Two patients at the 800 mg dose experienced two DLTs (G3 asthenia, and G3 nausea). The MTD for ipatasertib (and the dose that was used for expansion) was 600 mg orally QD on a 21/7 dosing schedule. The majority of AEs were Grade 1-2 in severity and could be managed with supportive care and/or dose holds so that patients could continue with ipatasertib. The most frequently reported Grade ≥2 AEs related to ipatasertib were diarrhea (35%), nausea (27%), asthenia (25%), hyperglycemia (10%), decreased appetite (6%), rash (6%), and vomiting (6%). Of note, a heavily pre-treated 68 year-old female patient with *AKT1* mutant (E17K), ER+/HER2-negative MBC had a complete metabolic PET response in Cycle 1 of treatment. Her CA15-3 tumor marker declined by more than 50%, and she remained on the study for 235 days.

Activation of AKT signaling has been associated with chemotherapy resistance in preclinical models[143]. For this reason, it was hypothesized that ipatasertib could overcome resistance to chemotherapy. In a pre-clinical study the combination of docetaxel and ipatasertib resulted in significantly increased inhibition of cell viability and tumor regression in xenograft models in both *PIK3CA* WT and H1047R mutants compared with each single agent alone[141]. Synergistic effects were also observed in the OVCAR3 ovarian cancer xenograft model when ipatasertib was combined with carboplatin.

These results prompted the launching of a Phase I study (NCT01362374) testing ipatasertib in combination with docetaxel (Arm A), FOLFOX6 (Arm B), paclitaxel (Arm C), and enzalutamide (Arm D). Nineteen patients with MBC were included in the taxane combination arms: 5 patients received docetaxel 75 mg/m$^2$ on Day 1 with escalating doses of ipatasertib QD on Days 2-15 every 21 days; and 14 patients received paclitaxel 90 mg/m$^2$ on Days 1, 8, and 15 with escalating doses of ipatasertib QD on Days 1-21, every 28 days. This study included an expansion

cohort of paclitaxel plus ipatasertib in HER2-negative MBC patients. Common G≥2 AEs related to ipatasertib in combination with docetaxel were diarrhea (80%), nausea (60%), and vomiting (40%). In the paclitaxel arm, common AEs were diarrhea (43%), fatigue (29%), and hyperglycemia (14%). Partial responses by RECIST v.1.1 were seen in 5 patients: 3 patients with ER+/HER2-negative tumors, and 3 patients with TNBC. Interestingly, among these 5 patients with PR, four had previously progressed to paclitaxel and two to PI3K inhibitors. Furthermore, four patients had an alteration in the PAM pathway (retrospectively determined in archival tissue): one patient had PTEN loss, two had a *PIK3CA* mutation, and one had an *AKT1* mutation[144].

Currently, there are two "twin" ongoing studies testing ipatasertib with paclitaxel in triple negative breast cancer, either in the metastatic (LOTUS, NCT02162719) or in the neoadjuvant setting (FAIRLANE, NCT02301988).

#### *1.7.4.1. AZD5363*

AZD5363 (AstraZeneca) is a potent, catalytic inhibitor of all three AKT isoforms (AKT1, AKT2 and AKT3). Preclinical data demonstrate inhibition of phosphorylation of AKT substrates (PRAS40 and GSK3β), tumor cell proliferation, and tumor growth in xenograft models[145].

In the Phase I study in Japanese patients with advanced solid tumors (NCT01353781), AZD5363 was administered orally QD, and the dose was then escalated to BID in separate continuous (every day) and intermittent (4 days on, 3 days off [4/3] or 2 days on, 5 days off [2/5]) dosing schedules[146]. Forty-one patients were treated, and DLTs were only experienced with continuous dosing. Most common AEs included diarrhea (78%), hyperglycemia (68.3%), nausea (56.1%), and maculopapular rash (56.1%). Confirmed partial responses were observed in two patients: an ovarian cancer patient in the 480mg BID 4/3 schedule, who experienced a 55% reduction in target lesions and was on study for more than 2 years, and a ER+/HER2-negative heavily pre-treated MBC patient who experienced a 61% reduction in target lesions for 12 months. Interestingly, in

both patients an *AKT1* E17K mutation was retrospectively identified in archival tumor tissue.

A parallel Phase I dose finding study in non-Japanese patients also escalated AZD5363 in both continuous and intermittent schedules (NCT01226316)[147]. In this study, 90 heavily pre-treated metastatic patients were enrolled to receive AZD5363 in escalating doses and different continuous and intermittent (4/3 and 2/5) schedules. The RP2D was AZD5363 480mg BID 4/3. At this dose, no DLTs were observed, and pharmacodynamics data were consistent with robust blockade of the PI3K pathway. Most common G3 or above toxicities in all patients and at the RP2D were, respectively: hyperglycemia (20% and 36.4%), diarrhea (10% and 9.1%), rash (10%, none at RP2D), and nausea (3.3% and 9.1%). In the escalation part of the trial, a cervical cancer patient with a *PIK3CA* mutation experienced a confirmed PR. Two expansion cohorts for breast and gynecological cancers with *PIK3CA* mutations were subsequently opened. Among 20 MBC patients with PIK3CA mutations, ORR was 10% (one ER+/HER2-negative and one ER+/HER2+ patient). Among 17 patients with gynecological cancers and a *PIK3CA* mutation, a PR was observed in an endometrial cancer patient with a *PIK3CA* mutation in the p85 adaptor binding domain.

Given the encouraging results in *AKT1* mutant tumors, a new expansion “basket” cohort started to enroll patients with breast cancer, gynecological cancer, or all solid tumors harboring an *AKT1* mutation to receive AZD5363 at a 480 mg BID on a 4/3 schedule (NCT01226316). In this part of the trial, serial plasma samples were collected for detection and tracking of AKT1 E17K mutation (expressed as the mutant allele fraction [MAF]) in ctDNA by droplet digital PCR (ddPCR). Preliminary results were presented at the 2015 AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics[148]. Besides *AKT1* mutation, key inclusion criteria included absence of known *RAS/RAF* mutations, and presence of measurable disease as per RECIST v.1.1. *AKT1* E17K mutation status was identified through local screening and confirmed retrospectively by central assay. Forty-five patients were enrolled: ER+/HER2-negative MBC 20 (44.4%), TNBC 4 (8.9%), gynecological 13 (28.9%), and other tumor types 8

(17.8%). Among the 18 ER+/HER2-negative MBC patients with available RECIST v.1.1 data, 14 demonstrated target lesion shrinkage, including 3 with confirmed PR (ORR 16.7%) and 2 with unconfirmed PR. All patients with TNBC had tumor shrinkage, and one of them had a confirmed PR. In this study, ctDNA enabled prediction of clinical and radiological response and eventual progression. Another study was recently reported using serial NGS of ctDNA from patients with *AKT1* mutations enrolled in the Phase I basket AZD5363 study[149]. In this exploratory study, serial NGS of ctDNA with a large gene panel demonstrated to be feasible and highly concordant with ddPCR approaches, capturing disease heterogeneity and permitting the monitoring of tumor clone dynamics. NCT01226316 continues to accrue patients with ER+/HER2-negative *AKT1*-mutant tumors to receive AZD5363 in combination with fulvestrant.

AZD5363 has also been combined with chemotherapy in the Beech Study: a Phase I/II study of AZD5363 combined with paclitaxel in patients with advanced or metastatic breast cancer[150]. In the part A of this trial, 36 MBC cancer patients were enrolled to receive AZD5363 BID in escalating doses intermittent 4/3 and 2/5 schedules. MTD for both schedules were 400mg BID and 560mg BID, respectively, combined with weekly paclitaxel 90mg/m$^2$ 3 out of 4 weeks. The recommended dose for part B was 400mg BID combined with weekly paclitaxel 90mg/m$^2$ 3 out of 4 weeks. Consistently with the Phase I single agent results, the most common AEs probably related to AZD5363 were diarrhea (63.9%, G≥3 16.7%), rash (52.8%, G≥3 11.1%), hyperglycemia (19.4%, no G≥3), and nausea (19.4%, no G≥3). Overall median PFS (as per RECIST v.1.1 criteria) was 8.2 months, despite 69% of patients had received previous taxane and 53% had been exposed to at least 1 prior line of chemotherapy in the metastatic setting. Part B of this trial subsequently randomized ER+/HER2-negative MBC patients that received no prior chemotherapy for advanced disease (including a cohort of prospectively identified *PIK3CA* mutant breast cancer patients) to receive weekly paclitaxel 90mg/m$^2$ 3 out of 4 weeks plus AZD5363 400mg BID 4/3 or placebo. The same combination is being tested in TNBC in the PAKT trial (NCT02423603).

## 1.8. Biomarkers of response to PAM pathway inhibitors in MBC

The success of precision medicine depends on the development of accurate and reliable predictive biomarkers. A genomic biomarker is defined by the FDA as "a measurable DNA and/or RNA characteristic that is an indicator of normal biologic processes, pathogenic processes, and/or response to therapeutic or other interventions"[151]. A prognostic biomarker informs about a likely cancer outcome (e.g., disease recurrence, disease progression, death) independently of treatment received. A predictive biomarker, in turn, can discriminate response to a given therapy (experimental compared with control) in biomarker-positive patients compared with biomarker-negative patients[152].

In the early-phase trials of PAM pathway inhibitors, some predictive biomarker hypotheses have been tested, such as the presence of *PIK3CA* mutations, PTEN dysregulation, or *AKT1* mutations. In the following sections, we will provide an overview of available results.

### *1.8.1. Biomarkers of response to everolimus*

#### *1.8.1.1. BOLERO-2*

The first systematic effort to find predictive biomarkers of efficacy to a PAM pathway inhibitor in a prospective randomized clinical trial came from the BOLERO-2 study[153]. In this exploratory study, exons of cancer-related genes were sequenced using NGS technology in archival tumor specimens from a subset of patients, and potential associations between PFS benefit from everolimus and genetic alterations in PAM and FGFR pathway genes were explored. The authors also developed an estimation metric for chromosomal instability using NGS data from a target gene panel and explored its correlation with everolimus PFS benefit. Tumor samples from 302 patients had NGS data available (41.7% of the whole trial population). The NGS subgroup was representative of the BOLERO-2 trial population in terms of patients' characteristics and randomization arm. The most frequently altered genes were *PIK3CA* (47.6%), *CCND1* (31.3%), *TP53* (23.3%), and *FGFR1* (18.1%).

Almost 90% of *PIK3CA* mutations occurred in one of the known hotspots in exon 9 (32.9%) or 20 (53.9%). Interestingly, *PIK3CA* mutations only minimally affected the efficacy of everolimus: HR 0.37 (95%CI 0.25-0.55) for *PIK3CA* WT and HR 0.51 (95%CI 0.34-0.77) for PIK3CA mutant (interaction P=0.35). Median PFS was longer in patients with *PIK3CA* WT in both treatment arms (Figure 9A). PFS benefit from everolimus appeared to be greater in patients with exon 9 mutations (HR 0.26, 95%CI 0.12-0.54) than in those with exon 20 mutations (HR 0.56, 95%CI 0.31-1.00).

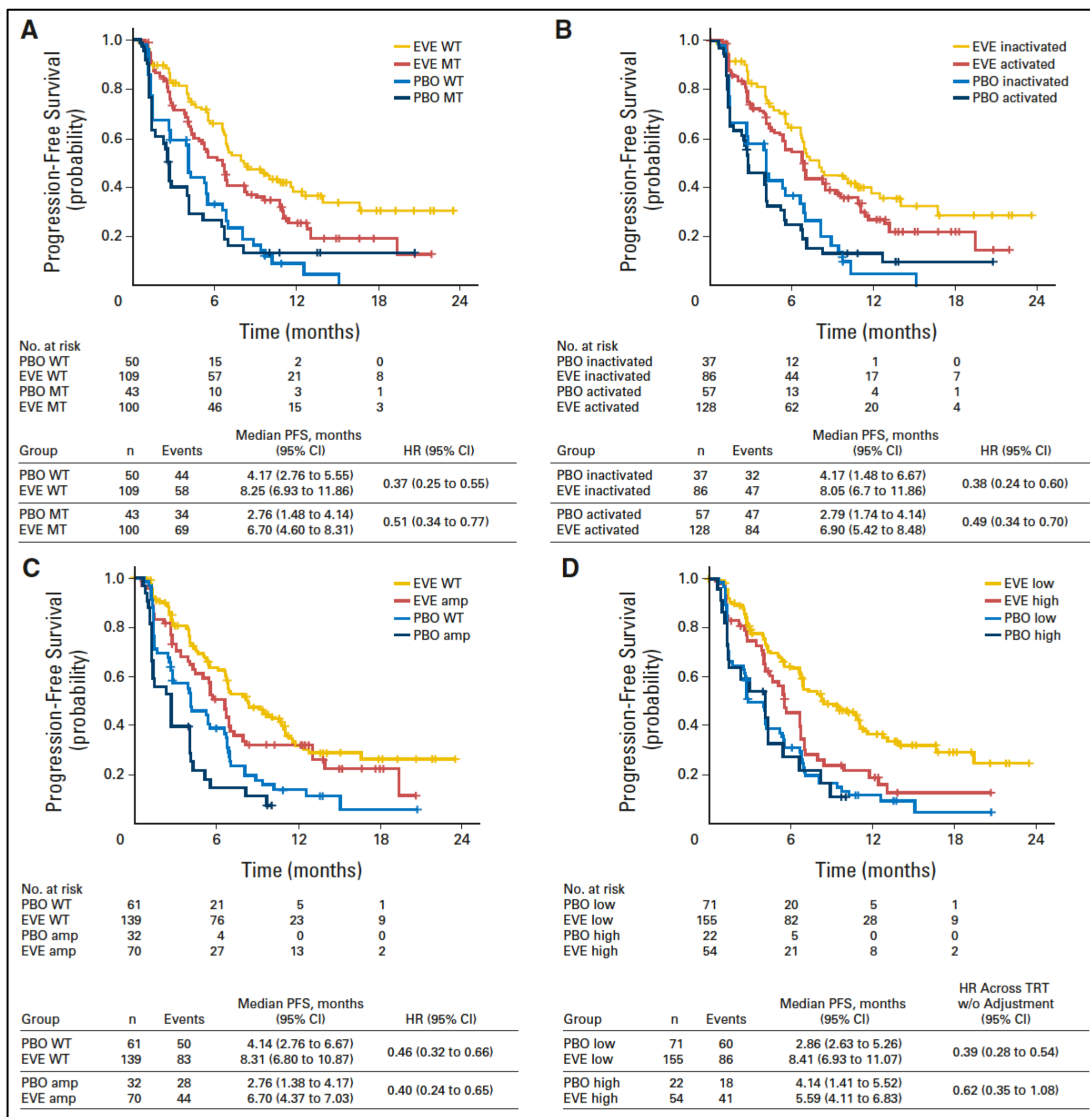


**Figure 9:** Kaplan-Meier curves for PFS by treatment arm for patient subgroups in the BOLERO-2. Subgroups were defined by gene mutation (MT) versus wild-type (WT), amplification (amp), chromosomal instability (CIN) score low or high, or pathway activity. **(A)** *PIK3CA* status. **(B)** PI3K pathway status. **(C)** Cell-cycle control genes. **(D)** CIN score in which the 75th percentile was used as the cutoff. EVE, everolimus; HR, hazard ratio; PBO, placebo; TRT, treatment; w/o, without. From Reference Hortobagyi G. et al[153].

The authors also studied whether activation of the PAM pathway would be predictive of everolimus benefit. For the purpose of this study, hyperactive PAM pathway was defined as the presence of either at least one mutation in *PIK3CA*, *PTEN*, *AKT1*, or *PIK3R1*, or low PTEN expression (IHC H-Score<10). Again, treatment with everolimus did not associate with better PFS in this population (interaction P=0.5, Figure 9B).

Regarding amplification of *CCND1* or genetic alterations in cell-cycle control genes *CCND1*, *CDK4*, *CDK6*, and *CDKN2A*, the authors found minimal or no effect on PFS gain with everolimus (Figure 9C).

Regarding *FGFR1* amplification, the PFS benefit with everolimus was similar in FGFR1-amplified (HR 0.39, 95%CI 0.21-0.72) and non-amplified (HR 0.43, 95%CI 0.31-0.6) cohorts, with a median PFS gain of approximately 4 months in each cohort.

Finally, tumors with lower chromosomal instability (as derived from NGS analysis) might derive greater benefit from the addition of everolimus (Figure 9D). Notably, the effect of chromosomal instability on PFS was only evident in patients in the everolimus arm and not the placebo arm, suggesting that the potential association between chromosomal instability and PFS was everolimus-specific.

Taken together, these results show that the efficacy of everolimus was well maintained independently of the presence of *PIK3CA* mutation, PAM pathway activation, cell-cycle pathway alteration, or *FGFR1* amplification. Interestingly, *PIK3CA* exon and the presence of chromosomal instability were potential predictive biomarkers of efficacy of everolimus in BOLERO-2.

#### *1.8.1.1. TAMRAD*

Treilleux I. et al. published an exploratory biomarker analysis using tumor samples collected from the TAMRAD trial, aiming at identifying potential predictive

biomarkers of response of everolimus plus tamoxifen[154]. Tissue samples could be obtained in 55 from the initial 111 patients enrolled in TAMRAD (49.5%): 20 patients from the tamoxifen alone arm, and 35 patients from the tamoxifen plus everolimus arm. The authors sought to analyze not only the presence of *PIK3CA* mutations, as well as downstream biomarkers of activation of the PAM pathway by IHC, including PI3Kp85, PTEN, LKB1, pAKT, eIF4E, 4EBP1, p4EBP1, S6RP, and pS6RP[154].

In the biomarker population, median TTP and HR for progression were consistent with the general population: 10 months with tamoxifen plus everolimus and 5 months with tamoxifen alone (HR 0.57, 95%CI 0.32-1.00). From the 55 available samples, DNA could be extracted in 45 (81.8%; 40.5% of the initial population). *PIK3CA* mutation was identified in 9/45 samples (20%; exon 9 N=2, exon 20 N=7), and *KRAS* mutation in 1/45 (2.2%). There was no correlation between *PIK3CA* mutational status and levels of expression of any of the biomarkers assessed by IHC. TTP of the nine patients with *PIK3CA*-mutant tumors was 7.5 months (95%CI 2.3-23.6) versus 6.8 months for the whole tumor population (N= 36; 95%CI 3.7-9.4). Given the small numbers of patients with *PIK3CA* mutations – 5 in the tamoxifen plus everolimus arm and 4 in the tamoxifen alone arm – the authors did not perform statistical analysis for TTP between these two groups.

Baseline expression levels of 4EBP1, p4EBP1, cytoplasmic LKB1, cytoplasmic pAKT, and PI3K – but not pS6RP or eIF4E – were potentially predictive of everolimus benefit (Figure 10).

One interesting finding in this analysis is the association of low LKB1 expression at baseline (consistent with mTORC1 activation through AKT-independent pathways) with benefit from everolimus. As the authors acknowledge, this observation needs further validation in larger trials.

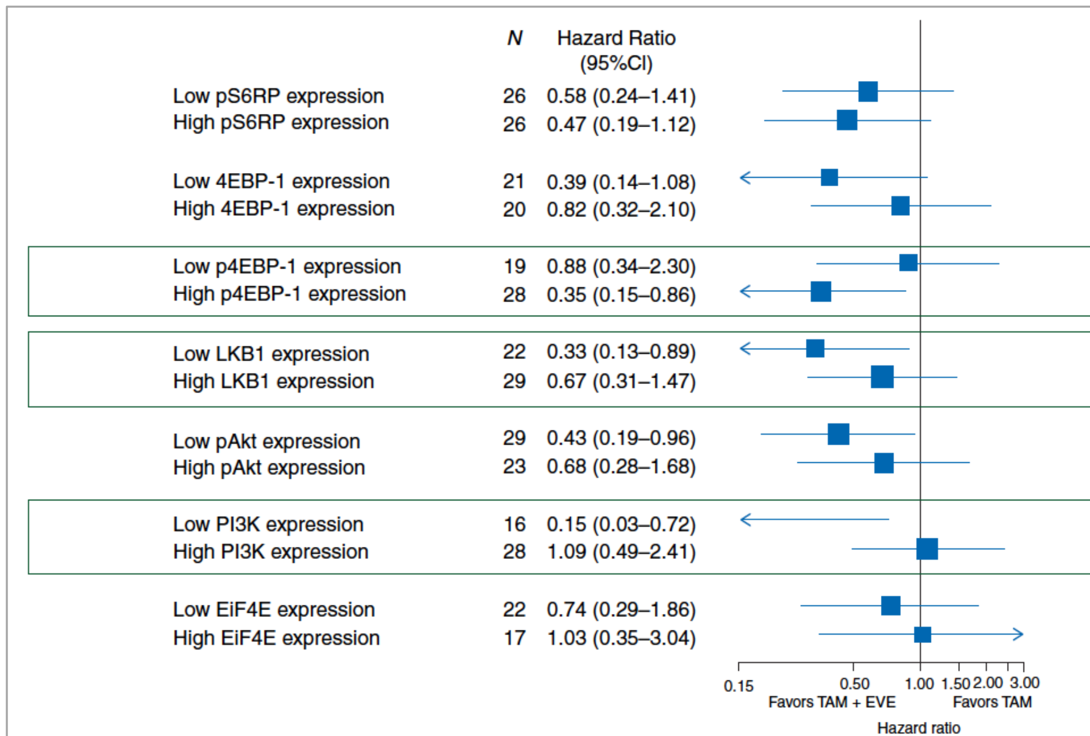


**Figure 10:** Forest plot of TTP with tamoxifen plus everolimus vs. tamoxifen alone for each biomarker tested. EVE: everolimus. TAM: tamoxifen. From Reference Treilleux I. et al[154].

### *1.8.1.2. BOLERO 1 and BOLERO 3*

BOLERO-1 and BOLERO-3 tested the hypothesis of whether the addition of everolimus would improve efficacy of standard anti-HER2 regimens with trastuzumab and paclitaxel or vinorelbine, respectively, in HER2+ MBC patients. As previously described, in BOLERO-1 the addition of everolimus to trastuzumab and paclitaxel as first-line treatment of HER2+ advanced breast cancer did not significantly prolong PFS (15 vs. 14.5 months; HR 0.89, 95%CI 0.73-1.08; P=0.117)[100]. In BOLERO-3, everolimus in combination with trastuzumab and vinorelbine significantly prolonged PFS in patients progressing on prior trastuzumab and a taxane (7.0 vs. 5.8 months; HR 0.78, 95%CI 0.65-0.95; P=0.007)[101]. Subset analysis of both trials suggested that some subpopulations (ER-negative, no visceral involvement, younger patients) might derive greater benefit from adding everolimus to standard trastuzumab plus chemotherapy. An exploratory biomarker analysis of these two trials was recently published, aimed at identifying predictive biomarkers of sensitivity to everolimus in HER2+ MBC[155].

Tumor samples from 549 patients (302 in BOLERO-1, 247 in BOLERO-3) had NGS or Sanger sequencing or IHC data (biomarker population) available for evaluation (42% of the BOLERO-1 and 43% of the BOLERO-3 trial populations). Overall, the biomarker population had similar demographics and baseline characteristics respect to the overall trial population. In BOLERO-1, PFS benefit with everolimus was similar between the biomarker population (HR 0.93, 95%CI 0.68-1.26) and the overall population (HR 0.89, 95%CI 0.73-1.08). In BOLERO-3, PFS benefit with everolimus was relatively lower in the biomarker population (HR 0.91, 95%CI 0.69-1.22) as compared with the overall trial population (HR 0.78, 95%CI 0.65-0.95).

Data from 195 samples in BOLERO-1 and 182 samples in BOLERO-3 (total, 377 samples) were evaluable for genomic analysis by NGS. Somatic mutations in *ERBB2* were detected in three samples (D769H, V774M, V777L) in BOLERO-1, and four samples (three patients with L755S, one with W825) in BOLERO-3. Other alterations in BOLERO-1 and BOLERO-3 were *TP53* mutation (65% and 70%, respectively), *PIK3CA* mutation (30% and 32%), PTEN dysregulation (defined as *PTEN* mutation or PTEN loss of expression; 16% and 12%), *CCND1* amplification (19% and 15%) and *FGFR1* amplification (9% and 13%). Hyperactive PI3K pathway (defined as PTEN dysregulation and/or known *PIK3CA* and/or *AKT1* E17K mutation) was reported in 101 (47%) samples in BOLERO-1 and 96 (41%) samples in BOLERO-3. Amplifications in *FGFR* and *CCND1* were more frequent in HER2+/ER+ than in HER2+/ER-negative tumors. Conversely, TP53 mutations were more frequent in HER2+/ER-negative than in HER2+/ER+ tumors.

A meta-analysis using pooled data from both trials showed statistically significant PFS benefit from everolimus in patients with *PIK3CA* mutations (HR 0.67, 95%CI 0.45-1.00; Figure 11A), PTEN low/loss (HR 0.54, 95%CI 0.31-0.96; Figure 11B), or hyperactive PI3K pathway (HR 0.67, 95%CI 0.48-0.93; Figure 11C). Patients without these molecular alterations showed essentially minimal PFS benefit from everolimus over placebo. In contrast, no significant or consistent effect on everolimus efficacy was found in either study according to the presence of alterations in genes regulating the cell cycle.

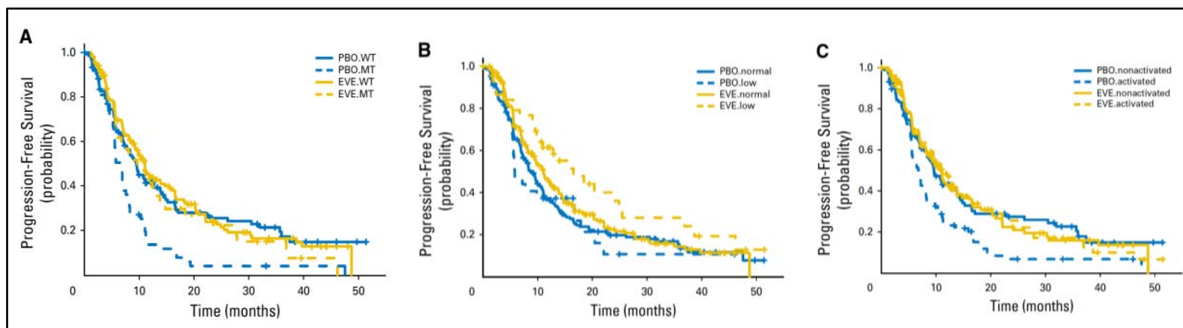


**Figure 11:** Kaplan-Meier curves for PFS by biomarker status in BOLERO-1 and BOLERO-3 (pooled data). **(A**) PIK3CA wild type (WT) versus mutant (MT). **(B)** PTEN normal versus low/loss. **(C)** PI3K pathway activity normal versus hyperactive. EVE, everolimus; PBO, placebo. From Reference Andre F. et al[155].

### *1.8.2. Biomarkers of response to PI3K inhibitors*

#### *1.8.2.1. Biomarkers of response to buparlisib*

The presence of *PIK3CA* mutation and/or PTEN dysregulation has been postulated to be predictive of response to PAM pathway inhibitors, especially PI3K inhibitors. For this reason, one of the co-primary objectives of the previously described Belle-2 trial was to assess whether the combination of fulvestrant plus buparlisib was superior than fulvestrant plus placebo in the subset of patients which tumors had a PI3K pathway activation (defined as the presence of a *PIK3CA* mutation and/or PTEN loss, as assessed by a Central laboratory in archival tissue). This issue was also studied in the Belle-3 trial.

In the Belle-2 trial, among the 372 patients with PI3K pathway activation assessed in archival tumor tissue, the addition of buparlisib to fulvestrant did not significantly increased PFS with respect to fulvestrant alone[116]. Median PFS in the buparlisib arm was 6.8 months, compared with 4.0 months in the placebo arm (HR 0.76, 95%CI 0.60-0.97; P=0.014, for one-sided α=0.01 level of significance). Interestingly enough, detection of a *PIK3CA* mutation in ctDNA just prior to the randomization did predict for better outcome, with larger differences in PFS in the buparlisib-treated patients than in the full population (7.0 vs. 3.2 months; HR 0.56, 95%CI 0.39-0.80; P<0.001).

In the Belle-3 trial, among the 109 patients with *PIK3CA* mutation detected in archival tumor tissue, the addition of buparlisib to fulvestrant significantly increased PFS with respect to fulvestrant alone (median PFS 4.7 vs. 1.4 months; HR 0.39, 95%CI 0.23-0.65; P<0.001)[117]. Conversely, among the 212 patients with no *PIK3CA* mutation detected in archival tumor, no difference was observed between treatment arms (2.8 vs. 2.7 months; HR 0.83, 95%CI 0.6-1.14, P=0.117). Similarly to Belle-2, detection of a *PIK3CA* mutation in ctDNA just prior to the randomization (N=136) also predicted for better outcome with buparlisib vs. placebo (median PFS 4.2 vs. 1.6 months; HR 0.46, 95%CI 0.29-0.73; P<0.001).

Taken together, these results suggest that the presence of a *PIK3CA* mutation, especially if detected in ctDNA just prior to the beginning of treatment, may be predictive of response to buparlisib plus fulvestrant in ER+/HER2-negative MBC patients whose disease progressed after AI or AI plus everolimus.

#### *1.8.2.2. Biomarkers of response to pictilisib*

The FERGI and PEGGY trials also tested the hypothesis of *PIK3CA* mutation as a predictive biomarker of response to pictilisib, as described above[106,121]. In these trials, patients with *PIK3CA* mutation detected in archival tissue did not benefit more from pictilisib plus fulvestrant or paclitaxel than those without *PIK3CA* mutation.

Pre-operative or window-of-opportunity trials are a validated strategy for evaluating the impact of targeted therapies and assess biomarkers of response to those therapies[156]. The OPPORTUNE trial randomized (2:1) 75 ER+/HER2-negative EBC patients to receive pictilisib plus anastrozole or anastrozole alone during 14 days before surgery[114]. The study had two primary endpoints: to detect an increase in Ki67 suppression with pictilisib, and to assess the treatment effects in subgroups defined by *PIK3CA* mutations, luminal A/B subtypes, and baseline Ki67 scores. When compared to anastrozole alone, the combination of pictilisib with anastrozole led to a higher mean percentage suppression of Ki67 (83.8% vs. 66.0%, P=0.004), a higher percentage of tumors with >50% fall in Ki67 expression

between baseline and day 15 (86.4% vs. 53.9%, P=0.003), and a higher end-of-treatment Ki67 expression suppression (2.9% vs. 6.1%, P=0.005). When looking at the association between Ki67 and *PIK3CA* mutations, the authors found that baseline Ki67 expression was comparable between WT and mutant samples (WT 23.3% vs. helical domain mutation 20.7% vs. kinase domain mutation 25.5%). Further, no association was found between *PIK3CA* mutation and antiproliferative response to anastrozole or anastrozole plus pictilisib. However, tumors with helical or kinase domain mutations appeared to respond differently to anastrozole plus pictilisib (Ki67 suppression ratio: helical 0.32 [95%CI, ≤0.73] vs. kinase 0.76 [95%CI, ≤1.63]). Subgroup analysis showed that patients with PAM50 luminal B tumors had a significantly higher antiproliferative response with anastrozole plus pictilisib compared to anastrozole alone (mean Ki67 suppression 86.5% vs. 63.6%, P=0.008), whereas adding pictilisib had no apparent benefit for luminal A tumors. Multivariable analysis confirmed significant interaction between treatment effect and molecular subtype (P=0.03), supporting the hypothesis that Ki67 suppression (measured as mean Ki-67 decrease at day 15) is higher with anastrozole plus pictilisib than with anastrozole alone for patients with luminal B tumors irrespective of PgR status or baseline Ki67 expression. Further genomic and transcriptomic analysis of these paired samples will be important to better characterize biomarkers of response to this drug.

#### ***1.8.2.3. Biomarkers of response to alpelisib***

An exploratory NGS analysis of predictive markers of sensitivity to alpelisib on baseline tumor samples was presented at the AACR 2013 Annual Meeting[157]. Analysis of 94 tumor samples from 79 patients enrolled in the first-in-human Phase I trial was conducted at Foundation Medicine and The Broad Institute, to characterize the genetic landscape of the tumors and investigate the potential association of genetic alterations with clinical efficacy as assessed by PFS (per RECIST v.1.0). In this study, preliminary clinical activity of alpelisib was mainly observed in patients with ER+ *PIK3CA* mutant MBC and in *PIK3CA* mutant head and neck cancer. Duration of treatment with alpelisib was shorter for patients with colorectal cancer (CRC). Mutations frequently found in CRC, such as APC and

TP53, associated with lack of clinical benefit to alpelisib, and there was a trend for a negative association for *KRAS* mutations and alpelisib efficacy. Interestingly, this finding supports the results from preclinical studies, where *KRAS*/*PIK3CA* double mutant cell lines are resistant to PAM pathway blockade[158].

#### *1.8.2.4. Biomarkers of response to taselisib*

Biomarker analysis from the escalation part Phase I study of taselisib were presented at the AACR 2014 Annual Meeting[159]. Among the 34 patients enrolled, metabolic partial responses via FDG-PET were observed in 6 out of 13 patients assessed, and clinical partial responses were observed in 6 patients, 5 of them with *PIK3CA* mutation. Tumor tissue was obtained from 30 out of the 34 patients enrolled. The proportion of PAM pathway alterations was as follows: *PIK3CA* mutation 41%, PTEN null 10% (with additional 10% with reduced PTEN expression based on an H-score assessment). *PIK3CA* mutations were largely mutually exclusive with mutations in the RAS pathway. However, three out of the 14 *PIK3CA* mutant patients had a coexisting mutation within *KRAS*. Interestingly, and similar to the findings with alpelisib, preliminary analysis showed lack of benefit in patients with *KRAS* mutations treated with taselisib single agent. Collectively, these results suggest that taselisib demonstrates single agent activity in patients with tumors harboring *PIK3CA* mutations and with unaltered PTEN or MAP-kinase pathways.

In the neoadjuvant LORELEI study (NCT02273973), a comprehensive analysis of biomarkers of response (and resistance) to taselisib plus letrozole is planned. Results are expected in 2017.

# 2
# Justification

## 2. Justification

Breast cancer is a heterogeneous disease, and adequate treatment of each tumor implies a thorough knowledge of its drivers and natural history. Identification of biologically important genes and pathways frequently disrupted in samples from MBC patients can generate clinically relevant diagnostic, prognostic, and therapeutic information. Routine molecular screening of primary or metastatic tumors is now a reality in several Centers around the world[3,61,160]. The major objective of this tremendous effort is to identify clinically relevant genes in order to deliver optimal cancer care for patients. In 2009, we initiated a prospective study aimed at determining genetic alterations present in samples from MBC patients, in order to enroll them in clinical trials with drugs targeting those alterations. Establishing the molecular portrait of our MBC patients is helpful to better match them to “rational” clinical trials as well as design and project next generation trials and combinations. One of the aims of this work was therefore to describe the molecular alterations present in a cohort of MBC patients treated at the Vall d’Hebron University Hospital / Vall d’Hebron Institute of Oncology (VHUH/VHIO).

Together with CDK4/6 inhibitors and anti-HER2 therapies, PAM pathway inhibitors are perhaps among the most studied drugs in breast cancer over the last decade. PAM pathway alterations are very frequent in breast cancer, and are also oncogenic. From the very outset, PAM pathway inhibitors have been one of the main areas of research led by our Early Clinical Drug Development Group at VHIO. For this reason, we have perhaps one of the most extensive populations of MBC patients treated with these drugs within the same Institution. We wanted to analyze the characteristics of these patients and the treatment they received, which is key to take advantage of this therapeutic expertise.

One relevant question when performing genotyping is whether this strategy improves patients’ outcomes. Most of the studies suggest that matching molecular alterations with targeted agents is better than giving targeted agents to unselected populations[161-163], although logistic hurdles may jeopardize inclusion rates in matched trials[63]. Another aim of this work is to establish the potential benefit of

PAM pathway inhibitors in MBC patients treated in the same Institution, where logistic problems related to genotyping efforts across different Institutions are minimized, and in a place where the drug portfolio in PAM pathway inhibitors is constantly updated.

The identification of predictive biomarkers to targeted therapies is an area of intense investigation and a clear clinical need. The main objective of selecting the right patients to offer them targeted drugs is to maximize treatment benefit while sparing them from unnecessary adverse events. *PIK3CA* mutations seem to predict efficacy for some PAM pathway inhibitors, but not all patients derive equal benefit[106,116]. One of the main purposes of this study is therefore to perform a comprehensive analysis of the potential predictive biomarkers of response to PAM pathway inhibitors. Specifically, we will focus on the presence of PAM pathway alterations, including *PIK3CA* mutations, PTEN dysregulation, and *AKT1* mutations. Furthermore, we will explore the clonality of *PIK3CA* mutations as a predictive factor of response to PAM pathway inhibitors.

# 3
# Hypothesis

# 3. Hypothesis

## 3.1. Primary Hypotheses

1. The presence of molecular alterations – especially those leading to PAM pathway dysregulation, including *PIK3CA* mutation, PTEN dysregulation, and *AKT1* mutation – is frequent in MBC patients.
2. The presence of PAM pathway alterations is predictive of efficacy to PAM pathway inhibitors.

## 3.2. Secondary Hypotheses

1. The prevalence of actionable molecular alterations in MBC, especially those in the PAM pathway, differs according to the breast cancer subtype.
2. Frequency of molecular alterations varies depending on the site of the sample used for genotyping.
3. *PIK3CA* and *AKT1* mutations are clonal events in MBC.
4. Multiple molecular alterations may co-occur in MBC.
5. The detection of an actionable molecular alteration increases the probability of a MBC patient entering a clinical trial.
6. The presence of a PAM pathway alteration increases the probability of a MBC patient receiving a PAM pathway inhibitor.
7. The presence of PIK3CA mutations is predictive of benefit to PI3Kα-specific inhibitors.

# 4
# Objectives

# 4. Objectives

## 4.1. Primary Objectives

1. Characterize the prevalence of molecular alterations in a cohort of MBC patients treated at VHUH/VHIO, with a special focus on PAM pathway alterations like *PIK3CA* mutation, PTEN dysregulation, and *AKT1* mutation.
2. Assess predictive factors of efficacy (measured as clinical benefit rate and time to treatment failure) of PAM pathway inhibitors in this population.

## 4.2. Secondary Objectives

1. Characterize the prevalence of actionable alterations and PAM pathway alterations, overall and according to breast cancer subtype.
2. Describe the differences in the molecular alterations according to the site of the sample used for genotyping.
3. Assess the clonality of *PIK3CA*, *AKT1*, and other somatic mutations.
4. Describe the co-occurrence of molecular alterations in this cohort of MBC patients.
5. Assess the rate of enrollment in clinical trials in this population according to the presence of an actionable alteration.
6. Evaluate whether the presence of PAM pathway alterations increases the probability of receiving PAM pathway inhibitors.
7. Analyze predictive factors of efficacy to PI3Kα-specific inhibitors.

# 5
# Patients and Methods

# 5. Patients and Methods

## 5.1. Study design

This is a retrospective cohort study using prospectively acquired data from several research projects and clinical trials.

## 5.2. Sample selection

Consecutive MBC patients screened for gene mutation by Sequenom® or AmpliconSeq within the VHUH/VHIO molecular screening program from January 2010 until December 2015 were identified from our Database. Data on FGFR1 amplification and PTEN IHC were also collected if available. All patients provided signed informed consent to the molecular determinations as part of routine molecular testing at our Institution. Patients were only included in the analysis if tumor tissue was available for mutation testing.

## 5.3. Clinical and pathologic variables

Registration of the patients in the database, pathology assessments, molecular determinations, and treatment were all performed at VHUH/VHIO. Medical charts of all patients were reviewed to confirm the accuracy of the variables recorded in the database.

Patients' demographics (age, sex), tumor characteristics (histologic type, grade, ER, PgR, HER2, Ki67), characteristics of metastatic disease at the time of initiating treatment with PI3K pathway inhibitor (number and type of metastatic sites, number of prior lines for MBC), outcome of the treatment (best response and reason for treatment discontinuation), and vital status (including date of death or last follow-up) were collected and recorded for each patient.

We used the following surrogate definitions for the breast cancer subtypes:

- **Luminal (LUM):** ER and/or PgR+ *and* HER2-negative, independently of Ki67;
- **HER2+:** HER2+ as per ASCO/CAP guidelines[164], independently of ER or PgR;
- **Triple negative (TN):** ER negative *and* PgR negative *and* HER2-negative.

Saint Gallen definitions[48] were not used because of missing data on grade and/or Ki67, which would have excluded many patients from the analysis.

Patients were classified as having a PAM pathway dysregulation in the presence of at least one of the following alterations: *PIK3CA* mutation, *AKT1* mutation, *NF2* mutation, and PTEN dysregulation (mutation or null/low expression). Otherwise, they were classified as having no pathway dysregulation.

### 5.4. Molecular analysis

Molecular analysis was performed according to the standards of the pre-screening program of VHUH/VHIO, briefly described below.

Upon signing the appropriate Ethics Committee approved informed consent form, a tumor sample was collected for each patient and underwent a quality check by an experienced pathologist. If more than 20% of tumor cells were present, the appropriate number of slides was cut for DNA extraction, IHC, and/or FISH analysis, depending on the clinician request.

The following targeted panels were used for the DNA mutation analysis: MassARRAY system (Sequenom®) until June 2014, and AmpliconSeq afterwards.

**Sequenom®** was performed using two assay panels: OncoCarta™ v1.0 and CLIA v2.2. This panel performs somatic mutation profiling of 273 mutations in 21 oncogenes (Table 2). The panel is based on the use of IPlex chemistry (Sequenom®). In brief, DNA was extracted from 5x10μm slices of FFPE tumor samples using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion). Six-

hundred nanograms (ng) of DNA were used for mutation profiling using OncoCarta v1.0 or CLIA v2.2. After quantification (nanodrop) and dilution of DNA to a 10ng/µl concentration, multiplexed polymerase chain reaction (PCR) was performed in order to amplify the genomic regions that contain the loci to be genotyped. Each mutation was then analyzed as the single-base extension product of a probe that anneals immediately contiguous to the mutation position. Gen II SpectroCHIPs were loaded into a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer (MassARRAY, Sequenom®) and spectra were obtained for each of the extension products. Data analysis and mutation reports were generated using the Typer Analyzer 4.0 software (Sequenom®). Manual assessing of spectra was performed on each of the PIK3CA assays included in the panel for all samples as well as on all reported mutations by the Sequenom software. According to the analytical sensitivity of this technique, a mutation call was done in the presence of >10% mutate alleles in a given sample.

**Table 2:** Genes in Oncocarta used with Sequenom®.

| | | |
|---|---|---|
| ABL1 | FGFR1 | MET |
| AKT1 | FGFR3 | NRAS |
| AKT2 | FLT3 | MET |
| BRAF | HRAS | NRAS |
| CDK | JAK2 | PDGFRA |
| EGFR | KIT | PIK3CA |
| ERBB2 | KRAS | RET |

**AmpliconSeq:** an initial multiplex-PCR with VHIO-Card, a custom panel, was performed on tumor FFPE-derived samples. The last version of the panel contains over 800 primer pairs targeting frequent mutations in oncogenes plus several tumor suppressors, totaling 61 genes (Table 3). Since the implementation of AmpliconSeq, this panel has been customized on an ongoing basis to include additional genes and mutations, according to the inclusion criteria of ongoing clinical trials at our Institution and the state of the art. For this reason, the number of samples analyzed for a given mutation may vary depending on the timing of the analysis. For instance, *TP53* mutations were only incorporated in the last version of the panel, whereas the number of detected mutations in *PIK3CA* has remained unchanged since the beginning. Indexed libraries were loaded onto a MiSeq

instrument and sequencing performed (2X100). After alignment and variant calling, SNPs were filtered out with dbSNP and 1000 genome datasets. All detected variants were manually checked. According to the analytical sensitivity of this technique, a mutation call was done in the presence of >5% mutant alleles in a given sample.

**Table 3:** Genes in VHIO-Card v2 used with AmpliconSeq.

| | | | | |
|---|---|---|---|---|
| ABL1 | ERBB3 | IDH1 | MYC | RNF43 |
| AKT1 | ESR1 | IDH2 | NF2 | RUNX1 |
| AKT2 | FBXW7 | JAK1 | NOTCH1 | SMAD4 |
| AKT3 | FGFR1 | JAK3 | NOTCH4 | SMARCB1 |
| ALK | FGFR2 | KIT | NRAS | SRC |
| APC | FGFR3 | KRAS | PDGFRA | STK11 |
| BRAF | FGFR4 | MAG | PIK3CA | TP53 |
| CDH1 | FLT3 | MAP2K1 | PIK3R1 | VHL |
| CDKN2A | GATA1 | MET | PIK3R5 | ZNRF3 |
| CSF1R | GNA11 | MLH1 | PTCH1 | |
| CTNNB1 | GNAQ | MPL | PTEN | |
| EGFR | GNAS | MSH6 | RB1 | |
| ERBB2 | HRAS | MTOR | RET | |

Whenever possible, information on **Mutant Allele Fraction (MAF)** for a given mutation call was collected. MAF was corrected for tumor purity (the fraction of neoplastic cells in the sample, excluding stroma and immune infiltrate), and this parameter was called adjusted MAF[165,166]. For instance, if the determined MAF was 20% (0.2) and tumor purity was 40% (0.4), adjusted MAF would be 0.5 (0.2 / 0.4). Mutations were then categorized according to adjusted MAF as clonal (adjusted MAF> 0.3) or subclonal (adjusted MAF≤ 0.3). Adjusted MAF was also analyzed as a continuous variable for the purpose of survival analysis.

**FGFR1 amplification** was determined using fluorescence in situ hybridization (FISH; Zytovision). FGFR1 probe was ZytoLight® SPEC FGFR1/centromere 8 Dual Color Probe (ref. Z-2072-200). Amplification was defined as a ratio gene/centromere ≥2.2, or an absolute number of gene copies equal or greater than 6, according to the published literature[167,168].

**PTEN expression** was determined by IHC. The antibody anti-PTEN from Cell Signaling Technologies® (Clone 138G6, cat# 9559, batch 7-12) was used in a 1:100 diluted incubated at room temperature for 60 minutes. An H-score was calculated based on the intensity of staining (0-3+) and percentage of staining-positive cells (0-100%) according to the following formula:

$$H - score = [1 \times (\%\, cells\ 1+) + 2 \times (\%\, cells\ 2+) + 3 \times (\%\, cells\ 3+)]$$

PTEN low expression was considered when H-score was ≤ 50 (0-300). PTEN null was assumed in case of absence of immunostaining (as compared with the internal control).

A given alteration was classified as **actionable** if there was a biologically reasonable matched treatment available. For the purpose of this work, the following alterations were considered actionable:

- *PIK3CA* mutation
- *AKT1* mutation
- PTEN dysregulation (mutation or low/null expression)
- *NF2* mutation
- *ESR1* mutation
- *ERBB2* mutation or amplification
- *FGFR1* amplification

### 5.5. Treatment and patient evaluation

We will consider 2 cohorts of patients:

- The **Complete Cohort of MBC patients** or **Complete Cohor**t refers to all the patients that had a molecular determination, independently of the treatments received.
- The **PAM pathway inhibitor cohort** refers to those patients that received a PAM pathway inhibitor at some point of their disease.

We did not separately analyze those patients with molecular testing that did not receive a PAM pathway inhibitor.

Data on type of inhibitor – pan-isoform PI3K, PI3Kα-specific, dual PI3K/mTOR, mTORC1/2 or AKT – was collected for each patient.

All efficacy and safety assessments were done according to each individual protocol the patient was enrolled to. Generally, this included physical examination, evaluation of AEs and laboratory work-up at baseline and at least prior to the beginning of each cycle of treatment (every 21- or 28-days).

Treatment was continued until progressive disease or presence of unacceptable toxicity, and the reason for discontinuation was collected and recorded.

Tumor assessments were done for all patients at baseline and every other cycle later on (every 6 or 8 weeks, depending on the length of cycles at each different clinical trial).

Response was assessed according to the RECIST v.1.1 and reported as the best response[169]:

- **Complete response (CR):** disappearance of all lesions.
- **Partial response (PR):** reduction of at least 30% in the sum of target lesions respect to the baseline, without evidence of appearance of new lesions.
- **Progressive disease (PD):** at least a 20% increase in the sum of largest diameters of target lesions, taking as reference the smallest sum of largest diameters recorded since the treatment started, or appearance of unequivocal new lesions.
- **Stable disease (SD):** neither sufficient decrease in sum of largest diameters to qualify for PR nor sufficient increase in sum of largest diameters to qualify for PD.

### 5.6. Statistical analysis

Statistical analysis was performed with R software (v. 3.2.4). Nonparametric tests were used for comparisons of discrete counts (Fisher's exact test). Cox Proportional Hazards modeling (univariate and multivariate) was performed using R package survival, with P values derived from Log-Rank test (significance level of ≤0.05).

Efficacy assessments were defined as follows:

- **Overall Response rate (ORR):** proportion of patients with CR or PR as best response.
- **Clinical benefit rate (CBR):** proportion of patients with CR, PR or SD at 16 weeks.
- **Time to treatment failure (TTF):** time (in months) from the date of beginning PAM pathway inhibitor until the date of discontinuation for any reason (PD, discontinuation due to toxicity, or death). Patients that were on treatment at the time of data cut-off were censored for the purpose of the TTF analysis.

We analyzed the following putative predictors of CBR and TTF to PAM pathway inhibitors (univariate analysis):

- PAM pathway dysregulation in the overall population: yes vs. no.
- *PIK3CA* mutation in the overall population: yes vs. no.
- *PIK3CA* mutation in patients treated with PI3Kα-specific inhibitors: yes vs. no.
- Adjusted MAF of *PIK3CA* in patients treated with PAM pathway inhibitors: clonal vs. subclonal.
- Adjusted MAF of *PIK3CA* in patients treated with PI3Kα-specific inhibitors: clonal vs. subclonal.
- Type of therapy: single agent vs. combo with endocrine treatment vs. combo with chemotherapy vs. combo with anti-HER2 therapy.

Then, we ran a multivariate model for TTF taking also into account several known or potential prognostic factors in this context:

- Presence of visceral metastasis: no vs. yes.
- Number of sites of metastasis: 1-2 vs. 3 or more.
- Breast cancer subtype: LUM vs. HER2 vs. TN.
- Number or prior chemotherapies: ≤2 vs. >2.
- Type of PAM pathway inhibitor: mTORC1/2 vs. AKT vs. pan-PI3K vs. PI3K/mTOR vs. PI3Kα-specific.

# 6 Results

# 6. Results

## 6.1. Complete cohort of MBC patients

### *6.1.1. Patients and samples characteristics*

From January 2010 to December 2015, 327 MBC patients were successfully screened for somatic mutations at our Hospital. Table 4 summarizes patients' and samples characteristics.

**Table 4:** Patients' and samples characteristics (Complete cohort).

| | | |
|---|---|---|
| **Age** median (range) | 56 (28-85) | |
| **Gender** | | |
| Female | 327 (100%) | |
| Male | - | |
| **Subtype** | | |
| LUM | 201 (61.5%) | |
| HER2 | 36 (11%) | |
| TN | 59 (18%) | |
| Unk | 31 (9.5%) | |
| **Tumor sample** | | |
| Primary tumor | 227 (69.4%) | |
| Metastasis | 100 (30.6%) | |
| **Panel used** | | |
| Sequenom | 213 (65.1%) | |
| AmpliconSeq | 114 (34.9%) | |
| **Tumor purity** median (IQR) | 60% (40%-80%) | |
| Metastatic sample | 70% (40%-85%) | P=0.036 |
| Primary tumor | 60% (40%-80%) | |
| AmpliconSeq | 70% (45%-84%) | P=0.014 |
| Sequenom | 60% (35%-80%) | |

IQR: Interquartile range. LUM: Luminal. TN: Triple negative. Unk: unknown.

Median age of the patients was 56 (range 28-85), all female. Most of the patients had LUM tumors (61.5%), followed by TN (18%), and HER2+ tumors (11%; Figure 12). Subtype could not be determined in 31 patients (9.5%).

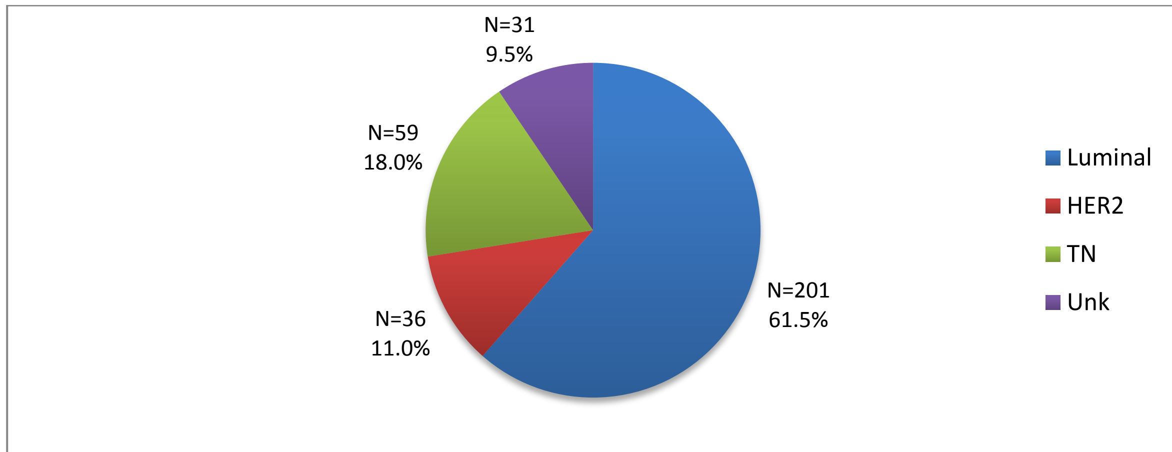


**Figure 12:** Subtype distribution of the samples in the Complete Cohort.
TN: triple negative. Unk: unknown.

Molecular analysis was performed in primary tumor samples in 227 cases (69.4%), and in metastatic samples in 100 (30.6%). Median tumor purity was higher in metastatic samples that in primary tumor samples (70% vs. 60%, P=0.036). Of note, minimum tumor purity required for the analysis with our targeted panels is 20%, and this criterion was met in all tested samples.

Sequenom® was used in 213 samples (65.1%), while AmpliconSeq was used in 114 (34.9%). Tumor purity was higher in samples tested by AmpliconSeq than by Sequenom (70% vs. 60%, P=0.014).

Figure 13 shows the evolution of the number of molecular determinations in MBC over the years. As depicted, there was an overall trend for an increased number of requests since the beginning of the pre-screening program until the present.

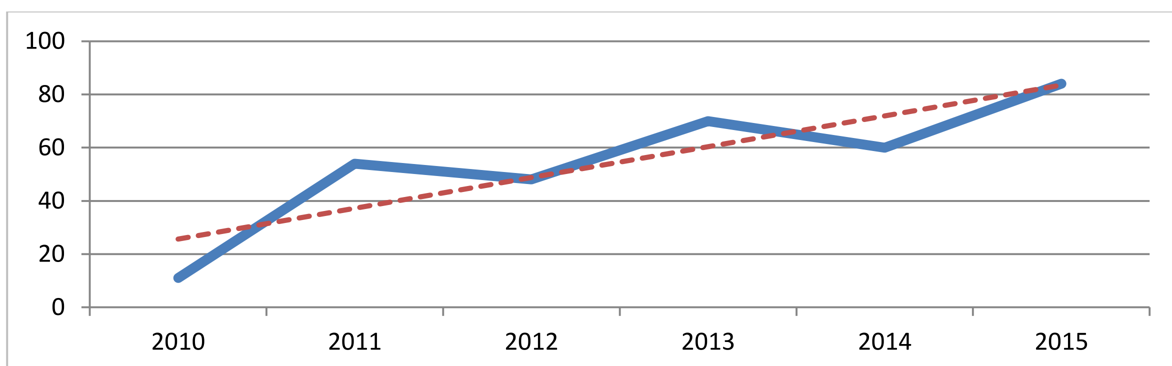


**Figure 13:** Evolution of the number of molecular determinations in MBC over the years.
Red dotted line denotes overall trend.

### 6.1.2. Distribution of molecular aberrations

#### 6.1.2.1. Overview

Figure 14 shows the overall distribution of molecular aberrations in the Complete cohort of MBC patients. *TP53* mutation was the most common alteration (34.2%), followed by *PIK3CA* mutation (24%), *FGFR1* amplification (15.8%), *HER2* amplification (12%), PTEN loss of expression (10.6%), *ESR1* mutation (6.3%), *PTEN* mutation (5.7%), *AKT1* mutation (4.1%), and *NF2* mutation (2.8%). Other genes with mutations included *ERBB3* (1.8%), *SMAD4* (1.8%), *ERBB2* (1.3%), *KRAS* (1.3%), *FGFR4* (0.9%), *RNF43* (0.9%), *FGFR2* (0.9%), *RUNX1* (0.9%), *EGFR* (0.6%), and *FGFR1* (0.6%).

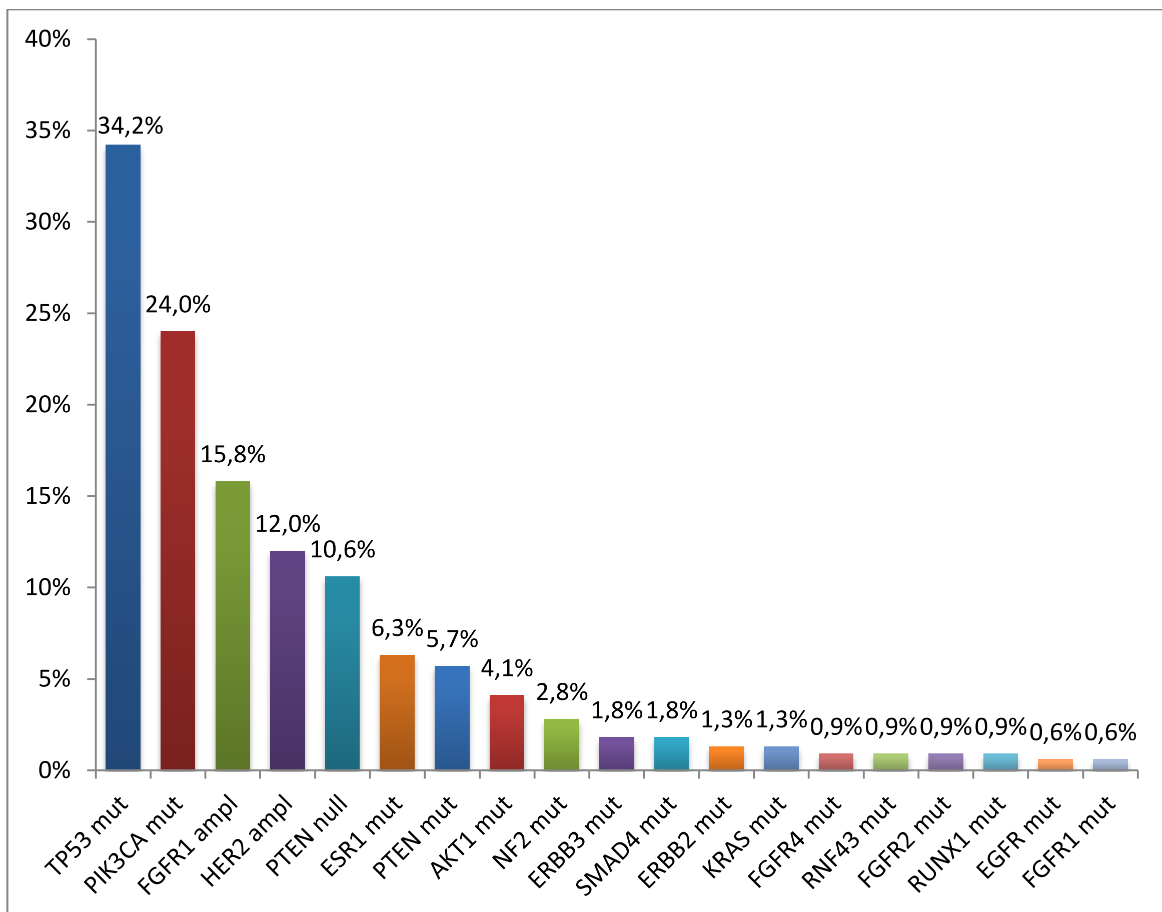


**Figure 14:** Overall distribution of molecular aberrations in the Complete cohort.

Figure 15 depicts the proportion of actionable alterations* within each subtype. Please note that the sum of the alterations may be higher than 100%, as several alterations may co-exist in the same sample.

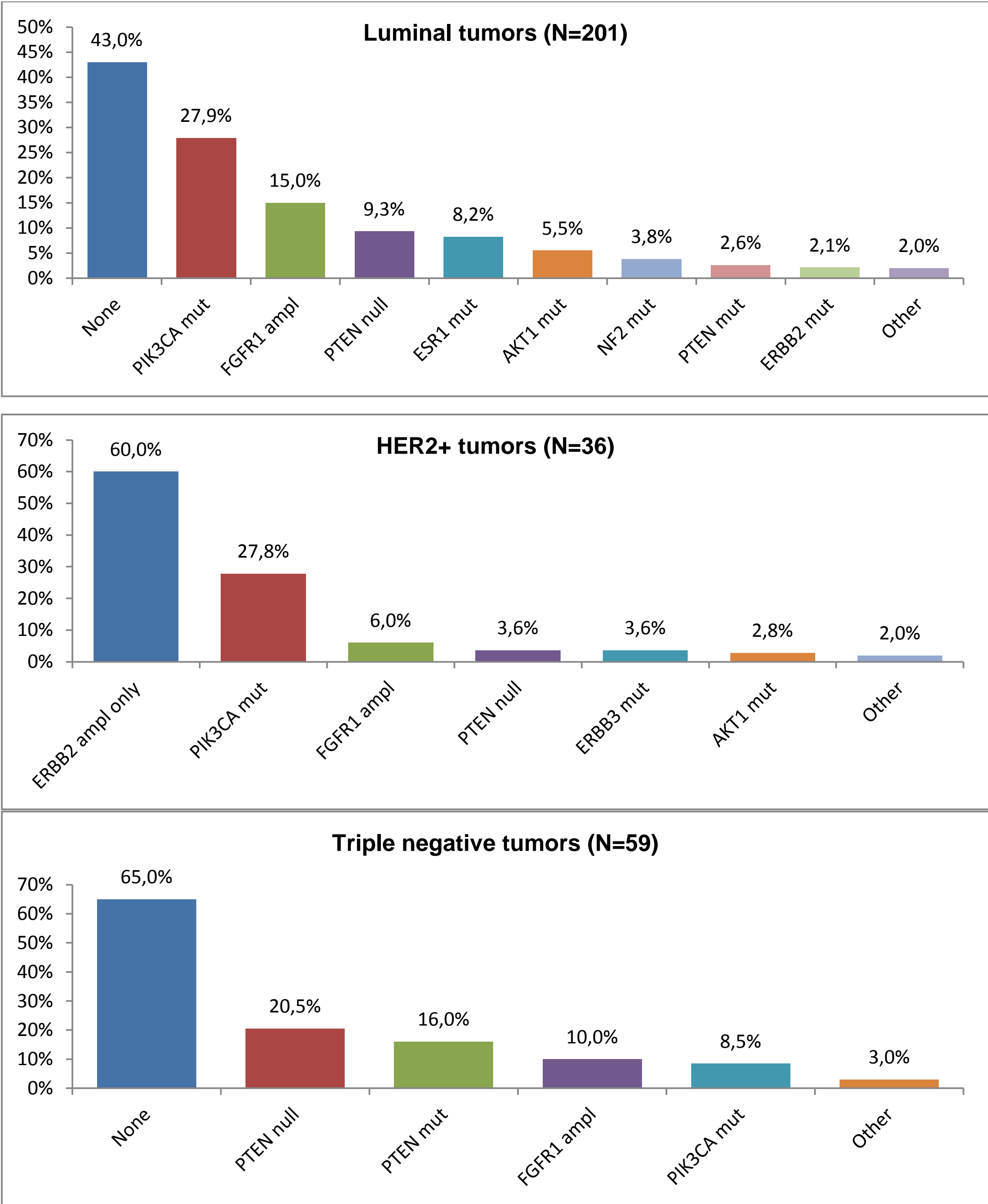


**Figure 15:** Actionable alterations within each subtype (Complete cohort). Ampl: amplification. Mut: mutation. Unk: unknown.

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*A given alteration was considered as **actionable** if there was a biologically reasonable matched treatment available. Please refer to Section 5.4 for details.

In luminal tumors, as expected, the most common actionable alteration was *PIK3CA* mutation (27.9%), followed by *FGFR1* amplification (15%), PTEN null expression (9.3%), *ESR1* mutation (8.2%), *AKT1* mutation (5.5%), and *NF2* mutation (3.8%). *ERBB2* mutations were found in 2.1% of luminal patients. In HER2+ tumors, the most common actionable alteration in addition to on top of *ERBB2* amplification was *PIK3CA* mutation (27.8%), followed by *FGFR1* amplification (6%) and PTEN null expression (3.6%). In TN samples, *TP53* mutation was the most common alteration (66.7%), but it is not actionable. The most common actionable alterations among TN tumors were PTEN null (20.5%), *PTEN* mutation (16%), *FGFR1* amplification (10%), and *PIK3CA* mutation (8.5%).

Overall, 21/327 (6.4%) of the samples had two or more actionable alterations (Figure 16).

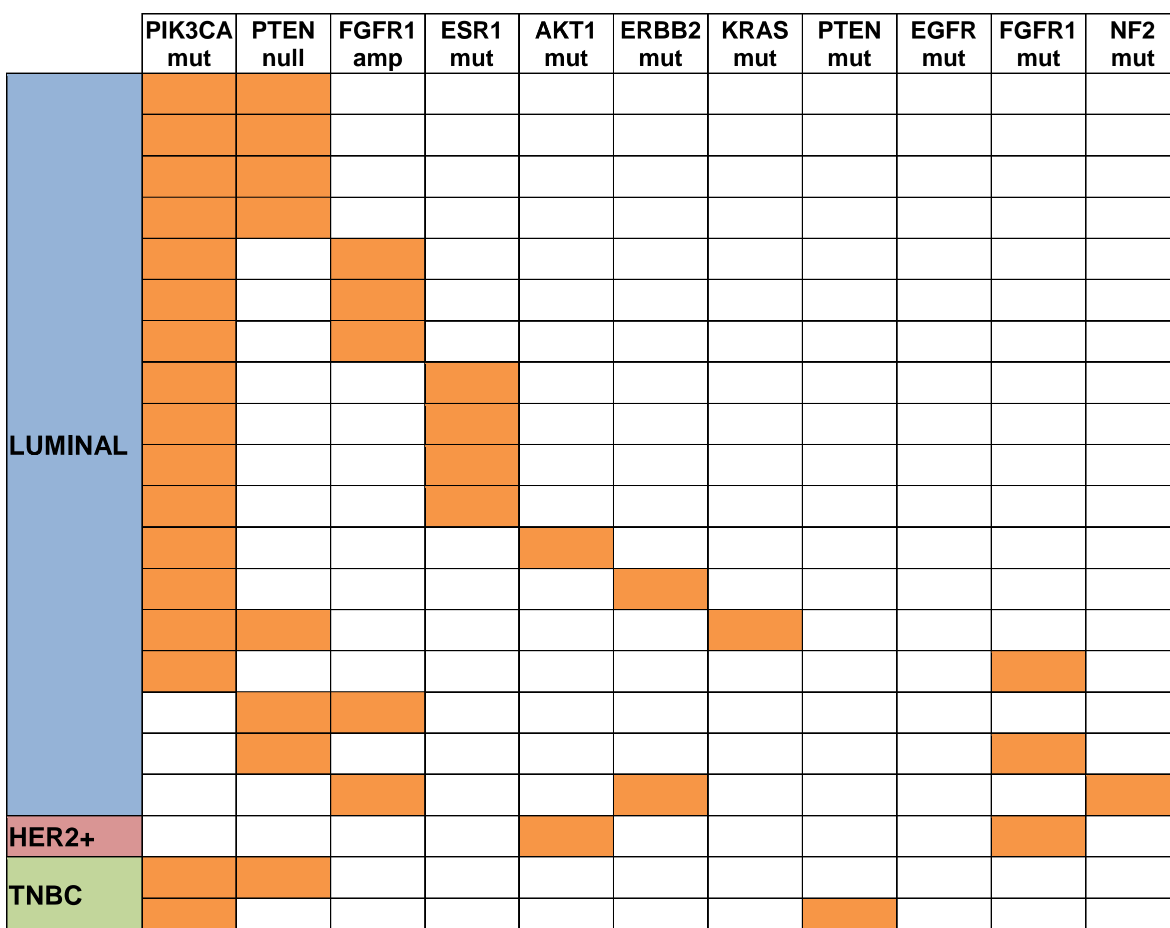


**Figure 16:** Heatmap of the samples with multiple actionable molecular alterations (Complete cohort). Orange fill denotes presence of alteration.

Table 5 shows the proportion of molecular alterations according to the site of the molecular determination. Interestingly, *PIK3CA*, *ESR1*, and *ERBB2* mutations occurred especially in metastatic samples. As these are not paired samples from the same patient, interpretation of these results should be made with caution (see discussion in Section 7.2).

**Table 5:** Distribution of molecular alterations according to site of determination (Complete Cohort).

| Gene | Primary | | | Metastasis | | | P |
|---|---|---|---|---|---|---|---|
| | N | n | % | N | n | % | |
| ***TP53*** | 73 | 26 | 35.6% | 41 | 13 | 31.7% | 0.837 |
| ***PIK3CA*** | 227 | 48 | 21.1% | 100 | 31 | 31.1% | 0.068 |
| **PTEN null** | 161 | 15 | 9.3% | 66 | 9 | 13.6% | 0.348 |
| ***ESR1*** | 71 | 2 | 2.8% | 41 | 5 | 12.2% | 0.097 |
| ***PTEN*** | 76 | 5 | 6.6% | 42 | 2 | 4.8% | 1.000 |
| ***AKT1*** | 227 | 8 | 3.5% | 100 | 6 | 6.0% | 0.374 |
| ***NF2*** | 76 | 3 | 3.9% | 42 | 1 | 2.4% | 1.000 |
| ***ERBB3*** | 71 | 2 | 2.8% | 41 | - | - | 0.532 |
| ***SMAD4*** | 73 | - | - | 41 | 2 | 4.9% | 0.127 |
| ***ERBB2*** | 216 | 1 | 0.5% | 92 | 3 | 3.3% | 0.081 |
| ***KRAS*** | 222 | 3 | 1.4% | 96 | 1 | 1.0% | 1.000 |
| ***FGFR4*** | 71 | 1 | 1.4% | 41 | - | - | 1.000 |
| ***RNF43*** | 71 | 1 | 1.4% | 41 | - | - | 1.000 |
| ***FGFR2*** | 73 | - | - | 41 | 1 | 2.4% | 0.360 |
| ***RUNX1*** | 73 | 1 | 1.4% | 41 | - | - | 1.000 |
| ***EGFR*** | 216 | 2 | 0.9% | 92 | - | - | 1.000 |
| ***FGFR1*** | 216 | 2 | 0.9% | 92 | - | - | 1.000 |

N: analyzed samples. n: samples with mutation/alteration.

#### 6.1.2.2. Aberrations in genes of the PAM pathway

PAM pathway dysregulation was detected in 35% of the samples. Table 6 shows the distribution of alterations in relevant genes in the PAM pathway, according to breast cancer subtype. *NF2* mutations were included because of some reports suggesting PI3K as a potential target for neurofibromatosis 2[170] and NF2-mutant breast tumors[171]. Patients with unknown breast cancer subtype (N=31) were excluded from this analysis.

**Table 6:** Alterations in relevant PAM pathway genes, according to breast cancer subtype (Complete cohort).

| | Overall N=296 | LUM N=201 | HER2+ N=36 | TN N=59 | P |
|---|---|---|---|---|---|
| ***PIK3CA*** | | | | | |
| MUT | 71 (24%) | 56 (27.9%) | 10 (27.8%) | 5 (8.5%) | 0.004 |
| WT | 225 (76%) | 145 (72.1%) | 26 (72.2%) | 54 (91.5%) | |
| ***PIK3CA* mut** | | | | | |
| Exon 20 | 36 (50.7%) | 23 (41.1%) | 8 (80%) | 5 (100%) | 0.051 |
| Exon 9 | 28 (39.4%) | 26 (46.4%) | 2 (20%) | - | |
| Other | 5 (7.1%) | 5 (8.9%) | - | - | |
| Double mut | 2 (2.8%) | 2 (3.6%) | - | - | |
| ***AKT1*** | | | | | |
| MUT (all E17K) | 12 (4.1%) | 11 (5.5%) | 1 (2.8%) | - | 0.142 |
| WT | 284 (95.9%) | 190 (94.5%) | 35 (97.2%) | 59 (100%) | |
| ***PTEN* (N=106*)** | | | | | |
| MUT | 6 (5.7%) | 2 (2.6%) | - | 4 (16%) | 0.047 |
| WT | 100 (94.3%) | 76 (97.4%) | 3 (100%) | 21 (84%) | |
| ***NF2* (N=106*)** | | | | | |
| MUT | 3 (2.8%) | 3 (3.8%) | - | - | ND |
| WT | 103 (97.2%) | 75 (96.2%) | 3 (100%) | 25 (100%) | |
| **PTEN IHC (N=207#)** | | | | | |
| Null | 22 (10.6%) | 13 (9.3%) | 1 (3.6%) | 8 (20.5%) | 0.064 |
| Intact | 185 (89.4%) | 127 (90.7%) | 27 (96.4%) | 31 (79,5%) | |

*Results for PTEN and NF2 mutation are available in 106 patients: LUM 78, HER2+ 3, and TN 25. #Results for PTEN IHC are available for 207 patients: LUM 140, HER2+ 28, and TN 39. IHC: immunohistochemistry. LUM: luminal. MUT: mutant. ND: not determined. TN: triple negative. WT: wild type.

Frequency of *PIK3CA* and *PTEN* mutations was significantly different between tumor subtypes (P=0.004 and P=0.047, respectively). Of note, all *PIK3CA* mutations in TN samples were found in exon 20 (all H1047R).

A double mutation in *PIK3CA* was identified in two LUM patients: E542A + Y1021H in one, and E545D + H1047R in another. In both these mutations were detected by AmpliconSeq in a metastatic biopsy.

The rate of *PIK3CA* mutations was similar independently of the targeted panel that was used (Sequenom 23.0% vs. AmpliconSeq 26.3%, P=0.506).

Adjusted MAF for *PIK3CA* mutation could be determined in 59/71 of the total samples (83.1%). Median adjusted MAF for *PIK3CA* was 0.43 (interquartile range [IQR] 0.28-0.63). *PIK3CA* mutations were clonal in 40 samples (67.8%), and subclonal in 19 (32.2%).

There was no difference on median adjusted MAF of *PIK3CA* according to breast cancer subtype (LUM 0.43, HER2+ 0.55, TN 0.20, P=0.611; Figure 17A), or according to the platform used for mutation detection (Sequenom 0.51 vs. AmpliconSeq 0.35, P=0.276; Figure 17B). Interestingly, there was a trend for a higher adjusted MAF when *PIK3CA* mutation was detected in the primary tumor, when compared to a metastatic site (0.49 vs. 0.29, P=0.072, Figure 18).

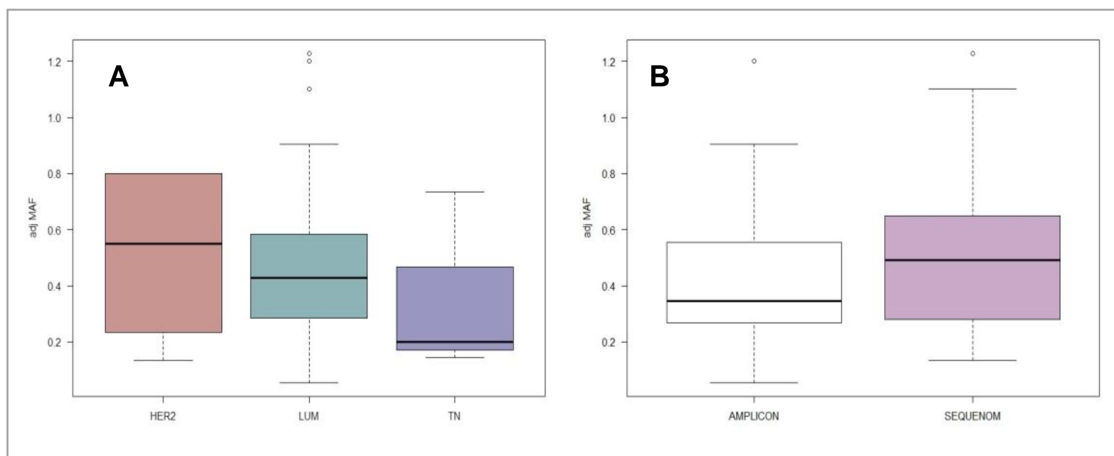


**Figure 17:** Adjusted MAF for *PIK3CA* (Complete Cohort).
**(A)** According to breast cancer subtype **(B)** According to the type of platform used for the analysis.

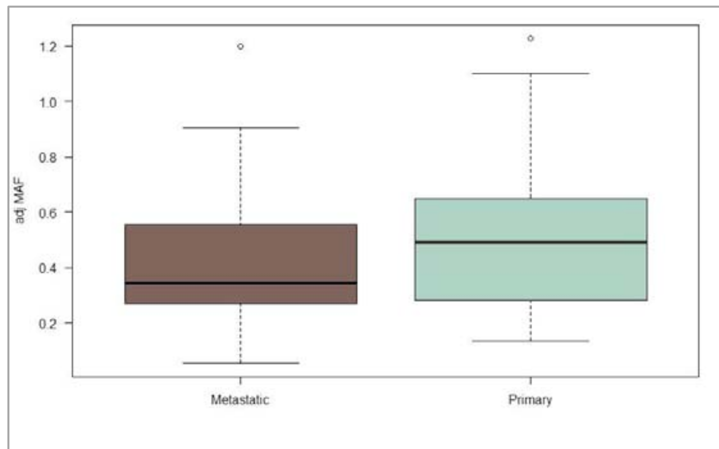


**Figure 18:** Adjusted MAF for *PIK3CA* (Complete Cohort), according to the site of the analysis.

Median adjusted MAF for *AKT1* E17K mutation was 0.58 (IQR 0.56-0.66). We found no differences in *AKT1* MAF according to the site of determination (metastasis 0.69 vs. primary 0.58, P=1.000).

#### ***6.1.2.3. Other aberrations***

##### *6.1.2.3.1. FGFR1 amplification*

*FGFR1* amplification was detected in 25/158 (15.8%) samples. There were no significant differences in *FGFR1* amplification according to subtype (LUM 15% vs. HER2+ 6% vs. TN 10%, P=0.687).

##### *6.1.2.3.2. Other somatic mutations*

***ESR1* mutations** were detected in 7/112 (6.3%) samples: D538G (N=2), L536H (N=2), Y537N (N=2), and Y537S (N=1). As expected, all mutations were detected in LUM tumors (8.2%). Median adjusted MAF for *ESR1* mutation was 0.71 (IQR 0.38-0.75). Of note, *ESR1* mutations were included in the AmpliconSeq panels, but not in those from Sequenom®. Five out of the seven *ESR1* mutations (71.4%) were identified in a metastatic sample, and two (25.6%) in the primary tumor. Interestingly, in 4/7 cases (57.1%) *ESR1* mutations co-occurred with a *PIK3CA* mutation.

***ERBB2* mutations** were detected in 4/308 (1.3%) samples: L755S (N=2), S310F (N=1), and V777delinsVGSP (N=1). All mutations were detected in LUM samples (2.1%). Median adjusted MAF for *ERBB2* mutation was 0.34 (IQR 0.29-0.48). In one sample, *ERBB2* mutation co-occurred with a *PIK3CA* mutation.

***TP53* mutations** were detected in 39/114 (34.2%) samples. *TP53* mutations were more frequent in TN (16/24, 66.7%) and HER2+ (3/3, 100%) than in LUM tumors (17/75, 22.7%), P<0.005. There was no difference in the detection of *TP53* according to the site of determination (primary tumor 35.6% vs. metastasis 31.7%, P=0.837).

Table 7 summarizes other less frequent mutations and variants also detected in these samples.

**Table 7**: Other less frequent mutations and variants (Complete cohort).

| Gene | Variant | Subtype |
|---|---|---|
| ***APC*** | M1413T | Unk |
| ***EGFR*** | F712L<br>H773_V774ins | TN<br>LUM |
| ***ERBB3*** | K356M<br>D297Y | HER2+<br>LUM |
| ***FGFR1*** | W4C<br>H253Y | LUM<br>LUM |
| ***FGFR2*** | N549K | LUM |
| ***FGFR4*** | R203H | LUM |
| ***KRAS*** | G12D<br>G12C<br>G12C<br>G12V | LUM<br>Unk<br>LUM<br>LUM |
| ***RNF43*** | G659fs | LUM |
| ***RUNX1*** | S100F | LUM |
| ***SMAD4*** | I240fs<br>Y353X | Unk<br>LUM |

LUM: luminal. TN: triple negative. Unk: unknown.

### *6.1.3. Enrollment in genotype-driven clinical trials*

Table 8 shows the prevalence of actionable alterations according to breast cancer subtype. Of note, 160 patients (54.1%) presented at least one actionable alteration, with significant differences between subtypes (P<0.001).

**Table 8:** Prevalence of actionable alterations according to breast cancer subtype (Complete cohort).

| | Number of actionable alterations | | | | |
|---|---|---|---|---|---|
| | **At least one** | **0** | **1** | **2** | **3** |
| **All patients** | 160 (54.1%) | 136 (45.9%) | 129 (43.6%) | 28 (9.5%) | 3 (1.0%) |
| **LUM** | 105 (52.2%) | 96 (47.8%) | 90 (44.8%) | 13 (6.5%) | 2 (0.9%) |
| **HER2+** | 36 (100%) | - | 23 (63.9%) | 12 (33.3%) | 1 (2.8%) |
| **TN** | 19 (32.2%) | 40 (67.8%) | 16 (27.1%) | 3 (5.1%) | - |

LUM: luminal. TN: triple negative.

From the 327 patients with a valid somatic mutation and/or *FGFR1* analysis, 184 (56.3%) received a targeted therapy. Of these, 74 (40.2%) were genotype-driven trials, i.e., trials with drugs matched to the alterations that were detected. Overall, 74/327 of the patients undergoing molecular pre-screening (22.6%) received a genotype-driven trial. Patients with actionable alterations had a trend towards receiving more targeted therapies (odds ratio [OR] 1.48, P=0.09; 33% vs. 23%).

As expected, patients with PAM pathway dysregulation were more likely to receive a PAM pathway inhibitor than patients without dysregulation (OR 1.98; 95%CI 1.19-3.26, P=0.006). Of note, 46% of patients with a *PIK3CA* mutation received a PAM pathway inhibitor, as compared to 30% of those without *PIK3CA* mutation.

## 6.2. Cohort of patients treated with PAM pathway inhibitors

From the 327 patients tested for molecular alterations that have just been described in Section 6.1, we identified 120 patients that were treated with at least one PAM pathway inhibitor at some point of their disease (PAM pathway inhibitor Cohort). In the next sections we will characterize this population and discuss the outcome of the treatment with PAM pathway inhibitors.

### *6.2.1. Patients and samples characteristics*

Table 9 summarizes patients' and samples characteristics.

**Table 9**: Patients' and samples characteristics (PAM pathway inhibitor Cohort).

| | N=120 | |
|---|---|---|
| **Age** median (range) | 56 (25-85) | |
| **Gender,** Female | 120 (100%) | |
| **Subtype** | | |
| LUM | 91 (75.8%) | |
| HER2+ | 22 (18.3%) | |
| TN | 7 (5.9%) | |
| **Number of prior lines for MBC,** median (range) | 3 (0-9) | |
| ≤2 | 59 (49.2%) | |
| >2 | 61 (50.8%) | |
| **Number of metastatic sites,** median (range) | 2 (1-7) | |
| ≤2 | 74 (61.7%) | |
| >2 | 46 (38.3%) | |
| **Visceral metastasis** | 79 (65.8%) | |
| **Sites of metastasis** | | |
| Bone | 79 (65.8%) | |
| Nodes | 56 (46.7%) | |
| Liver | 54 (45.0%) | |
| Lung / pleural | 39 (32.5%) | |
| Skin / soft tissue | 30 (25.0%) | |
| CNS | 5 (4.2%) | |
| Other | 11 (9.2%) | |
| **PAM pathway dysregulation** | | |
| Yes | 52 (43.3%) | |
| No | 68 (56.7%) | |
| **Tumor sample** | | |
| Primary tumor | 70 (58.3%) | |
| Metastasis | 50 (41.7%) | |
| **Panel used** | | |
| Sequenom | 85 (70.8%) | |
| AmpliconSeq | 35 (29.2%) | |
| **Tumor purity** median (IQR) | | |
| Metastatic sample | 80% (72.5-80) | P=0.051 |
| Primary | 60% (35-76.3) | |
| AmpliconSeq | 70% (53.8-80) | P=0.675 |
| Sequenom | 65% (35-80) | |

CNS: central nervous system. IQR: Interquartile range. LUM: luminal. MBC: metastatic breast cancer. TN: triple negative.

Median age of the patients was 56 (range 25-85), all female. Most of the patients had LUM tumors (75.8%), followed by HER2+ (18.3%), and TN (5.9%). Nineteen patients (15.8%) were diagnosed with *de novo* MBC (Stage IV). Most of the patients had visceral disease (65.8%), mainly in liver (45%) and lung or pleura (32.5%). Median time that elapsed from MBC diagnosis until the treatment with a PAM pathway inhibitor was 31.4 months (95%CI 27.2-41.4). Median number of prior lines for MBC before receiving a PAM pathway inhibitor was 3 (range 0-9).

One hundred and twenty MBC patients received 130 treatments with PAM pathway inhibitors. This means that 10 patients received more than one agent targeting the same signaling pathway in different time points in their metastatic disease: three received an mTOR and later on a PI3Kα-specific inhibitor, two an mTOR and a dual PI3K/mTOR inhibitor, two others an mTOR and an AKT inhibitor, one received two different mTOR inhibitors, another a pan-PI3K and a PI3Kα-specific inhibitor, and one received a dual PI3K and a PI3Kα-specific inhibitor.

The majority of the patients received PI3Kα-specific inhibitor-based therapy (41.5%), followed by mTOR inhibitor-based therapy (20.8%), dual PI3K/mTOR inhibitor-based therapy (16.1%), and pan-PI3K and AKT inhibitor-based therapy (10.8% each; Table 10).

**Table 10:** Treatment characteristics (PAM pathway inhibitor Cohort).

| | **N (%)** |
|---|---|
| **Targeted-based treatment** | |
| PI3Kα | 54 (41.5%) |
| Pan-PI3K | 14 (10.8%) |
| PI3K-mTOR | 21 (16.1%) |
| mTOR | 27 (20.8%) |
| AKT | 14 (10.8%) |
| **Combination therapy** | |
| None | 21 (16.1%) |
| Endocrine therapy | 70 (53.9%) |
| Anti-HER2 therapy | 21 (16.1%) |
| Chemotherapy | 17 (13.1%) |
| Other | 1 (0.8%) |

Of note, only a small proportion of patients (16.1%) received a PAM pathway inhibitor as single agent. The most common combination was with endocrine therapy (53.9%), followed by anti-HER2 therapy (16.1%) and chemotherapy (13.1%).

### *6.2.2. Distribution of molecular aberrations*

#### *6.2.2.1. Overview*

Figure 18 shows the overall distribution of the molecular aberrations in the PAM pathway inhibitor Cohort. The most common alteration was *PIK3CA* mutation (31.7%), followed by *TP53* mutation (21%), PTEN IHC null (10.8%), *ESR1* mutation (8.1%), AKT1 mutation (5.8%), NF2 mutation (5.3%), PTEN mutation (4.8%), and *ERBB2* mutation (2.8%). Other mutations, such as *ERBB3*, *FGFR4*, *RNF43*, *RUNX1*, *FGFR2*, *SMAD4*, *KRAS*, *EGFR*, and *FGFR1*, occurred at a lower proportion in this cohort.

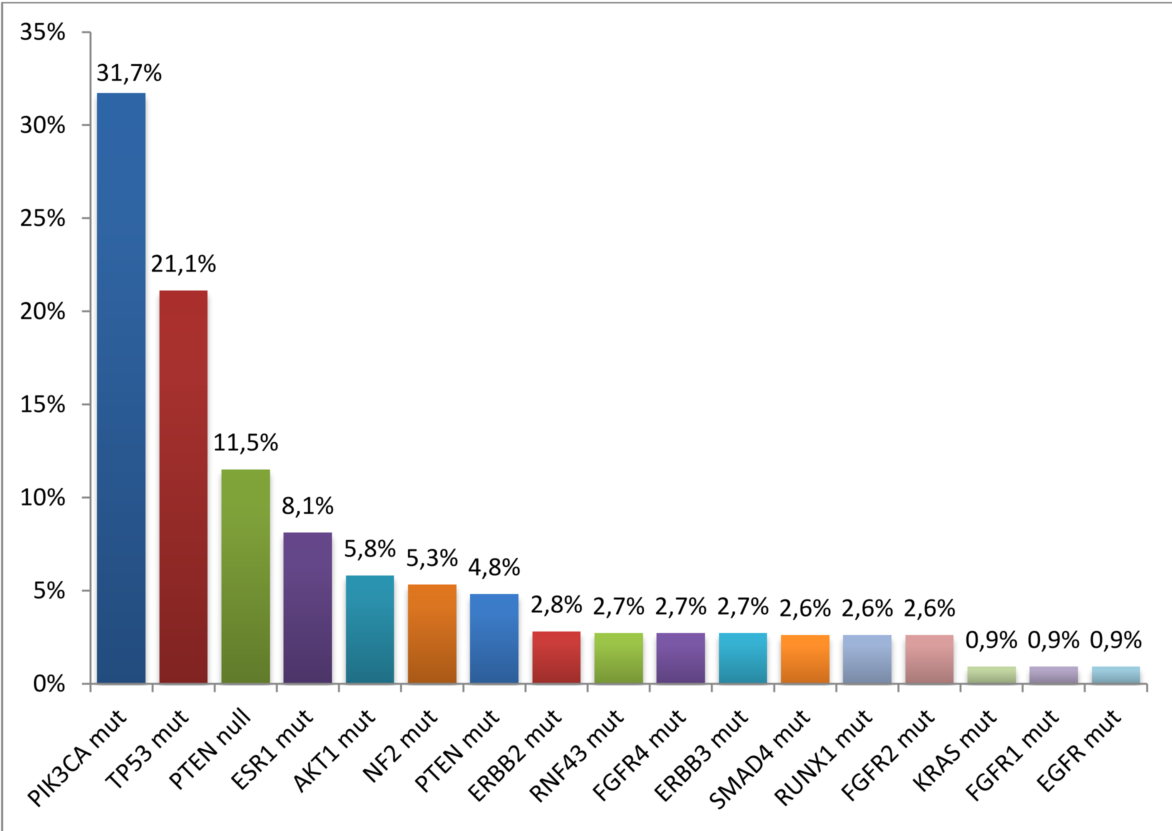


**Figure 19:** Overall distribution of molecular alterations in the PAM pathway inhibitor Cohort.

#### *6.2.2.2. Aberrations in genes of the PAM pathway*

Overall, 52 patients (43.3%) had at least one alteration in the PAM pathway (Table 11): *PIK3CA* mutation 38 patients (31.7%), PTEN null 9 (11.5%), *AKT1* mutation 7 (5.8%), and *NF2* mutation 2 (5.3%).

Mutations in the kinase domain of *PIK3CA* (exon 20) were more frequent (57.9%) than mutations in the helical domain (exon 9, 28.9%). Interestingly, two patients had a double *PIK3CA* mutation in the same sample.

**Table 11**: Aberrations in genes of the PAM pathway (PAM pathway inhibitor Cohort).

| | **N (%)** |
|---|---|
| ***PIK3CA* mutation** | |
| MUT | 38 (31.7) |
| WT | 82 (68.3) |
| ***PIK3CA* Exon 20 (single mutation)** | **22 (57.9)** |
| H1047R | 20 (52.7) |
| H1047L | 1 (2.6) |
| G1049R | 1 (2.6) |
| ***PIK3CA* Exon 9 (single mutation)** | **11 (28.9)** |
| E545K | 8 (21.2) |
| E542K | 2 (5.3) |
| E545G | 1 (2.6) |
| ***PIK3CA* Other (single mutation)** | **3 (7.9)** |
| E110K | 1 (2.6) |
| N345K | 1 (2.6) |
| C420R | 1 (2.6) |
| ***PIK3CA* Double mutation** | **2 (5.3)** |
| E545D + H1047R | 1 (2.6) |
| E542A + Y1021H | 1 (2.6) |
| ***AKT1* mutation** | |
| MUT (all E17K) | 7 (5.8) |
| WT | 113 (94.2) |
| ***NF2* mutation (N=38)** | |
| MUT | 2 (5.3) |
| WT | 36 (94.7) |
| **PTEN IHC (N=78)** | |
| Null | 9 (11.5) |
| Intact | 69 (88.5) |

IHC: immunohistochemistry. MUT: mutant. WT: wild type.

Adjusted MAF for *PIK3CA* mutation could be determined in 31/38 samples (81.6%). Median adjusted MAF was 0.49 (IQR 0.28-0.62). *PIK3CA* mutations were clonal in 21 samples (67.7%), and subclonal in 10 (32.3%).

Interestingly, while median adjusted MAF did not vary according to breast cancer subtype [LUM 0.49 (IQR 0.38-0.63), HER2 0.55 (IQR 0.30-0.75), and TN 0.14 (IQR 0.14-0.14), P=0.460; Figure 20A], *PIK3CA* mutation was more frequently clonal in primary tumors than in metastatic samples [0.53 (IQR 0.46-0.64) vs. 0.25 (IQR 0.14-0.54), P=0.04; Figure 20B].

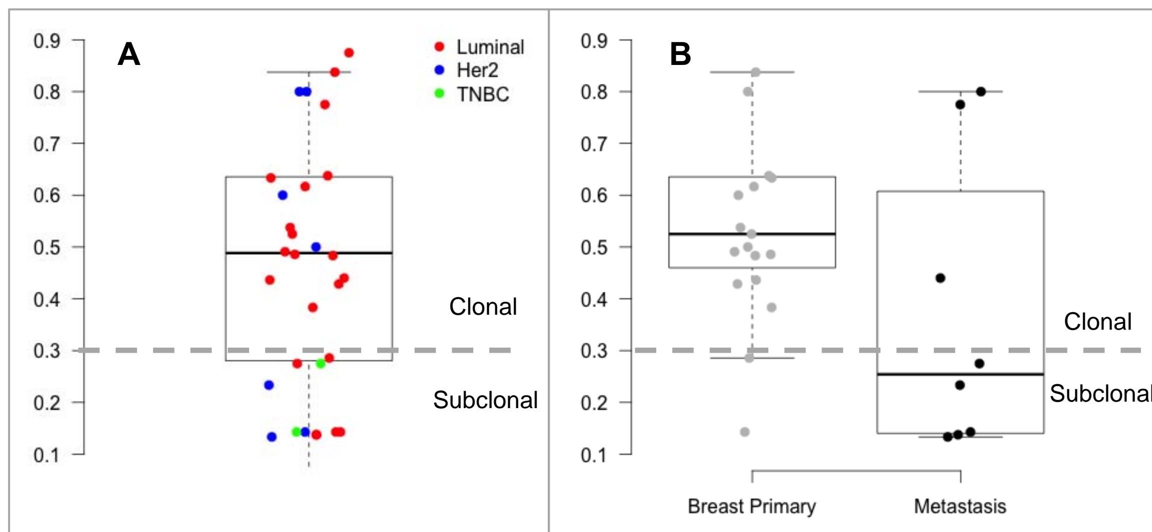


**Figure 20**: *PIK3CA* adjusted MAF.
**(A)** According to breast cancer subtype; **(B)** According to the site of analysis.

AKT1 mutations were detected in 7 patients (5.8%), and were clonal in 100% of the samples (median adjusted MAF 0.83, IQR 0.59-0.99).

Figure 21 shows the distribution of PAM pathway alterations according to breast cancer subtype, and the type of treatment these patients received.

Of note, the majority of patients with LUM or HER2+ tumors received a PAM pathway inhibitor in combination with other treatment, whilst TN patients received it mainly as a single agent.

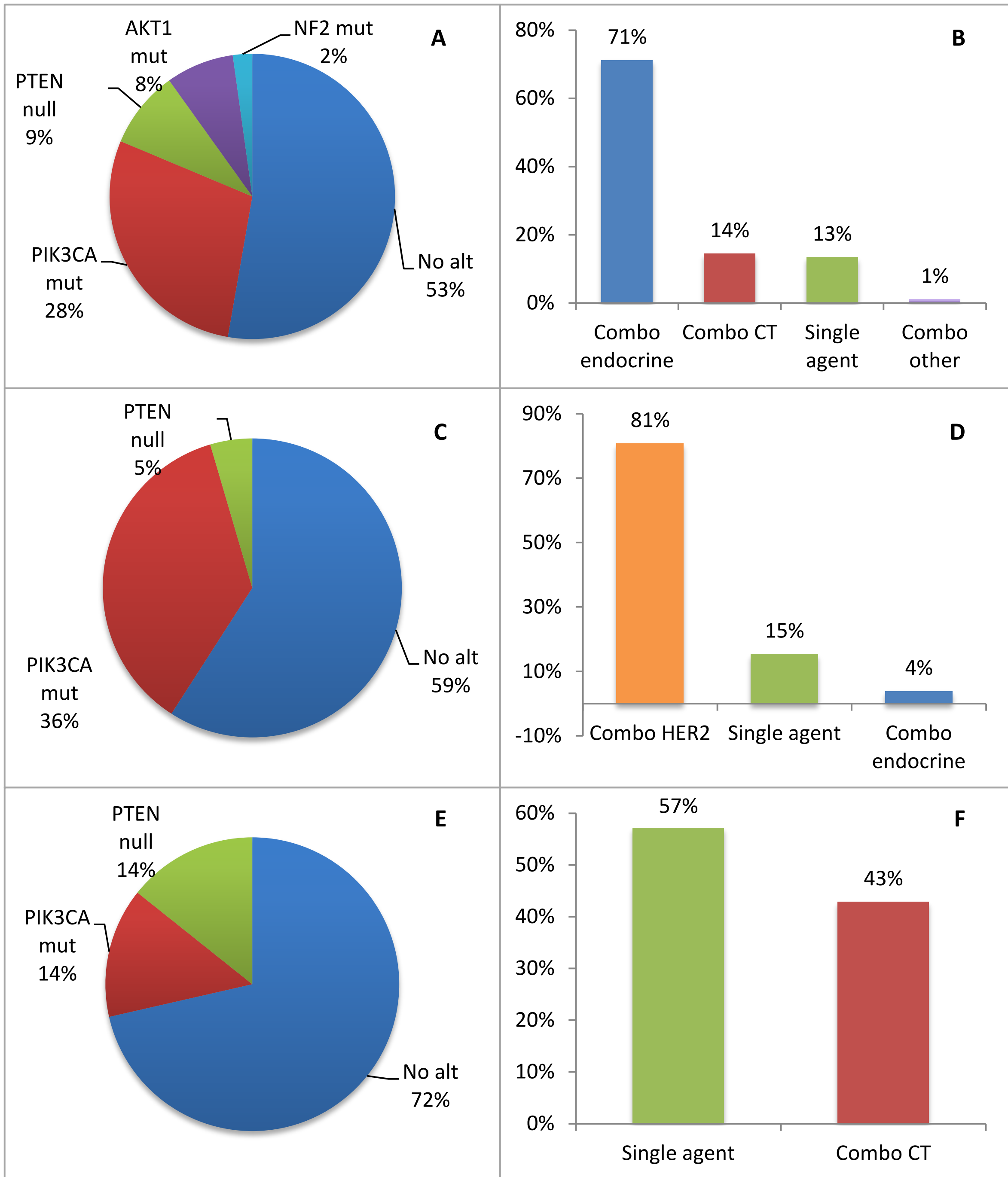


**Figure 21:** PAM pathway alterations and treatments received, according to breast cancer subtype (PAM pathway inhibitor Cohort).
**A** and **B**: LUM patients; **C** and **D**: HER2+ patients; **E** and **F**: TN patients.
Alt: alteration. Combo: combination therapy. CT: chemotherapy. Mut: mutation.

### 6.2.3. *Efficacy of PAM pathway inhibitors*

Table 12 summarizes some parameters of efficacy of PAM pathway inhibitors. Of note, CBR (the proportion of patients with CR, PR, or SD for at least 16 weeks) was 57.7%. Median TTF in this pre-treated population was 4.9 months (95%CI 3.9-6.1).

**Table 12**: Efficacy of treatment with PAM pathway inhibitors.

| | **N (%)** |
|---|---|
| **Best response** | |
| CR | 2 (1.5%) |
| PR | 29 (22.3%) |
| SD | 70 (53.9%) |
| PD | 29 (22.3%) |
| **ORR** | 23.8% |
| **Clinical benefit rate** | 57.7% |
| **Reason for treatment discontinuation** | |
| PD | 98 (80.3%) |
| Toxicity | 15 (12.3%) |
| Other (including ongoing patients) | 9 (7.4%) |
| **Median TTF** (months) | 4.9 (95%CI 3.9-6.1) |

CI: confidence interval. CR: complete response. MBC: metastatic breast cancer. ORR: overall response rate. PD: progressive disease. PR: partial response. SD: stable disease. TTF: time to treatment failure.

#### 6.2.3.1. *Clinical benefit*

For the overall population, having a PAM pathway dysregulation did not significantly increase the likelihood of benefit to PAM pathway inhibitors (OR 1.42, P=0.375; Table 13).

In contrast, having a *PIK3CA* mutation significantly increased the likelihood of clinical benefit to PAM pathway inhibitors (OR 2.95, P=0.008).

Having a clonal *PIK3CA* mutation did not seem to impact the response (OR 0.57, P=0.682).

**Table 13**: Univariate analysis for clinical benefit to PAM pathway inhibitors.

| | | CBR to PAM pathway inhibitor | | OR (95%CI) | P |
|---|---|---|---|---|---|
| | | **No** | **Yes** | | |
| **PAM pathway dysregulation** | **No**<br>**Yes** | 46.4%<br>37.7% | 53.6%<br>62.3% | 1.42 (0.67-3.06) | 0.375 |
| ***PIK3CA* mut** | **No**<br>**Yes** | 50.6%<br>25.6% | 49.4%<br>74.4% | **2.95 (1.26-7.36)** | **0.008** |
| **Clonality** | **WT**<br>**Subcl**<br>**Clonal** | 45.9%<br>40.0%<br>27.3% | 54.1%<br>60.0%<br>72.7% | 0.57 (0.09-3.78) | 0.682 |

CBR: clinical benefit rate. CI: confidence interval. OR: odds ratio. Subcl: subclonal. WT: wild type.

Focusing in the group of patients treated with PI3Kα-specific inhibitors (Table 14), having a *PIK3CA* mutation significantly increased the likelihood of clinical benefit to these drugs (OR 4.37, P=0.038). However, again, clonality does not seem to impact response.

**Table 14**: Univariate analysis for clinical benefit to PI3Kα-specific inhibitors.

| | | CBR to PI3Kα-specific inhibitor | | OR (95%CI) | P |
|---|---|---|---|---|---|
| | | **No** | **Yes** | | |
| ***PIK3CA* mut** | **No**<br>**Yes** | 45.7%<br>15.8% | 54.3%<br>84.2% | **4.37 (0.99-27.61)** | **0.038** |
| **Clonality** | **WT**<br>**Subcl**<br>**Clonal** | 41.0%<br>16.7%<br>22.2% | 59%<br>83.3%<br>77.8% | 1.40 (0.06-100.83) | 1.000 |

CBR: clinical benefit rate. OR: odds ratio. Subcl: subclonal. WT: wild type.

#### 6.2.3.2. *Time to Treatment Failure*

As previously mentioned, TTF in the overall population was 4.9 months (95%CI 3.9-6.1; Figure 22).

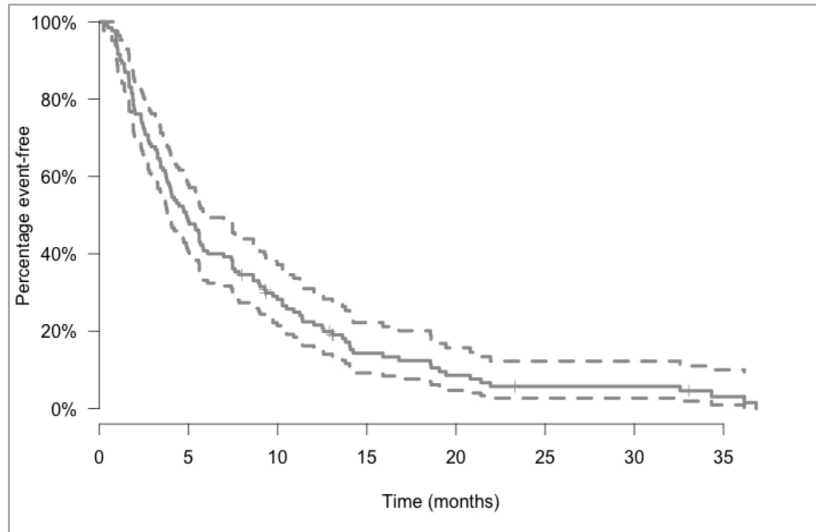


**Figure 22:** TTF to PAM pathway inhibitors.
Dotted lines represent the limits of the 95% confidence interval.

Having a PAM pathway dysregulation did not significantly increase the likelihood of treatment benefit with PAM pathway inhibitors (HR 0.83, 95%CI 0.58-1.20; P=0.325, Figure 23).

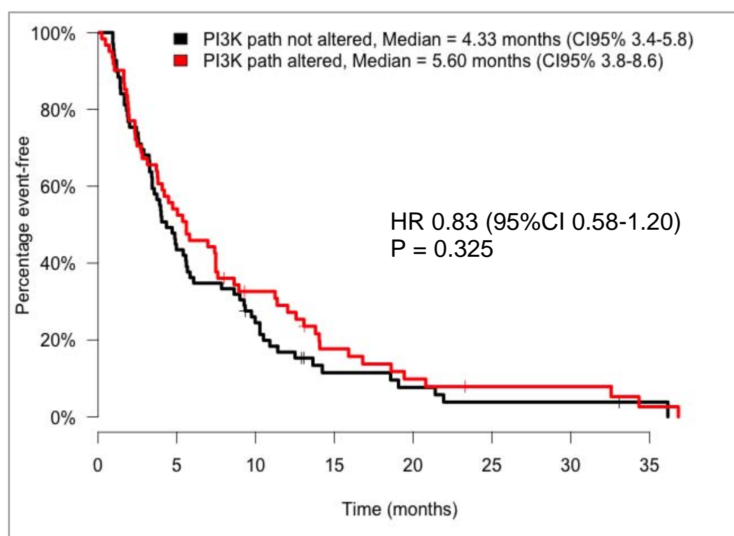


**Figure 23:** TTF according to the presence of PAM pathway dysregulation.

In contrast, the presence of a *PIK3CA* mutation significantly increased the likelihood of treatment benefit with PAM pathway inhibitors (HR 0.66, 95%CI 0.45-0.96; P=0.031, Figure 24).

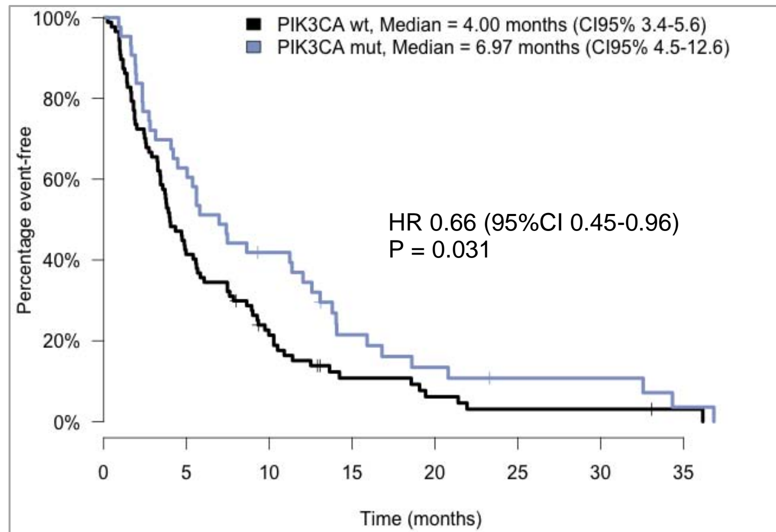


**Figure 24**: TTF according to the presence of *PIK3CA* mutation.

Patients treated with PAM pathway inhibitors in combination with chemotherapy, anti-HER2, or endocrine therapy had significantly better TTF than those treated with PAM pathway inhibitors as single agents (P < 0.05 for all comparisons; Figure 25)*.

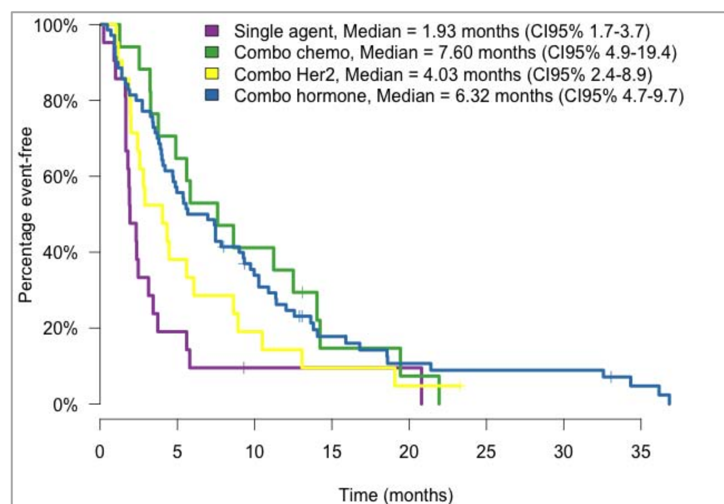


**Figure 25**: TTF according to type of treatment received.

*One patient treated with a combination of an mTOR plus an AKT inhibitor was excluded from this analysis (total N=129).

In a multivariate model including other known prognostic factors, such as breast cancer subtype, number of prior lines of treatment for MBC, or presence of visceral metastasis (Table 15), *PIK3CA* mutation was an independent predictive factor of benefit to PAM pathway inhibitors (P=0.003). Additionally, combination treatment with endocrine treatment (P<0.001) or chemotherapy (P=0.001), and having received 2 or less prior regimens of treatment for metastatic disease (P=0.002) were also predictive of benefit for the treatment with PAM pathway inhibitors.

**Table 15**: Multivariate model for TTF to PAM pathway inhibitors.

| | | HR (95%CI) | P |
|---|---|---|---|
| ***PIK3CA* mutation** | **Yes vs. No** | **0.50 (0.32-0.79)** | **0.003** |
| **Breast Cancer Subtype** | HER2 vs. LUM<br>TN vs. LUM | 0.71 (0.24-2.09)<br>0.61 (0.23-1.64) | 0.534<br>0.328 |
| **Combo vs. Monotherapy** | **ET**<br>**CT**<br>Anti-HER2<br>Other | **0.24 (0.11-0.52)**<br>**0.20 (0.07-0.53)**<br>0.53 (0.16-1.70)<br>0.95 (0.11-7.91) | **<0.001**<br>**0.001**<br>0.283<br>0.963 |
| **Visceral metastasis** | No vs. Yes | 1.32 (0.84-2.10) | 0.229 |
| **Number of metastatic sites** | ≥3 vs. <3 | 1.41 (0.89-2.24) | 0.140 |
| **Number of prior lines for MBC** | **>2 vs. ≤2** | **1.95 (1.29-2.96)** | **0.002** |
| **Type of inhibitor (ref. mTOR)** | AKT<br>Pan-PI3K<br>PI3K/mTOR<br>PI3Kα | 0.98 (0.34-2.80)<br>0.73 (0.34-1.57)<br>0.72 (0.36-1.45)<br>0.91 (0.54-1.54) | 0.968<br>0.417<br>0.363<br>0.724 |

CT: chemotherapy. ET: endocrine therapy. HR: hazard ratio. LUM: luminal. MBC: metastatic breast cancer. TN: triple negative.

In the subset of patients with known *PIK3CA* adjusted MAF, clonality of *PIK3CA* was not a determinant of TTF to PAM pathway inhibitors, either as a dichotomous variable (HR 1.09, 95%CI 0.48-2.52; P=0.828, Figure 26), or a continuous variable (HR 0.81, 95%CI 0.24-2.78; P=0.745).

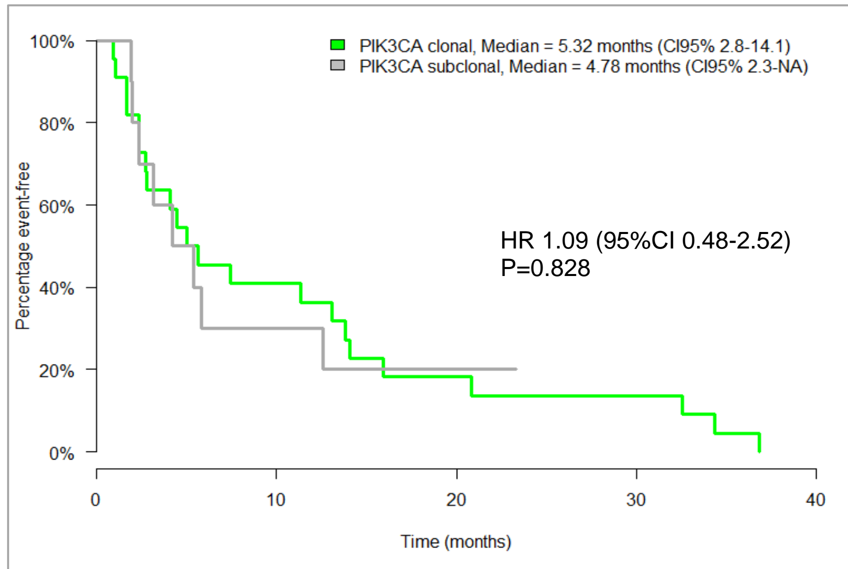


**Figure 26:** TTF according to clonality of *PIK3CA* mutation.

In the population treated with PI3Kα-specific inhibitors (N=54), the presence of a PIK3CA mutation showed a non-significant trend to increased TTF respect to non-mutant patients (Figure 27).

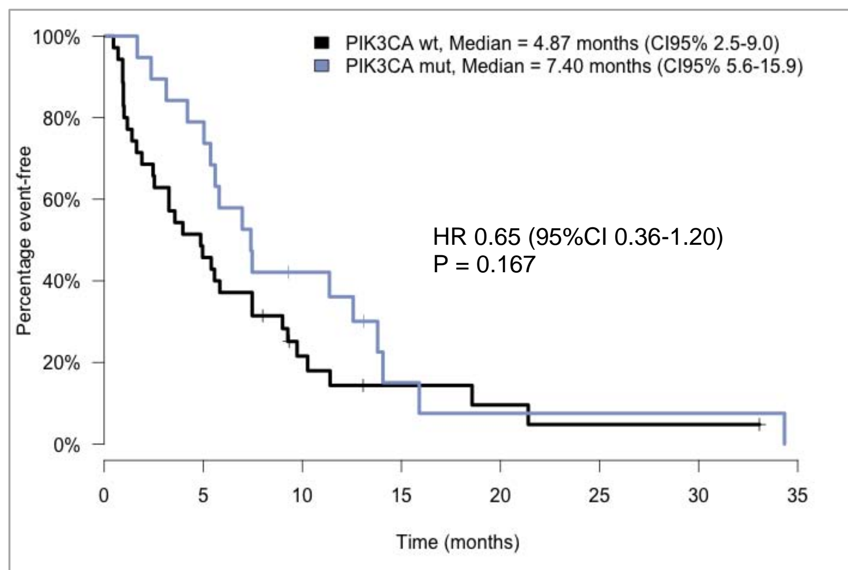


**Figure 27**: TTF in patients treated with PI3Kα-specific inhibitors.

Lastly, we performed exploratory analysis to assess whether clonality of *PIK3CA* mutation influenced efficacy of PI3Kα-specific inhibitors. In those patients with *PIK3CA* mutation, known adjusted MAF of *PIK3CA*, and treated with PI3Kα-specific inhibitors (N=15), clonality of *PIK3CA* mutation did not significantly

influence TTF (HR 0.57, 95%CI 0.16-2.01; P=0.380). However, patients with clonal *PIK3CA* had numerically longer median TTF than those with subclonal events (11.4 months vs. 5.5 months, Figure 28).

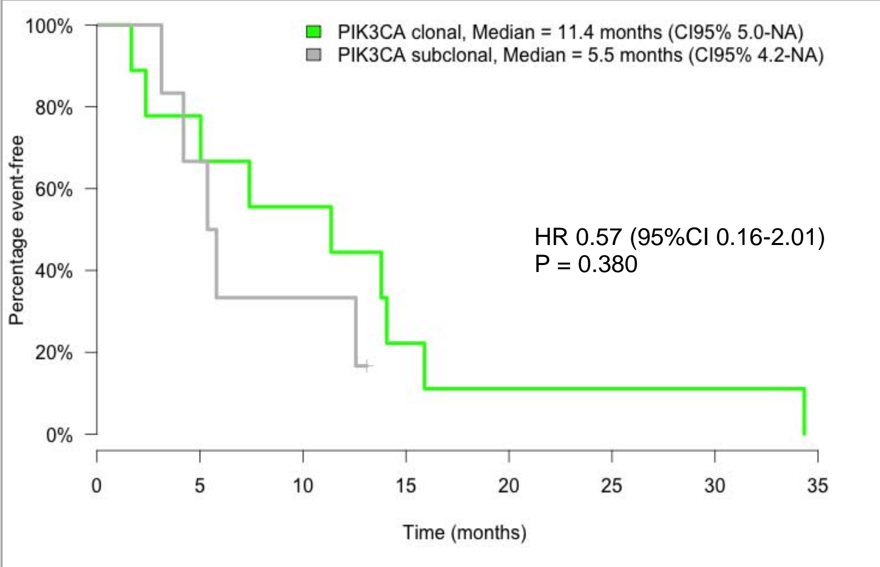


**Figure 28:** TTF in patients with *PIK3CA* mutation treated with PI3Kα-specific inhibitors.

# 7
# Discussion

# 7. Discussion

Recent advances in molecular biology and sequencing techniques have shed light on our understanding of breast cancer biology. Besides ER, PgR, and HER2, new targets for drug intervention have been identified and novel drugs have been incorporated into our anti-cancer armamentaria. Additionally, the application of genomics in clinical research and, to some extent, in clinical practice, has made the concept of precision medicine a reality in many Cancer Centers across the globe. Our Institution embraced this vision of oncology many years ago, and routine molecular pre-screening is performed for our MBC patients. The first main objective of our study was therefore to analyze the results of this genotyping effort.

PAM pathway alterations such as *PIK3CA* mutation, PTEN dysregulation, and *AKT1* mutations, are among the most frequent molecular alterations in MBC. PAM pathway inhibitors entered clinical trials more than a decade ago, and a growing body of evidence suggests that they are useful drugs in the treatment of MBC. However, knowledge regarding biomarkers of response (and resistance) to these agents, as well as the clinical contexts in which they would be most useful in, is incomplete. Since we have participated in the development of several PAM pathway inhibitors from the very outset, the second main objective of our study was to assess predictive factors of efficacy to these agents in MBC patients treated at our Institution.

One pioneering aspect of our study is the analysis of clonality of *PIK3CA* and *AKT1* mutations in a clinical setting. To the best of our knowledge, this has not yet been described in breast cancer outside a purely research environment. We also study whether *PIK3CA* clonality is a predictor of efficacy to PI3Kα-specific inhibitors.

Since this is a retrospective study results must be interpreted with caution: it is difficult to completely control selection biases, data collection biases, and biases related to missing clinical information in patients' medical records. Some factors, however, may have mitigated these issues. First, we collected the genomic data

directly from our Institution's genomic database, where all the molecular information is prospectively registered. Second, patients included in the PAM pathway inhibitor population were treated in the context of a clinical trial, and for this reason, medical records are generally more accurate and complete than those from patients treated within routine care. Lastly, all medical records were re-checked for accuracy on clinical information and on the response to PAM pathway inhibitors.

### 7.1. Molecular alterations in MBC patients

We present the molecular characterization of 327 MBC patients screened in our Unit from January 2010 to December 2015. This sample size is comparable to some of the clinical series published in the literature, although some of the studies have more patients and other encompass shorter periods of time. For instance, at the MD Anderson Cancer Center (MDACC) 2000 patients, including more than 850 MBC patients, were successfully screened over an 18-month period[62]. At the Princess Margaret Hospital, 1640 patients underwent successful screening over the course of 29-months, including 310 MBC patients[172]. At the Mass General Hospital (MGH), Juric and colleagues report their 4-year experience of MBC genotyping, with 347 patients enrolled[173]. These differences in numbers probably relate to the strategy of the different Institutions regarding the selection of patients for molecular screening. At our Institution, we do not perform massive genotyping, but rather offer tumor genotyping only to those patients that can be potential candidates for enrollment in our clinical trials. The reason for this is twofold. First, it allows us to optimize Laboratory and human resources. The second reason relates to financial constraints – in a public healthcare system environment it is difficult to obtain funding to perform these kinds of studies. This pragmatic approach, while not optimal for research/discovery, allows us to rationally use available resources and thus promote sustainability. It is also in line with a recent consensus panel on personalized medicine, which recognized that molecular screening programs should not aim to discover new cancer-related genes, but instead offer first validation steps about their clinical relevance[174]. As a result of this strategy, we have a higher patient inclusion rate in genotype matched clinical

trials at our Institution when compared to other Hospitals. This will be further discussed later on.

Most of the patients included in our analysis have LUM tumors (61.5%), followed by TN (18%), and HER2+ (11%). This distribution is similar to other studies such as the MDACC experience, where the proportion of LUM, TN, and HER2+ tumors was 70.4%, 18.3%, and 11.3%, respectively[175]. Of note, we and others report a higher proportion of TN tumors with respect to the general population of breast cancer patients. This probably reflects the search for novel therapeutic targets in population with a dismal prognosis and limited treatment options.

*TP53* mutation was the most frequent mutation in our series (34.2%). Interestingly, 100% of HER2+ samples tested for *TP53* had a *TP53* mutation, while in TN the proportion was 66.7%. In the Basal-like subset included in TCGA, this proportion was 86.0% (Table 16)[54]. In the MDACC series, 23.8% of patients had a *TP53* mutation, especially if TN (58.3%)[175]. Importantly, *TP53* mutations were not included in the panel that was used with Sequenom® or in the first versions of the AmpliconSeq panel, and hence the true prevalence of *TP53* mutations in our complete cohort is very likely underestimated. Currently, while there are no specific drugs targeting this alteration, we are running a trial at our Institution enrolling patients with WT *TP53* (NCT02143635), which was one of the reasons why *TP53* mutation was included in the AmpliconSeq panel.

*PIK3CA* mutation was the second most frequent alteration in our cohort (24%). This percentage is similar to other published series (Table 16). In the TCGA dataset the proportion of *PIK3CA* mutations is 34.3%[54], in the SAFIR01 trial 24.9%[63], in the MGH series 23.3%[173], and in the MDACC cohort 27.1%[175]. *PIK3CA* mutation is more frequent in LUM tumors (especially if LUM A, according to TCGA), followed by HER2+ and then TN tumors, where it has a frequency of 7-9%. Of note, the identification of a small subset of TN patients with a targetable alteration such as *PIK3CA* mutation may have therapeutic implications. In our Hospital, some of these TN, *PIK3CA* mutant, MBC patients were offered treatment in a basket study with taselisib, a PI3K β-sparing inhibitor (NCT01296555, see

Section 1.7.3.2.2). Another potential therapeutic implication is the association that has been described between *PIK3CA* mutations and the expression of androgen receptor (AR) in TN tumors (40% in AR-positive vs. 4% in AR-negative)[176]. Currently, there is a Phase Ib/II clinical trial ongoing of taselisib in combination with enzalutamide in advanced TNBC (NCT02457910). We did not test our samples for AR expression, but if results from this trial are encouraging, determining AR expression in *PIK3CA* mutant TN patients may be clinically relevant. Taselisib is also being tested in combination with palbociclib in TNBC with PAM pathway dysregulation (NCT02389842).

**Table 16:** Molecular alterations in breast cancer across different studies.

| Values in % | Our study | TCGA* | SAFIR01 | Lefebvre et al. | MDACC | MGH |
|---|---|---|---|---|---|---|
| ***TP53*** | **34.2** | **34.3** | NR | **39.6** | **23.8** | NR |
| LUM | 22.7 | 13.9/42.6 | | 26.6 | 21.7 | |
| HER2+ | 100 | 72.5 | | 64.3 | 42.4 | |
| TN | 66.7 | 86.0 | | 74.5 | 58.3 | |
| ***PIK3CA*** | **24** | **34.5** | **24.9** | **35.2** | **27.1** | **23.3** |
| LUM | 27.9 | 46.3/35.2 | NR | 38.4 | 31.1 | 29.1 |
| HER2+ | 27.8 | 37.3 | NR | 14.3 | 30.9 | 21.5 |
| TN | 8.5 | 6.5 | NR | 11.8 | 9.0 | 8.3 |
| ***AKT1*** | **4.1** | **2.4** | **4** | **5.1** | **3.9** | NR |
| LUM | 5.5 | 5.5/2.6 | NR | 6.3 | 5.2 | |
| HER2+ | 2.8 | 2 | NR | - | - | |
| TN | - | - | NR | 3.9 | 1.1 | |
| ***ERBB2*** | **1.3** | 2.2 | NR | **3.7** | **1.3** | NR |
| LUM | 2.1 | 1.5/0.8 | | 4.9 | 2.3 | |
| HER2+ | - | 3.9 | | - | 3.4 | |
| TN | - | 0.9 | | 2 | - | |
| ***ESR1*** | **6.3** | NR | NR | **11.1** | NR | NR |
| LUM | 8.2 | | | 14 | | |
| HER2+ | - | | | - | | |
| TN | - | | | - | | |
| ***PTEN*** | **5.7** | **5.1** | NR | **4.2** | **1** | NR |
| LUM | 2.6 | 3.0/9.0 | | 3.5 | 1.9 | |
| HER2+ | - | 7.8 | | - | - | |
| TN | 16 | 5.6 | | 5.9 | - | |

*Data for IDC, obtained from Reference Ciriello G. et al[54]. In TCGA, proportion of mutations is described according to PAM50 intrinsic subtypes (Luminal A / Luminal B, HER2-E, Basal-like). LUM: luminal. MDACC: MD Anderson Cancer Center. MGH: Mass General Hospital. NR: not reported. TCGA: The Cancer Genome Atlas. TN: Triple negative.

The frequency of *AKT1* mutations was also similar to the literature (Table 16). Although *AKT1*-mutant tumors represent a small segment of MBC (around 4-5%, mainly LUM tumors), detection of this alteration has potential therapeutic impact, as there is an ongoing basket trial with AZD5363, an ATP-competitive pan-AKT inhibitor (NCT01226316), for patients with ER+/*AKT1* mutant MBC. Preliminary results suggest that breast tumors with *AKT1* mutation have high response rates with this drug[148], and our AKT-mutant patients are offered treatment in this trial.

In LUM tumors, *FGFR1* amplification was the second most common actionable alteration (15%). FGFR signaling plays a crucial role in cancer cell proliferation, migration, angiogenesis, and survival. The FGF family consists of 18 ligands which signal through 4 high-affinity receptors[177]. FGFR signaling may be activated in breast cancer through FGFR amplification or mutation, or by amplification of FGFR ligands (located in 11q). *FGFR1* amplification is the most common FGFR alteration in breast cancer, and it associates with worse prognosis and resistance to endocrine therapy[178,179]. In our series, the frequency of *FGFR1* amplification was in line with other reported studies. In the biomarker analysis from the BOLERO-2 trial, for instance, *FGFR1* amplification was found in 18.1% of the samples[153]. In the SAFIR01 trial, this proportion was 13%[63], and in the TCGA 11% (13% in LUM patients)[56]. The variation may be due to the type of tissue analyzed, as well as slightly different definitions of FGFR1 amplification positivity across trials.

Several drugs targeting FGFR have been developed over recent years. They can be divided into tyrosine kinase inhibitors (TKI) with dominant pharmacological activity in other kinases (such as VEGFR, PDGFR, FLT3, RET, KIT, or BCR-ABL), and selective FGFR inhibitors. The first group includes agents such as dovitinib and lucitanib, while BGJ398, AZD4547, LY2874455, and JNJ-42756493 are examples of drugs from the second group. In breast cancer, results from early clinical trials suggest that selective FGFR inhibitors may not be optimal in treating *FGFR1* amplified tumors, while pan-TKI drugs may prove more efficacious. Results from trials testing FGFR inhibitors in breast cancer have been disappointing so far, either because of low efficacy[167], or high toxicity rates[168].

Nonetheless, this is an alteration that – at least for the moment – we keep prospectively screening in our MBC patients.

Mutations in *ESR1*, the gene encoding for ERα, have been detected in 6.3% of our patients, all with LUM tumors. These mutations have been considered very infrequent in breast cancer for years, probably due to the use of low sensitivity methods, coupled with analysis focused only in primary tumors. In the METABRIC study, for instance, no *ESR1* mutation was reported[81]. However, when NGS is used in the genotyping of metastatic biopsies, the proportion of *ESR1* mutations may rise to up to 20% of the cases[180]. In our series, we found a proportion of *ESR1* mutations of 6.3%, especially if the determination was done in a metastatic sample (12.2% vs. 2.8% in the primary). Please refer to Section 7.2 below to additional discussion of this alteration.

The EGFR/HER2 pathway plays a critical role in the pathogenesis of breast cancer, especially in HER2+ subtype (10-15% of the total of breast cancer cases). In these tumors, HER2 (or *ERBB2*) amplification drives tumorigenesis, and drugs targeting HER2 have changed the natural history of the disease[8,9]. It was not until recently that activating mutations in *ERBB2* were identified in MBC patients lacking *ERBB2* gene amplification or overexpression[181]. *ERBB2* mutation occurs in around 1.5-2.5% of MBC patients[181,182]. In our cohort, *ERBB2* mutation was detected in 1.3% of the patients (all LUM tumors), which is in line with the available literature. These patients are not candidates for HER2-targeted drugs under current standards of treatment, but some reports suggest that they may benefit from anti-HER2 small molecule inhibitor drugs, such as neratinib[181,183]. A basket trial with neratinib is available at our Institution (NCT01953926), and patients with *ERBB2* mutations are offered enrollment in this study.

*PTEN* is a tumor suppressor gene that encodes a dual specific phosphatase that dephosphorylates PIP3 to PIP2. Intact PTEN plays a key role in the negative regulation of the PAM pathway[82]. PTEN can be downregulated by several mechanisms including genetic alterations (like mutations or gene deletions), transcription, translation, and post-translational modification. In our cohort, we

analyzed both PTEN expression and *PTEN* mutations. Loss of PTEN expression by IHC is probably a sign of impaired PTEN function, leading to the consequent PAM pathway activation. The best cut-off to measure PTEN dysregulation by IHC is not well established, but probably the best read-out for loss of PTEN function is the absence of PTEN staining (PTEN null), and this is the definition we used as a surrogate for pathway dysregulation. Overall, 10.6% of our patients had PTEN null, especially if TN (20.5%, compared with 9.3% in LUM and 3.6% in HER2+).

PTEN can also be dysregulated by the presence of inactivating mutations. In our study, *PTEN* mutation was present in 5.7% of the samples, with significant differences between subtypes (LUM 2.6%, HER2+ no *PTEN* mutation detected, TN 16%, P=0.047). The overall *PTEN* mutation rate is similar to the one found in the study by Lefebvre et al. (4.2%), although no differences were observed in this study according to breast cancer subtype (LUM 3.5% vs. TN 5.9%). Our results are however aligned with the findings of Nik-Zeinal et al., who described an enrichment for PTEN mutations in ER-negative breast tumors[55]. Importantly, not all PTEN mutations or alterations are deleterious to PTEN function. It is crucial to have a good annotating procedure in order to call PTEN variants as pathogenic or non-pathogenic, with the consequent therapeutic implications. PTEN alterations that ablate function of the gene – such as point or multi-nucleotide substitutions, insertions and deletions, rearrangements and loss of part of or the entire gene locus, coding or non-coding variants – are generally considered to be pathogenic. Trials with PI3K β-specific inhibitors, such as AZD8186 (NCT01884285), and with AKT-inhibitors, like AZD5363 (NCT01226316), are enrolling PTEN-mutant TN and ER+ patients, respectively.

In HER2+ tumors, the most common actionable alteration on top of *ERBB2* amplification was *PIK3CA* mutation, detected in 27.8% of the samples. These numbers are similar to the reported in the literature. Of note, *PIK3CA* mutations confer resistance to some anti-HER2 drugs such as trastuzumab and lapatinib[184,185], but not to others, e.g. T-DM1 or pertuzumab[185,186]. For this reason, it is clinically relevant to obtain data regarding the *PIK3CA* mutation status in a HER2+ MBC patient. However, for the time being, the presence of a *PIK3CA*

mutation should not *per se* prevent these patients from receiving any effective anti-HER2 drug, if otherwise candidates to that treatment.

Actionable alterations in the population of TN patients were infrequent. Indeed, no actionable alteration could be identified in 65% of these patients, suggesting that new genomically agnostic treatment strategies, such as immunotherapy[187] or the use of antibody-drug conjugates[188], are urgently needed in this bad prognosis MBC subgroup.

In our study, we have detected a long tail of alterations occurring in less than 2% of the samples, including *EGFR*, *ERBB3*, *FGFR1*, *FGFR2*, *FGFR3*, or *KRAS* mutations. These results are in line with several published works[63,172,175]. This raises the question whether it will ever be feasible to design clinical trials for these small segments of MBC, even if they represent potentially actionable alterations. For instance, frequency of *EGFR* mutation in our population was 0.6%, which suggests that the logistics of a specific trial with anti-EGFR drugs in *EGFR*-mutant breast cancer are at least challenging. However, EGFR TKIs are active drugs in lung cancer[12], and we may preclude EGFR-mutant MBC patients to receive a potentially active treatment. Umbrella trials and basket trials are options to circumvent this issue.

Umbrella trials assess the effect of different drugs in different molecular alterations within the same tumor type. They typically include a central infrastructure for screening and identification of patients, and have multiple sub-trials within the umbrella framework. By continuously gaining insights into the molecular complexity of the disease and the efficacy of the different arms of the trials, they have the flexibility to drop-off or add additional arms as needed. Some examples are the BATTLE trial in lung cancer[189], or the SAFIR-02 trial in breast cancer (NCT02299999).

Basket trials, in turn, test the effect of a single drug on a molecular alteration in a variety of cancer types[190]. Intense translational work can be done in basket trials in order to study mechanisms of resistance to targeted therapies in the different

tumor types, with the possibility of subsequently adding new arms of treatment with novel, more active, combinations. For instance, it was observed that *BRAF* V600E inhibition causes a rapid feedback activation of EGFR in *BRAF*-mutant CRC, which supports continued proliferation despite treatment[191]. Based on these findings, an arm of vemurafenib plus cetuximab for *BRAF*-mutant CRC was included in the basket trial recruiting patients with *BRAF*-mutant tumors. In the cohort of patients with CRC who received single agent vemurafenib, no responses were observed. In the cohort of patients with CRC treated with vemurafenib and cetuximab, one response was observed, and approximately half the patients had tumor regression[192].

We identified 21 samples with two or more molecular alterations. This information is useful in better guiding treatment decisions. First, it may lead us to consider the upfront use of combination therapies, if available. For instance, the patient with *PIK3CA* plus *ERBB2* mutation could potentially benefit from dual PAM pathway and ERBB2 blockade. Second, it may influence the decision in selecting a given therapy over another. The patients with *ESR1* plus *PIK3CA* mutation won't be good candidates to receive an AI plus a PAM pathway inhibitor, while the combination of a PAM pathway inhibitor with fulvestrant could be a better option. Lastly, it may allow us to exclude upfront some treatment options. Based on pre-clinical as well as some clinical data, the *PIK3CA* mutant patient with a concomitant *KRAS* mutation will probably not respond to a PI3K inhibitor, and we could spare potential toxicities to a patient that probably will not benefit from the treatment.

### 7.2. Genomic alterations in primary tumors and metastasis

Sequencing of EBC has provided insight into the biology of early malignancy, but only around 20-25% of the patients will ever present with overt metastatic disease[22,23]. Furthermore, as a result of tumor clonal evolution, the genetic landscape of a metastasis from breast cancer is different from the primary[193-195]. For this reason, we believe that genotyping a metastatic sample rather than the primary tumor may be more informative to steer therapeutic decisions and, if

feasible, this is our preferred approach. In our cohort, 100 samples (30.6%) derived from a metastatic site. Generally, these patients undergo a biopsy specifically to perform molecular pre-screening, or in the context of a clinical trial. Interestingly, median tumor purity was higher in metastatic samples than in primary tumor samples (70% vs. 60%, P=0.04). This probably reflects more stringent criteria for collection of metastatic biopsies in a research environment.

As previously mentioned, we found a higher proportion of *ESR1* mutations in metastatic samples than in primary tumors (12.2% vs. 2.8%, P=0.097), which is in line with the current literature[196]. Of note, these samples were not matched, i.e. coming from the same patient, so the results are merely hypothesis generating. The proportion of *ESR1* mutations appears to increase with successive exposure to endocrine therapies, especially AIs. Recent studies have reported detection rates of *ESR1* mutations in plasma samples from patients previously treated with AIs of 26.8-30.6%, suggesting that they arise as a mechanism of resistance to these therapies[197,198]. Since this is a heavily pre-treated cohort – with a median of 3 prior lines of treatment for MBC – it is conceivable that most, if not all of our patients had received an AI as part of their adjuvant or metastatic treatment. Detection of *ESR1* mutation has also been correlated with worse prognosis and resistance to AIs, but not to fulvestrant. In one study investigating the presence of *ESR1* mutations in ctDNA, the authors found that LUM patients with *ESR1* mutations have a substantially shorter PFS on AI-based therapy than those patients without *ESR1* mutations (HR 3.10, 95%CI 1.9-23.1; P=0.004)[198]. Additionally, in the SOFEA trial, patients with *ESR1* mutations had improved PFS after taking fulvestrant compared with exemestane (HR 0.52, 95%CI 0.30-0.92; P=0.02), whereas patients with WT *ESR1* had similar PFS after receiving either treatment (HR 1.07, 95%CI 0.68-1.67; P=0.77)[199]. This analysis is outside the scope of our work, but these data reinforce the importance of real-time detection of *ESR1* mutations to optimize the treatment of ER+ MBC, either by the acquisition of biopsies from metastatic sites prior to initiation of a new therapy, or by the determination of *ESR1* mutation in plasma ctDNA. Of note, detection of *ESR1* mutations in ctDNA is currently being incorporated in the prescreening of our MBC patients.

Interestingly, there was also a trend for *PIK3CA* and *ERBB2* mutations to be more frequent in samples from metastatic sites than in those from primary tumors: 31.1% vs. 21.1% (P=0.068) and 3.3% vs. 0.5% (P=0.081), respectively. This may suggest that *PIK3CA* and *ERBB2* have a role in the metastatic process and drug resistance, and hence could be good candidates for drug targeting in the metastatic setting.

The landscape of MBC has been better characterized in a recent paper from Lefebvre et al[200]. In this study, the authors performed whole-exome sequencing (WES) on 216 metastatic samples from MBC patients who underwent a biopsy in the context of the SAFIR-01[63], SAFIR-02 (NCT02299999), SHIVA[201], or the MOSCATO (NCT01566019) prospective trials. Taking the TCGA dataset as a reference, the authors found similar frequencies of mutations in LUM MBC as compared to LUM EBC in some genes, like *PIK3CA* (37% vs. 40%) or *TP53* (27% vs. 20%). In contrast, LUM MBC had a higher proportion of mutations in other genes like *ESR1* (14% vs. <1%) and *RB1* (6.3% vs. 2%). Other genes frequently mutant in MBC samples were *PALB2*, *TSC1*, *TSC2*, *ERBB4*, *NOTCH3*, and *ALK*. Interestingly, *TSC1* and *TSC2* mutations have been described as sensitivity mutations to mTOR inhibitors, and this finding may also have therapeutic implications[202]. The panels we used for our analysis do not include these genes, and we may have missed these mutations in our population.

### 7.3. Clonality of *PIK3CA* and *AKT1*

Tumors often evolve through a process of branched evolution, involving genetically distinct subclones[203,204]. This means that the level of knowledge of a patient's genomic alterations at a given time point should not merely be based on a catalog of cancer genes mutations, but also on the understanding of their spatial and temporal dynamics during the tumor's evolution. While it is difficult to rigorously assess the clonal evolution of a patient's tumor outside a purely research-based environment, real-time information about the presence of dominant somatic events could be therapeutically relevant[205].

The use of NGS techniques in molecular pre-screening programs allows us to assess the MAF of a given mutation in a patient's sample. MAF is the number of mutant reads divided by the total number of reads – coverage – at the specific genomic position of interest. It is largely influenced by tumor purity (fraction of neoplastic cells in the sample) and copy number alterations (gene amplifications and deletions)[165]. MAF of driver genes may be used to infer the mutational timeline, intra-tumor genomic heterogeneity, and dynamic clonal evolution[166]. However, this information is seldom reported, despite its potential impact on response to targeted drugs.

To the best of our knowledge, characterization of MAF of recurrent mutations in breast cancer within a clinical setting is lacking so far. We therefore proposed to analyze the clonality of *PIK3CA* and *AKT1* mutations in our MBC cohort, and to assess whether the clonality of *PIK3CA* was predictive of response to PAM pathway inhibitors, namely PI3Kα-specific inhibitors.

In our study, clonality was determined as described in section 5.4. In brief, the number of mutant alleles in the sample was corrected for tumor purity, resulting in a value that we called adjusted MAF. We considered an adjusted MAF of >0.3 as clonal, and subclonal otherwise. In the absence of an established cut-off to define clonality, the presence of at least 30% mutant alleles was considered biologically relevant. Given the small numbers, we did not look at other cut-offs, but we did analyze adjusted MAF as a continuous variable. Of note, we did not consider the copy number variants of the gene, or the ploidy, to correct the MAF for a given mutation – in most of the cases this information was not available. As a consequence, there might be an overestimation of adjusted MAF if a gene amplification coexisted with a gene mutation. It is unlikely that our results were altered for this reason, since the percentage of breast cancer samples with both *PIK3CA* mutation and amplification is typically low (1.9% in METABRIC and 2.3% in TCGA[79,80]).

Median adjusted MAF for *PIK3CA* in the complete cohort and in the PAM pathway inhibitor cohort was 0.43 (IQR 0.28-0.63) and 0.49 (IQR 0.28-0.62), respectively. *PIK3CA* adjusted MAF was higher in primary tumor samples when compared to samples coming from a metastatic site (complete cohort 0.49 vs. 0.29, p=0.072; PAM pathway inhibitor cohort 0.53 vs. 0.25, P=0.04). In contrast, no differences were found across breast cancer subtypes in both cohorts (complete cohort: LUM 0.43 vs. HER2 0.55 vs. TN 0.20, P=0.611; PAM pathway inhibitor cohort: LUM 0.49 vs. HER2 0.55 vs. TN 0.14, P=0.460). Of note, in TN tumors *PIK3CA* adjusted MAF was found to be in the subclonal range in both cohorts (0.2 and 0.14, respectively), while in LUM and HER2+ it was always in the clonal range. Regarding *AKT1*, it was a clonal event in both cohorts, independently of the type of sample.

Taken together, all these data suggest that *PIK3CA* and *AKT1* mutations are truncal events in breast cancer (or at least in LUM and HER2+ subtypes), and there may be a selection for *PIK3CA* WT subclones in the metastasis probably due to therapeutic pressure. In a comprehensive analysis of TCGA data in nine solid tumors, McGranahan et al. found a clear tendency for mutations in driver genes to be clonal compared with mutations in non-cancer genes. However, PAM pathway-related genes (such as *PIK3CA*) harbored a higher proportion of subclonal mutations compared with genes associated with RAS-MAPK pathway, including KRAS, NRAS and BRAF[206]. These data are in line with our findings, which certainly require further validation.

### 7.4. Rate of enrollment in clinical trials

From the 327 patients with a valid somatic mutation and/or *FGFR1* analysis, 184 (56.3%) received a targeted therapy. Of these, 74 (40.2%) were genotype-driven trials, i.e., trials with drugs matched to the alterations that were detected. Overall, 22.6% (74/327) of the patients undergoing molecular pre-screening received treatment within a genotype-driven trial.

The number of patients that received a matched drug is globally higher than in the literature. For instance, in the Princess Margaret IMPACT/COMPACT trial, only 5% of patients who underwent successful molecular profiling (i.e. 84 patients out of 1640 patients tested, all cancer types) were subsequently treated on genotype-matched clinical trials[172]. The MDACC institutional genomic testing protocol matched 83/2000 (4%) of patients[62], while the British Columbia Cancer Agency Personalized Oncogenomics Trial matched 1/100 (1%)[207]. Specifically in breast cancer, the SAFIR-01 trial matched 48/423 (11.3%) patients, although only 28/423 (6.6%) received a matched treatment within a Phase I/II clinical trial[63].

One reason that can explain the high enrollment rate at our Institution is patient selection for genomic testing. As previously discussed, we restrict genotyping to those patients that can potentially be good candidates for clinical trials. Additionally, the genotyping request is made directly by the treating physician, not by an investigator not directly involved in treatment decisions. In this way, genotyping information is generally not lost nor missed, and can be used for real-time treatment decisions.

### 7.5. Characteristics of the PAM pathway inhibitor cohort

From the 327 patients that were tested for molecular alterations, 120 received a PAM pathway inhibitor (PAM pathway inhibitor Cohort). Patients in the PAM pathway inhibitor Cohort were heavily pre-treated, with a median of 3 prior lines of therapy for metastatic disease (range 0-9). This information is important when comparing outcomes of our patients with those from trials testing mTOR and PI3K inhibitors. In the latter, patients generally have received less lines of therapy, typically an AI and a maximum of one prior chemotherapy for metastatic disease.

Hyperactivation of the PAM pathway induces resistance to endocrine therapy[208], anti-HER2 treatment[209], and chemotherapy[210]. Conversely, inhibition of the pathway reverts the resistance to endocrine therapies in ER+ breast cancer cells and xenografts[211,212], restores the sensitivity to HER2 blockade in preclinical models resistant to anti-HER2 therapy[213], and induces tumor regression when

combined to chemotherapy in models resistant to the same chemotherapy backbone[210,214]. Over the years, the drug development strategy of PAM pathway inhibitors in breast cancer has evolved from trials testing single agents to trials testing combinations with standard therapies. It is not surprising, though, that most of the patients in this cohort received a combination of PAM pathway inhibitor with endocrine (53.9%), anti-HER2 (16.1%), or chemotherapy (13.1%). Of note, 3 out of 4 patients with LUM tumors received a combination with endocrine therapy, and over 80% of patients with HER2+ tumors received a combination with anti-HER2 blockade. Regarding TN patients, 57% received a PAM pathway inhibitor as a single agent, and the remaining in combination with chemotherapy.

Interestingly, most of the patients in this cohort (54/120, 41.5%) were treated with PI3Kα-specific inhibitors, followed by mTOR inhibitors (20.8%), which traduces the availability of drugs in clinical trials during the considered period of time at our Institution.

The proportion of molecular aberrations in the PAM pathway inhibitor cohort was similar to that of the complete cohort (Figure 29). The main differences were a higher proportion of *PIK3CA* mutations (31.7% vs. 24%) and a lower proportion of TP53 mutations (21.1% vs. 34.2%) in the PAM pathway inhibitor cohort. Notably, the proportion of patients with PAM pathway dysregulation was higher in the PAM pathway inhibitor cohort than in the complete cohort (43.3% vs. 35%). Overall, this reflects the matching effort described above.

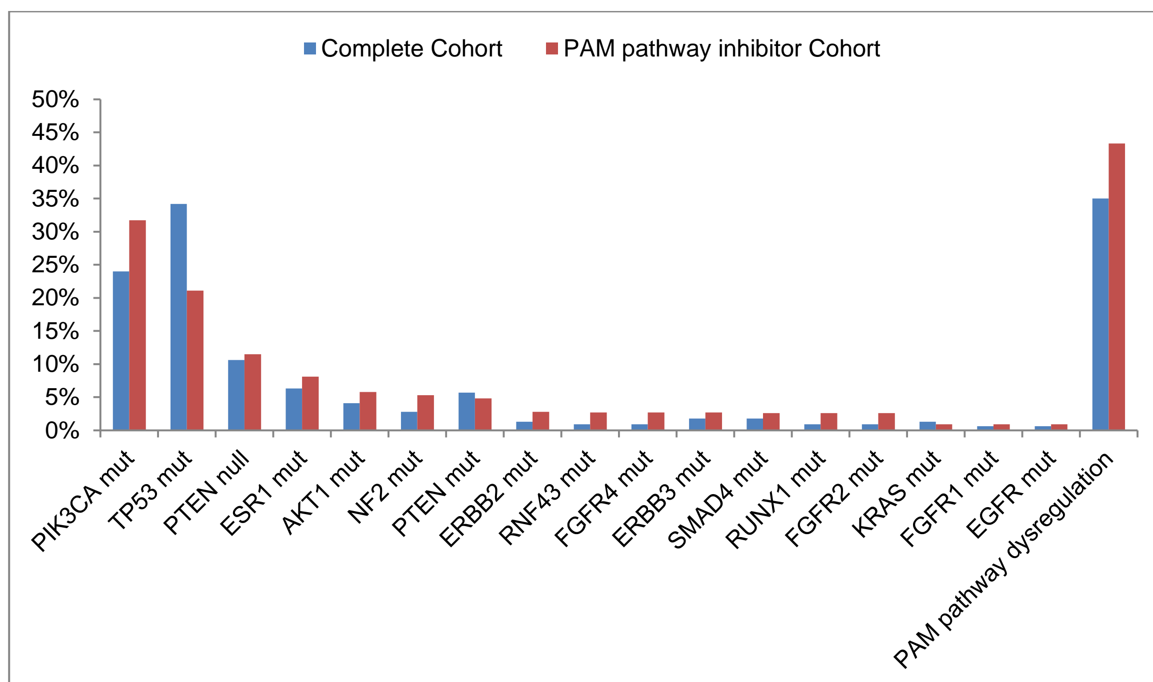


**Figure 29:** Molecular alterations in the Complete Cohort and in the PAM pathway inhibitor Cohort.

### 7.6. Predictive factors of efficacy of PAM pathway inhibitors

Routine genomic profiling in the clinic aims at the real-time identification of targetable, genetic driven, tumor dependencies and vulnerabilities, with the ultimate goal of offering the patient the optimal drug at the right time during his/her natural history. Over recent years, caution has been raised regarding this approach[215,216]. The main criticism is that although feasible, it is currently unknown whether massive genomic profiling and matching treatments to aberrations actually improve patient outcomes. SHIVA is the only study published so far that prospectively explored this question. In this study, the authors found no differences in the outcomes of patients randomized to receive a genomically matched therapy versus conventional therapy[201]. However, it is important to point out some limitations of the trial that can preclude definitive conclusions regarding this issue. First of all, the trial was underpowered to identify any differences in specific tumor types, or in patients with genomic alterations on top of AR/ER/PgR positivity. Second, the genomic alterations that were considered had not been validated as optimal targets (like *STK11* deletion, *ABL1/2* mutation, or *YES1* amplification). Lastly, the drugs that were used were not the best in class (e.g.

everolimus for *AKT1* mutation, or lapatinib for *ERBB2* mutation). All these factors may have influenced the negative results of SHIVA. Next-generation prospective trials, such as SAFIR-02, may more reliably address this question.

Given the limited evidence regarding the success of matching genomic alterations with targeted treatments in terms of patient benefit, it is important to study predictive factors of efficacy to novel drugs in real-world data. PAM pathway inhibitors are one of the main areas of research and drug development in our Unit, and our patients with PAM pathway dysregulation are more likely to receive a PAM pathway inhibitor than those patients without dysregulation (OR 1.98, 95%CI 1.19-3.26; P=0.006). We took advantage of the large number of patients treated with PAM pathway inhibitors – mTOR, dual PI3K/mTOR, pan-PI3K, PI3Kα, and AKT inhibitors – and the fact that most of them were treated in genotype-driven trials, to analyze predictive factors of efficacy to these drugs.

In our population, 57.7% of the patients showed clinical benefit –achieving a CR, PR, or SD for at least 16 weeks – when treated with PAM pathway inhibitors, and the ORR was 23.8%. Median TTF in this pre-treated population was 4.9 months (95%CI 3.9-6.1).

In our study the presence of a *PIK3CA* mutation, but not its clonality or the presence of PAM pathway dysregulation, was predictive of clinical benefit to PAM pathway inhibitors, measured both as CBR and TTF. In the multivariate model including other prognostic factors for TTF, *PIK3CA* mutation stood as an independent predictor of better TTF. Other factors that positively correlated with TTF in the multivariate model were receiving combination therapy with endocrine and chemotherapy, and having received the PAM pathway inhibitor in up to third line of treatment for MBC. Breast cancer subtype, the presence of visceral metastasis, or the type of PAM pathway inhibitor, were not independent predictors of TTF in our population.

Globally, these results of CBR and TTF compare favorably with trials testing different PAM pathway inhibitors. However, comparison with these prospective

trials is limited and must be made with caution in view of the retrospective design of our study. Additionally, ours is a heterogeneous population with different breast cancer subtypes, treated with different agents that inhibit the PAM pathway at different levels, and given in different combinations. For these reasons, the following next paragraphs are merely descriptive and don't aim at direct comparisons. They are intended to put our results into a broader context. We will also try to highlight some predictive factors of response to each of the drugs tested in these trials.

Table 17 shows some data of the combination arms of several trials testing PAM pathway inhibitors together with endocrine therapy.

**Table 17:** Efficacy results of combination arms of trials with PAM pathway inhibitors plus endocrine therapy.

| Trial | Drug (class) | Prior therapies Median (range) | ORR % | CBR % | TTF / PFS (months) Median (95%CI) |
|---|---|---|---|---|---|
| Our study | Miscellaneous | 3 (0-9) | 23.8 | 57.7 | 4.9 (3.9-6.1) |
| BOLERO-2[96] | Everolimus (mTOR) | 3 (-) | 9.5 | NR | 6.9 (6.4-8.1) |
| BOLERO-4[95] | Everolimus (mTOR) | 0 | 42.6 | 74.3 | - |
| TAMRAD[99] | Everolimus (mTOR) | 24% prior CT | 9.3 | 61 | 8.6 (5.9-13.9) |
| AZD2014 + Fulv[§103] | AZD2014 (mTOR) | 6 (1-26) | 14 | 30* | - |
| BELLE-2[116] | Buparlisib (pan-PI3K) | 72.6% prior ET | | - | |
| Full population | | | 11.8 | | 6.9 (6.8-7.8) |
| PI3K activated | | | 10.6 | | 6.8 (4.9-7.1) |
| *PIK3CA* ctDNA | | | 18.4 | | 7.0 (5.0-10.0) |
| BELLE-3[117] | Buparlisib (pan-PI3K) | 70% ≥2 lines | | | |
| Full population | | | 7.6 | 24.6 | 3.9 (2.8-4.2) |
| PIK3CA tissue | | | - | - | 4.7 (2.9-6.7) |
| PIK3CA ctDNA | | | - | - | 4.2 (2.8-6.7) |
| FERGI[106] | Pictilisib (pan-PI3K) | 1 (0-≥3) | | | |
| Part 1 | | | 7.9 | 24.7 | 6.6 (3.9-9.8) |
| *PIK3CA* mut | | | 7.3 | 19.5 | 5.4 (3.8-8.3) |
| MK-2206 + ET[§132] | MK-2206 (AKT) | 0.5 (0-3) | 7.7 | 36.7* | 5.8 (IQR 3-14) |
| Alpelisib + Let[§124] | Alpelisib (PI3Kα) | 2 (1-4) | 19 | 35* | - |
| Taselisib + Fulv[128] | Taselisib (PI3Kα) | | | | - |
| Full population | | 2 (1-5) | 22.7 | 29.5 | |
| *PIK3CA* mut | | 2 (1-5) | 38.5 | 38.5 | |

§Single arm Phase I trial. *Defined as no progressive disease at 6 months. Dash (-) denotes not reported. CBR: clinical benefit rate. CI: confidence interval. CT: chemotherapy. ET: endocrine therapy. Fulv: Fulvestrant. IQR: Interquartile range. Let: letrozole. Mut: mutant. ORR: overall response rate. PFS: progression-free survival. TTF: time to treatment failure.

In the BOLERO-2 trial, ORR was 9.5% and median PFS in the initial publication and the final analysis was 6.9 months and 7.8 months, respectively[96,97]. Notably, benefit from everolimus was observed across all clinical subgroups. *PIK3CA* mutation was not predictive of everolimus benefit, nor were PAM pathway activation, cell-cycle pathway alterations, or *FGFR1* amplification[153]. In the TAMRAD trial, the best predictor of CBR and TTP was the presence of secondary resistance to endocrine treatment, defined as relapse more than 6 months after stopping adjuvant AIs or progressing after ≥6 months to AIs in the metastatic setting[99]. We did not look into this particular factor in our study. Given the very small numbers (N=5 in everolimus plus tamoxifen arm and N=4 in tamoxifen alone arm), the predictive value of *PIK3CA* mutations in the response to everolimus was not assessed in TAMRAD[154]. However, the authors studied pathway activation with several IHC tests, and found that baseline expression levels of 4EBP1, p4EBP1, cytoplasmic LKB1, cytoplasmic pAKT, and PI3K – but not pS6RP or eIF4E – were potentially predictive of everolimus benefit. We did not perform IHC analyses in our study, but fully acknowledge that measuring downstream substrates of the PAM pathway, such as p4EBP1 or pAKT, may be a better way to assess activation of the pathway.

The BELLE-2 and the BELLE-3 trials, testing buparlisib in combination with fulvestrant, showed a statistically significant increase in PFS with the addition of buparlisib to fulvestrant in those patients with a *PIK3CA* mutation detected in ctDNA[116,117]. In BELLE-3, the same findings were observed if *PIK3CA* mutation was detected in archival tissue, although this difference did not reach statistical significance in BELLE-2. It must be noted that BELLE-2 and BELLE-3 have not been published yet, and there is limited information regarding other predictive factors of response to buparlisib, either clinical or molecular. In the FERGI trial, testing pictilisib in combination with fulvestrant, *PIK3CA* mutation did not associate with pictilisib benefit as measured by PFS or ORR[106]. The authors remark that pictilisib exposure was limited by tolerability, and this may be a confounding factor in interpretation of the data.

In the trial testing MK-2206, an allosteric AKT inhibitor, plus endocrine therapy, TTP was similar independently of prior therapy, presence or absence of visceral metastasis, or treatment-induced hyperglycemia[132]. There was a trend towards longer TTP in patients who received study drug as first-line endocrine therapy, which is in line with our own results. Among the 30 patients included, there was no clear association between *PIK3CA* mutation status and TTP.

Table 18 shows some data of the combination arms of several trials testing PAM pathway inhibitors together with chemotherapy.

**Table 18:** Efficacy results of combination arms of trials with PAM pathway inhibitors plus chemotherapy.

| Trial | Drug (class) | Prior therapies Median (range) | ORR % | CBR % | TTF / PFS (months) Median (95%CI) |
|---|---|---|---|---|---|
| Our study | Miscellaneous | 3 (0-9) | 23.8 | 57.7 | 4.9 (3.9-6.1) |
| BELLE-4[119] | Buparlisib (pan-PI3K) | 16.4% prior ET | 22.6 | 26.2 | 8.0 (7.2-9.2) |
| PEGGY[121] | Pictilisib (pan-PI3K) | | | | |
| ITT | | - | 22 | - | 8.2 |
| *PIK3CA* mut | | - | - | - | 7.3 |
| BEECH Part A[150] | AZD5363 (AKT) | 1 CT (0-2) | 44.4 | - | 8.2 |
| Ipatasertib + taxane[§144] | Ipatasertib (AKT) | - | 26.3 | - | - |

[§]Single arm Phase I trial. Dash (-) denotes not reported. CBR: clinical benefit rate. CI: confidence interval. CT: chemotherapy. ET: endocrine therapy. ITT: intention-to-treat. MBC: metastatic breast cancer. Mut: mutant. ORR: overall response rate. PFS: progression-free survival. TTF: time to treatment failure.

In the BELLE-4 trial, there was no benefit from addition of buparlisib to paclitaxel in either the full or the PI3K pathway-activated population (defined as those patients with either a *PIK3CA* mutation and/or loss of PTEN expression. i.e. 1+ in ≤ 10% of tumor cells by IHC)[119]. Similar results were observed in the PEGGY trial[121]. Strikingly, in BELLE-4 the buparlisib arm performed generally worse than the placebo arm, although this difference did not reach statistical significance.

The randomized Phase II part of the BEECH trial tested the addition of AZD5363, an AKT inhibitor, to paclitaxel as front line treatment to ER+/HER2-negative MBC. While the results of this randomized trial are awaited, in the Phase I of the combination there was an encouraging ORR of 44% and PFS of 8.2 months[150]. Thorough biomarker analyses are being performed in order to identify biomarkers

of sensitivity and resistance to AZD5363, and the combination of AZD5363 with paclitaxel.

In the Phase I trial testing ipatasertib plus docetaxel or paclitaxel ORR was 26.3%, and objective responses were seen in patients with prior progression on taxanes and PAM pathway inhibitors[144]. Results from the LOTUS trial, testing the addition of ipatasertib to paclitaxel as front line treatment to TN MBC patients, are expected mid-2017.

In our study, neither HER2+ subtype nor combination treatment with anti-HER2 drugs was an independent predictor for TTF in the multivariate model. We did not have enough numbers to look at predictive factors of response to PAM pathway inhibitors specifically within the HER2+ subset. Table 19 shows some data of the combination arms of several trials testing PAM pathway inhibitors together with anti-HER2 therapy.

**Table 19:** Efficacy results of combination arms of trials with PAM pathway inhibitors plus anti-HER2 therapy.

| Trial | Drug (class) | Prior therapies Median (range) | ORR % | CBR % | TTF / PFS (months) Median (95%CI) |
|---|---|---|---|---|---|
| Our study | Miscellaneous | 3 (0-9) | 23.8 | 57.7 | 4.9 (3.9-6.1) |
| BOLERO-1[100] | Everolimus (mTOR) | 0 | 67.1 | 75.8 | 15 (14.6-17.9) |
| BOLERO-3[101] | Everolimus (mTOR) | 1 (0-≥4) | 41.0 | 59.0 | 7.0 (6.7-8.2) |
| Buparlisib + Trast[§111] | Buparlisib (pan-PI3K) | 4 (1-8) | 11.8 | 17.6* | - |

[§]Single arm Phase I trial. Dash (-) denotes not reported. CBR: clinical benefit rate. CI: confidence interval. ORR: overall response rate. PFS: progression-free survival. TTF: time to treatment failure.

In the BOLERO-3 trial, testing the addition of everolimus to a backbone of vinorelbine and trastuzumab in HER2+ MBC patients progressing to trastuzumab and a taxane, the efficacy of everolimus was more pronounced in patients with ER-negative tumors[101]. We did not perform this analysis in our study. In BOLERO-3, low PTEN expression (considered as an H-score <20$^{th}$ percentile) and high pS6 seemed to be significantly associated with higher sensitivity to everolimus. *PIK3CA* mutations, in turn, had a much less profound effect on treatment benefit[155]. Interestingly, in a pooled analysis including data from BOLERO-1 and

BOLERO-3 (both in the setting of HER2+ MBC), *PIK3CA* mutation, PTEN loss, and PAM pathway activation predicted efficacy of everolimus plus trastuzumab plus chemotherapy (CT)[155]. In line with BOLERO-3 and BOLERO-1, in the Phase I trial testing trastuzumab plus buparlisib all the patients experiencing a PR or SD for more than 6 months had PAM pathway activation[111].

Focusing on the 54 patients treated with PI3Kα-specific inhibitors in our study, *PIK3CA* mutation was predictive of clinical benefit (84.2% vs. 54.3% in patients with no *PIK3CA* mutation, P=0.038). In contrast, *PIK3CA* mutation did not correlate to increased TTF, although numerical TTF was higher in *PIK3CA* mutant patients treated with PI3Kα-specific inhibitors than in *PIK3CA* WT patients (7.4 vs. 4.9 months, HR 0.65, 95%CI 0.36-1.20; P=0.167). The small number of patients within each subgroup may have diluted the potential magnitude of the TTF benefit of PI3Kα-specific inhibitors in *PIK3CA* mutant patients in this population.

In line to our results, the Phase I trial testing alpelisib plus letrozole showed increased benefit from the combination in those patients with *PIK3CA* mutation[124]. In this trial, patients with *PIK3CA*-mutant MBC had numerically higher CBR equal or greater than 6 months and 12 months than patients with WT *PIK3CA* tumors (44% vs. 20% and 38% vs. 20%, respectively). Additionally, from the five PR observed, four were in patients with *PIK3CA* mutation. Similarly, in the Phase II trial testing taselisib plus fulvestrant, ORR was 38.1% (95%CI 18.1-61.6) in *PIK3CA*-mutant patients and 8.7% (95%CI 1.1-28.0) in *PIK3CA* WT patients[128].

In summary, in large prospective trials testing PAM pathway inhibitors, *PIK3CA* mutations seem to be predictive of benefit to some pan-PI3K or PI3Kα-specific inhibitors, especially when combined with endocrine therapy or anti-HER2 blockade, but not with mTOR inhibitors, or PAM pathway inhibitors when combined with chemotherapy. In our cohort, the presence of *PIK3CA* mutation was predictive of benefit to PAM pathway inhibitors, especially in patients that received these agents in combination with endocrine therapy and chemotherapy early on during the course of metastatic disease. Again, it must be noted that the

different sample size and the huge heterogeneity of these trials make these comparisons merely descriptive.

### 7.7. Clonality of *PIK3CA* mutation and efficacy of PAM inhibitors

The MAFs of driver genes may be used to infer mutational timelines, intra-tumor genomic heterogeneity, and dynamic clonal evolution. However, despite their potential impact on tumor progression and response to targeted agents, they are often not reported. To the best of our knowledge, the influence of *PIK3CA* mutation clonality on response to PAM pathway inhibitors has not yet been described in breast cancer.

In our study, clonality of *PIK3CA* mutation did not impact response to PAM pathway inhibitors or to PI3Kα-specific inhibitors. Similarly, clonality of *PIK3CA* mutation was not a determinant of TTF to PAM pathway inhibitors – either as continuous or dichotomous variable – or to PI3Kα-specific inhibitors.

Our results are in contrast with some published data in NSCLC and CRC. In NSCLC, different studies have found that the abundance of *EGFR* mutations in primary tumors predicts for benefit from treatment with EGFR TKIs in the advanced setting[217,218]. Zhou et al. tested 100 samples from NSCLC patients treated with gefitinib for the presence of *EGFR* mutations and their abundance[217]. They used both Sanger sequencing and Scorpion amplification refractory mutation system (ARMS; DxS, Manchester, United Kingdom), which have a sensitivity cut-off for mutation call of 10% and 1%, respectively. In this study, MAF was not directly evaluated, but patients were categorized in 3 groups according to the detection of *EGFR* mutation by the two techniques: high abundance of EGFR (group H, N=51), i.e. those patients with mutation-positive tumors by both methods; low abundance of EGFR (group L, N=18), i.e. those patients with mutation positive by ARMS but negative by sequencing; and WT group (group W, N=31), i.e., those patients with mutation-negative tumors by both methods. In group L, tumors were also tested for *KRAS*, *BRAF*, *PI3KCA*, *ALK*, and *cMET*, in order to exclude their potential effect on the benefit from gefitinib treatment, and

no alteration was detected in 10/18 patients. Median PFS of patients in group H (11.3 months, 95%CI 7.4-15.2) was significantly longer than that in group L (6.9 months, 95%CI, 5.5-8.4; P=0.014). Additionally, median PFS of patients in group L was significantly longer than that in group W (2.1 months, 95%CI 1.0-3.2; P=0.010). This study provided the first evidence that the relative abundance of *EGFR* mutations could predict the extent of benefit from EGFR-TKI therapy.

A subsequent study from Ono et al. assessed the predictive implications of the MAF of a point mutation at codon 858 in exon 21 (L858R) for the usefulness of treatment with EGFR-TKIs (gefitinib and erlotinib) in 29 patients with advanced mutant EGFR lung adenocarcinoma[218]. EGFR mutation was analyzed by pyrosequencing, Cycleave and ARMS. The MAF for L858R was 18.5% (range 8-82%). The authors found that the ORR to EGFR-TKI was significantly higher in the group with MAF >9% (79.1%, N=24) than in the group with a MAF of ≤9% (20%, N=5; P=0.022). Also, PFS was longer in the group with MAF >9% than in the group with MAF of ≤9% (92 vs. 284 days, P=0.0027).

Conversely, the presence of subclonal *KRAS* mutations may reduce the clinical benefit of cetuximab in CRC[219]. For instance, Laurent-Puig et al. studied the role of minor mutant *KRAS* subclones in patients with advanced CRC treated with anti-EGFR antibodies in the resistance to these drugs[220]. They used an ultra-sensitive method, picoliter multiplex droplet PCR (dPCR), to detect mutant *KRAS* subclones among 136 patients WT for *KRAS*, *NRAS*, and *BRAF*, and 41 patients with *KRAS*-mutant tumors – all treated with anti-EGFR agents. The use of dPCR allowed for the detection of 22 additional tumors with *KRAS* mutation (median MAF 0.04%, range 0.01%-12.4%), and 2 with BRAF mutation (MAF of 6.9% and 0.07%). The authors observed an inverse correlation between the proportion of mutant DNA and the frequency of anti-EGFR response (P < 0.001). Considering a cutoff value for *EGFR* mutation MAF of 1%, only 2/46 patients (4.3%) with >1% of KRAS-mutant allele were responders as compared with 62.5% of the patients with 1% or less of KRAS-mutant allele. This association with response was translated in terms of PFS and OS. When comparing patients with WT tumors or with tumors presenting ≤1% of a mutant allele with patients with tumor with >1% of mutant

allele, the HRs were 3.2 (95%CI 1.3-7.9; P=0.014) and 4.7 (95%CI 1.6-14.3; P=0.006) for PFS respectively, and 4.1 (95%CI 1.6-10.4; P=0.003) and 5.6 (95%CI 1.8-16.8; P=0.002) for OS.

In another study, Azuara et al. sought to evaluate the added value of using dPCR to analyze a panel of 34 hotspots, including *RAS* (*KRAS* and *NRAS* exons 2/3/4) and *BRAF* (V600E), in tumor FFPE samples from 102 metastatic CRC patients treated with anti-EGFR therapy[221]. Frequency of mutations by conventional techniques was 22%, and using dPCR the number of patients bearing mutations increased to 47%. Interestingly, dPCR identified multiple additional low-frequency mutant alleles in 12 cases. The percentage of mutant alleles in this set of 26 patients detected only by dPCR ranged from 0.04% to 10.8% (median 1.26%). An inverse correlation between the fraction of mutant alleles and radiologic response was observed (P<0.005). Consistent with the previous study, ROC analysis showed that a fraction of 1% or higher of any mutant alleles offered the best predictive value for all combinations of RAS and BRAF analysis, and optimized prediction of both PFS and OS to EGFR-inhibitors.

It could be speculated that not only MAF, but also the level of gene amplification, could influence the sensitivity to targeted therapy. Using data from a translational clinical trial to assess whether cancers with amplification of *FGFR1* or *FGFR2* respond to the selective FGFR inhibitor AZD4547[222], Pearson et al correlated the levels of amplification of each gene with response to the targeted agent. They found that gastric cancers with high-level clonal *FGFR2* amplification (ratio FGFR2:CEP10 ≥5.0) had a high response rate to AZD4547, whereas cancers with subclonal or low-level amplification did not respond[223]. In contrast to this observation, it had previously been described that the level of *ERBB2* amplification does not affect sensitivity to the HER2-targeting antibody trastuzumab[224], which highlights the need to learn the underlying biology of each molecular alteration. Importantly, Pearson et al. also described the mechanistic model behind their observation. They showed that high-level *FGFR2* amplification initiates a distinct oncogene addiction phenotype, characterized by *FGFR2*-mediated transactivation of alternative receptor kinases, bringing PI3K/mTOR signaling under FGFR

control. In contrast, in low-level *FGFR1*-amplified cancers PI3K/mTOR signaling is not blocked by FGFR inhibition, which limits the sensitivity of these cell lines to FGFR inhibition. We did not analyze whether the levels of amplification of *FGFR1* or *FGFR2* were predictive of benefit to FGFR inhibitors since this is outside of the scope of our study.

Among our 15 patients with *PIK3CA* mutation, with information regarding MAF, and treated with a PI3Kα-specific inhibitor, patients with clonal *PIK3CA* had numerically higher TTF than those with subclonal *PIK3CA* (11.4 vs. 5.5 months), but this difference did not reach statistical significance (HR 0.57, 95%CI 0.16-2.01; P=0.380). This finding, in contrast to the studies previously described, may have several explanations. The most likely explanation is that the very small sample size in our study (15 patients, 9 with clonal and 6 with subclonal *PIK3CA*) is not enough to give statistical power for the comparison. This is suggested by the wide 95%CI associated with an HR of 0.57. If the sample size is increased, a statistically significant difference may emerge. Another explanation is that clonality of *PIK3CA* is indeed not a determinant of response to PI3Kα-specific inhibitors. There are some studies that suggest that PAM pathway activation signatures, rather than the presence of *PIK3CA* mutation *per se*, is determinant to the response to other PAM pathway inhibitors such as everolimus[225,226]. If this were the case, the relative abundance of *PIK3CA* mutant clones would have no influence on the treatment with PAM pathway inhibitors whatsoever, unless it also correlated with PAM pathway activation as measured by RNA or RPPA signatures. It must be noted that in our series, *PIK3CA* mutation correlates with better CBR and marginally with better TTF to PI3Kα-specific inhibitors, and is an independent predictor of benefit to PAM pathway inhibitors in general, so we believe these findings do not support the above explanation. Finally, the cut-off we used to define clonality may have missed a significant benefit of PI3Kα-specific inhibitors. However, with so small numbers, it was not possible to test other cut-offs. Nonetheless, this is an issue that merits further investigation.

### 7.8. Summary of findings and future directions

This study had two main objectives: characterize the prevalence of molecular alterations in a cohort of MBC patients treated in our Institution, and assess predictive factors of efficacy to PAM pathway inhibitors in our population.

We have shown that the prevalence of molecular alterations in our cohort is similar to that previously reported in the literature (with *TP53* mutation being the most frequent alteration, followed by *PIK3CA* mutation), and have characterized the distribution of molecular alterations across breast cancer subtypes. We have also evidenced that additional alterations in the PAM pathway – such as PTEN dysregulation (either by the presence of missense mutations or null expression by IHC) and *AKT1* mutation – are frequent in breast cancer. We have identified samples with two or more actionable alterations and discussed the potential therapeutic implications of this finding. We have also described the differences between the alterations that were detected in primary tumor samples and metastatic samples. These were not paired biopsies, and hence conclusions must be cautious. Although the differences were not statistically significant, we have found a trend for *ESR1*, *ERBB2*, and *PIK3CA* mutation to be more frequently detected when the sample came from a metastatic lesion.

We have described that both *PIK3CA* and *AKT1* mutations are clonal events in our population. To the best of our knowledge, this analysis has not previously been performed in samples coming from a clinical setting in breast cancer. Interestingly, we have found that *PIK3CA* mutation was a subclonal event when detected in a metastatic sample, and have discussed the potential implications of this finding.

We have presented the rate of enrollment in clinical trials among the patients tested for molecular alterations, and have discussed the reasons for the generally higher numbers in our Institution as compared to other series.

We then focused on the outcomes of the patients treated with PAM pathway inhibitors and analyzed putative predictive factors for clinical benefit to these

agents. We found that patients with *PIK3CA* mutation, treated early in their course of metastatic disease in trials testing combinations with endocrine or chemotherapy had better outcomes than the rest.

Finally, given the previous results in other tumor types such as NSCLC and CRC[217,220], we studied whether the clonality of *PIK3CA* mutation was predictive of benefit to treatment with PI3Kα-specific inhibitors. To the best of our knowledge this issue had not previously been explored in MBC. In our series we found no such association and have discussed potential reasons for these results.

The introduction of NGS in clinical practice has allowed the routine genomic study of a patient's tumor at the point of care, bringing important advances in the clinical management of breast cancer patients and translational research. Crucially, a good annotation of the variables that are found in routine genotyping with NGS will better distinguish between actionable variations and passenger alterations with no biological importance. Several efforts are ongoing to facilitate the clinical interpretation of variants. Some examples include the Gene Drug Knowledge Database[227], the Database of Curated Mutations[228], ClinVar[229], ClinGen[230], PharmGKB[231], OncoKB (http://oncokb.org), My Cancer Genome[232], and CiVIC[233], among others. These initiatives are important to narrow the gap between the identification of increasing numbers of somatic mutations and the scarcity of biological or clinical information – and validation – for each of those alterations, as well as translating this biological information into better patient care.

Despite all the genotyping efforts, there are relatively few actionable mutations in breast cancer (*PIK3CA*, *AKT1*, *ERBB2*, *ESR1*), and the response to targeted agents towards those alterations, although promising, is less than ideal. Moving forward, it will be important to expand the druggable genome in breast cancer, either by identifying additional actionable missense mutations, or by identifying other targetable alterations, such as driver copy number alterations or gene fusions. The latter have dramatically changed the natural history of NSCLC (ALK and ROS1 translocations[17,234]), and sarcomas and other tumor types (NTRK translocations[235]). The introduction of NGS techniques in the clinical setting may

provide us with knowledge about these and other alterations in breast cancer, ultimately leading to improvement to the treatment and care of patients.

Despite acknowledging that the mutational landscape of the metastatic disease is not identical to that of the primary tumor, not all patients are amenable to undergoing a biopsy from a metastatic site. It is therefore important to implement the genotyping of the so-called “liquid biopsies” in the clinic; a test that only requires a blood draw, and can be repeated as needed, with little or no risk to patients. Liquid biopsies include ctDNA, circulating tumor cells (CTCs), exosomes, and other circulating biomarkers. ctDNA may better capture tumor heterogeneity by detecting mutations that are both shared and private to metastasis sites, and can also be used to monitor response to targeted therapy[236]. We envision that the routine use of ctDNA will change clinical care of MBC patients.

Another area of required improvement is the accurate identification of those patients who will benefit from targeted agents. The quest for biomarkers of response to novel drugs seems a long and hard path, but there are some tools that may help us our endeavors. As postulated by Hyman et al. in a recent manuscript, clinical studies evaluating a genomic-driven hypotheses should be designed to learn from each case in an unprecedented way[237]. This includes a systematic analysis of patient-derived biospecimens and potential clinical strategies to overcome adaptive or acquired resistance in real-time. The authors propose that the hallmarks of a modern precision-oncology study should include the following primary scientific objectives: 1) Identification of the target, by collection of pre-treatment biopsy and ctDNA; 2) Confirmation of target inhibition, with collection of an on-treatment biopsy, liquid biopsy (ctDNA, exosomes, circulating tumor cells), and functional imaging with 18F-PET-CT; 3) Biologic target validation, through the generation of patient-derived xenograft (PDX) models or organoids at the time of tumor biopsies; and 4) Description of the mechanisms underlying acquired resistance, by comparative analysis of pre- and post-treatment tumor biopsies (using DNA and RNA-sequencing, phosphoprotein analysis, and immune profiling), acquisition of liquid biopsies upon progression, generation of PDX models with the progressing biopsy, or by performing rapid

autopsies in those patients who ultimately succumb to their disease. While such an effort can only be done in specialized and dedicated Cancer Centers, the analysis and integration of huge amounts of data that can potentially come from all this biological material will certainly help to inform regarding biomarkers to response and resistance to targeted agents.

As a final remark, all that we have discussed until now relates mainly to DNA changes and their influence in cancer genesis, progression, and response to targeted agents. This was the main focus of our study. However, an increasing body of evidence suggests that other factors such as immune-mediated phenomena, epigenetic changes, and metabolic dysregulations are essential in the development of breast cancer and response to therapy[238-240]. An integrative approach of genomic oncology, immunogenomics, epigenomics, and metabolomics will be key to understanding, and hence target, additional susceptibilities in breast cancer.

# 8
# Conclusions

# 8. Conclusions

1. Molecular alterations are frequent events in MBC patients treated at our Institution. The most common molecular alteration across subtypes is *TP53* mutation, followed by *PIK3CA* mutation.
2. PAM pathway dysregulation is frequent in MBC, and *PIK3CA* mutation is the most common alteration leading to PAM pathway dysregulation.
3. The most common actionable alteration in LUM and HER2+ breast cancer on top of ER/PgR expression and *ERBB2* amplification, respectively, is *PIK3CA* mutation. In TN, the most common actionable alteration is PTEN null expression, but most of the tumors have no actionable alteration.
4. There is a trend for more frequent detection of *ESR1*, *ERBB2*, and *PIK3CA* mutations in metastatic samples than in primary tumors.
5. Overall, *PIK3CA* mutation and *AKT1* mutation are clonal events in our population of MBC.
6. Co-occurrence of molecular alterations is an infrequent event in MBC, but if present can have relevant therapeutic implications.
7. Patients with actionable alterations are enrolled in clinical trials with matched agents more frequently at our Institution than in others, and the presence of a PAM pathway alteration increases the probability of enrollment in a trial with a PAM pathway inhibitor.
8. Patients with *PIK3CA* mutation, treated early during their metastatic disease in trials testing combinations with endocrine or chemotherapy, benefit most from PAM pathway inhibitors.
9. Although it associates with better response, *PIK3CA* mutation is not predictive of TTF to PI3Kα-specific inhibitors.
10. Clonality of *PIK3CA* mutation does not correlate with efficacy of PAM pathway inhibitors or PI3Kα-specific inhibitors.

# 9
# References

## 9. References


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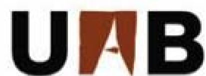
## Index of Abbreviations

| | |
|---|---|
| AE | Adverse Event |
| AI | Aromatase Inhibitor |
| Alt | Alteration |
| AR | Androgen Receptor |
| ARMS | Amplification Refractory Mutation System |
| BID | Twice a Day |
| BIG | Breast International Group |
| CBR | Clinical Benefit Rate |
| CGH | Comparative Genomic Hybridization |
| CI | Confidence Interval |
| CNA | Copy Number Aberration |
| CNS | Central Nervous System |
| CNV | Copy Number Variation |
| CR | Complete Response |
| CRC | Colorectal Cancer |
| CT | Chemotherapy |
| CTC | Circulating Tumor Cell |
| ctDNA | Circulating tumor DNA |
| DLT | Dose Limiting Toxicity |
| ddPCR | Digital droplet PCR |
| dPCR | droplet PCR |
| EBC | Early Breast Cancer |
| EBCTCG | Early Breast Cancer Trialists Collaborative Group |
| EGFR | Epidermal Growth Factor Receptor |
| ER | Estrogen Receptor |
| FFPE | Formalin-Fixed Paraffin Embedded |
| GCIG | Gynecologic Cancer InterGroup |
| HER2 | Human Epidermal growth factor Receptor 2 |
| HER2-E | HER2-Enriched |
| HR | Hazard Ratio |
| ICGC | International Cancer Genome Consortium |
| IDC | Invasive Ductal Carcinoma |
| IHC | Immunohistochemistry |
| ILC | Invasive Lobular Carcinoma |
| INPP4B | Inositol polyphosphate-4-phosphatase |
| IntClust | Internal Cluster |
| IQR | Interquartile range |
| IRS1 | Insulin receptor substrate 1 |
| ITT | Intention To Treat |
| LUM | Luminal |

| | |
|---|---|
| MAF | Mutant Allele Fraction |
| MBC | Metastatic Breast Cancer |
| MDACC | MD Anderson Cancer Center |
| MGH | Mass General Hospital |
| mRNA | messenger RNA |
| MTD | Maximum Tolerated Dose |
| mTOR | mammalian target of rapamycin |
| MUT | Mutant |
| NGS | Next Generation Sequencing |
| NSCLC | Non-small Cell Lung Cancer |
| OR | Odds Ratio |
| ORR | Overall Response Rate |
| OS | Overall Survival |
| PAM | PI3K/AKT/mTOR |
| PCR | Polymerase Chain Reaction |
| PD | Progressive Disease |
| PDK1 | 3-phosphoinositide dependent protein kinase 1 |
| PDX | Patient-derived xenograft |
| PFS | Progression Free Survival |
| PgR | Progesterone Receptor |
| PI3K | Phosphatidylinositol-3-kinases |
| PIP2 or PIP(4,5)2 | Phosphatidylinositol 4,5-bisphosphate |
| PIP3 or PIP(3,4,5)3 | Phosphatidylinositol (3,4,5)-trisphosphate |
| PR | Partial Response |
| PTEN | Phosphatase and tensin homolog in chromosome 10 |
| QD | Once a day |
| RECIST | Response Evaluation Criteria In Solid Tumors |
| RP2D | Recommended Phase 2 Dose |
| RPPA | Reverse Protein Phase Array |
| RR | Relative Risk |
| RTK | Receptor Tyrosine Kinase |
| SD | Stable Disease |
| TCGA | The Cancer Genome Atlas |
| TKI | Tyrosine-Kinase Inhibitor |
| TN | Triple Negative |
| TNBC | Triple Negative Breast Cancer |
| TPC | Treatment of Physician's Choice |
| TTP | Time to Progression |
| US | United States |
| VPS34 | Vacuolar Sorting-associated Protein 34 |
| WES | Whole-exome Sequencing |
| WT | Wild Type |

TESIS DOCTORAL

# MOLECULAR ALTERATIONS IN METASTATIC BREAST CANCER AND EFFICACY OF PI3K/AKT/MTOR INHIBITORS IN EARLY PHASE CLINICAL TRIALS

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