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Programa de doctorado en Medicina 3042

TESIS DOCTORAL

Estudio de las alteraciones genéticas asociadas con la resistencia al tratamiento neoadjuvante de deprivación estrogénica en cáncer de mama

D. ÁNGEL LUIS GUERRERO ZOTANO

DIRIGIDA POR:

DRA. DÑA. ANA LLUCH HERNÁNDEZ

DR. D. VICENTE GUILLEM PORTA

DR. D. JOSÉ ANTONIO LÓPEZ GUERRERO

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Doctorando:

D. Ángel Luis Guerrero Zotano

Directores:

Dr. Dña. Ana Lluch Hernández

Dr. D. Vicente Guillem Porta

Dr. D. José Antonio López Guerrero

Dña. Ana Lluch Hernández, Doctora en Medicina y Catedrática del Departamento de Medicina de la Universidad de Valencia. Hemato-oncóloga y jefa del Servicio de Hematología y Oncología del Hospital Clínico Universitario de Valencia.

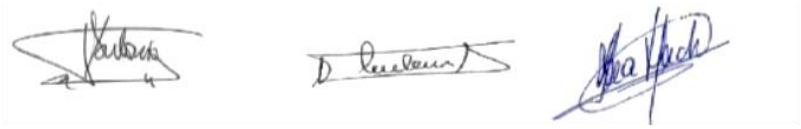
D. Vicente Guillem Porta, Doctor en Medicina. Jefe del Servicio de Oncología Médica de la Fundación Instituto Valenciano de Oncología.

D. José Antonio López Guerrero, Doctor Biología. Jefe del Servicio de Biología Molecular de la Fundación Instituto Valenciano de Oncología.

CERTIFICAN:

Que la presente memoria**, "Estudio de las alteraciones genéticas asociadas con la resistencia al tratamiento neoadjuvante de deprivación estrogénica en cáncer de mama",** corresponde al trabajo realizado bajo su dirección por D. Ángel Luis Guerrero Zotano y constituye su Tesis para optar al grado de Doctor.

Y para que conste y en cumplimiento de la legislación vigente, firman el presente certificado en Valencia, a 23 de Marzo de 2020.



Fdo: Dr. D. Jose A. López Guerrero Fdo: Dr.D. Vicente Guillem Fdo: Dra. Dña Ana Lluch

A mi padre, que fue un hombre bueno, honrado, trabajador,humilde y luchador. Me enseñaste con tu ejemplo el valor del trabajo diario, de las cosas terminadas y bien hechas. Papá te quiero mucho, me acuerdo de ti todos los días. Gracias papa por haber cuidado de nosotros, haberte preocupado de que estudiaramos. Gracias papá por la infancia y la educación que he tenido.

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American Joint Committee On Cancer	AJCC
Aromatase Inhibitor	AI
Bicinchoninic Acid Assay	BCA
CDK4/6 Inhibitors	CDK4/6i
cDNA	Complementary DNA
Chemotherapy	CT
Chromatin Immunoprecipitation	ChiP
Circulating Tumor DNA	ctDNA
Complete Cell Cycle Arrest	CCCA
Copy Number Alteration	CNA
Differentially Expressed	DE
Disease-Free Survival	DFS
DNA Binding Domain	DBD
Double Strand DNA	dsDNA
Ductal Carcinoma In Situ	DCIS
EIF4E Binding Protein 1	4E-BP1
Endocrine Therapy	ET
Estrogen Receptor	ER
Estrogen Receptor Element	ERE
False Discovery Rate	FDR
Fold Change	FC
Formalin-Fixed Paraffin-Embedded	FFPE
Gene Ontology	GO

Genetically Engineered Mice	GEM
Growth Factor Receptor	GFR
Grupo Español De Investigación En Cáncer De Mama	GEICAM
Hazard Ratio	HR
Heat Shock Proteins	HSP
High Developing Index	HDI
Hormone Receptor	HR
Human Epidermal Growth Factor Receptor 2	HER2
Immunohistochemistry	IHQ
Invasive Ductal Carcinomas Not Otherwise Specified	IDC-NOS
Ligand-Binding Domain	LBD
Long Term Estrogen Deprived	LTED
Low Developing Index	LDI
Luminal A	Lum-A
Luminal B	Lum-B
Major Histocompatibility Complex	MHC
Menopausal Hormone Therapy	MHT
Molecular Taxonomy Of Breast Cancer International Consortium	METABRIC
Neoadjuvant Chemotherapy	NAC
Neoadjuvant Endocrine Therapy	NET
Next Gen Sequencing	NGS
Organisation For Economic Co-Operation And Development	OECD
Ovarian Function Suppression	OFS
Overall Survival	OS
Pathologic Complete Response	pCR

Phospahete Buffer Salin	PBS
Phosphoinositide 3-Kinases	PI3K
Polyvinylidene Difluoride	PVDF
Prediction Analysis Of Microarray 50	PAM50
Preoperative Endocrine Prognostic Index	PEPI
Principal Component	PC
Progesterone Receptor	PgR
Programmed Death-Ligand 1	PD-L1
Ras Homolog Enriched In Brain	RHEB
Razón De Mortalidad Estandarizada Suavizada	RMES
Recurrence Score	RS
Residual Cancer Burden	RCB
Ribosomal Protein S6 Kinase	S6K1
Risk Of Recurrence	ROR
Short Hairpin RNA	shRNA
Single Nucleotide Variant	SNV
Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis	SDS-PAGE
The Cancer Genome Atlas	TCGA
Triple Negative	TPN
Tris Buffered Saline With Tween 20	TBST
Tumor Infiltrating Lymphocytes	TILs
Tumor, Node, Metastasis	TNM Staging System
Whole Genome Sequencing	WGS

1 .INTRODUCTION

1.1 Breast cancer overview

1.1.1 Cancer epidemiology in the World

Cancer remains one of the leading causes of morbidity and mortality in the world. According to the latest available data estimated within the GLOBOCAN project, the number of tumors continues to grow, having increased from the estimated 14 million cases in the world in 2012 to 18.1 million in 2018. Population estimates indicate that the number of new cases will increase in the next two decades, reaching 29.5 million in 2040. The most frequently diagnosed tumors in the world in 2018 were lung, breast, colon and rectum, prostate and stomach. One in 5 men and one in 6 women worldwide develop cancer during their lifetime, and one in 8 men and one in 11 women die from the disease. Worldwide, the total number of people who are alive within 5 years of a cancer diagnosis, called the 5-year prevalence, is estimated to be 43.8 million (1) **[Figure 1]**.

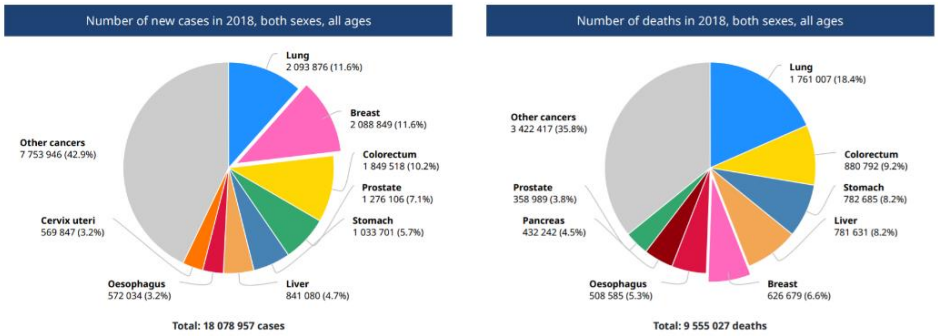


Figure 1: Estimated number of new cases and death in 2018, worldwide, all cancer, both sexes all ages. Source GLOBOCAN 2018

1.1.2 Breast cancer epidemiology in the world

Breast cancer is currently the most frequent tumor in the female population, both in High Developing Index (HDI) and Low Developing Index (LDI) countries. In 2018, an estimated 2.1 million women were newly diagnosed with breast cancer worldwide, and 626,679 women with breast cancer died (1) **[Figure 2,Figure 3].** The global incidence of breast cancer has been rising with annual increases of 3.1%, beginning with 641,000 cases in 1980 and increasing to >1.6 million in 2010 (2). Indeed, the global cancer burden in women is increasing in countries regardless of income level, owing to population growth and an ageing population. Incidence varies worldwide, with higher incidence in HDI regions (92 per 100,000 in North America) than in LDI regions (27 per 100,000 in middle Africa and eastern Asia) . Despite lower incidence of breast cancer in LDI countries, mortality is higher. The estimated age-standardized breast cancer mortality rates per 100000 habitants in the World in 2018 was 17.1 and 10.3 for HDI and LDI countries, respectively. This difference could be attributed to due to delayed presentation, late stage at diagnosis and limited access to treatment **[Figure 4].**

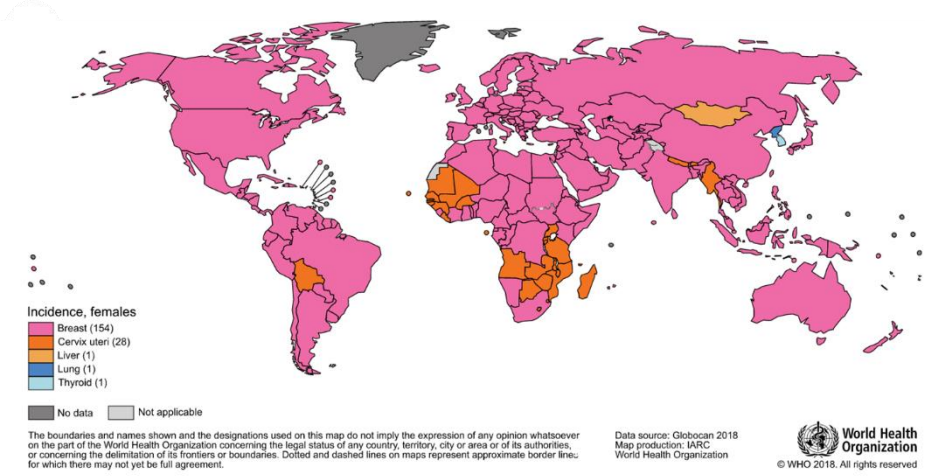


Figure 2: Global map presenting the most common type of cancer incidence in 2018 in each country among women. The numbers of countries represented in each ranking group are included in the legend. Source: GLOBOCAN 2018.

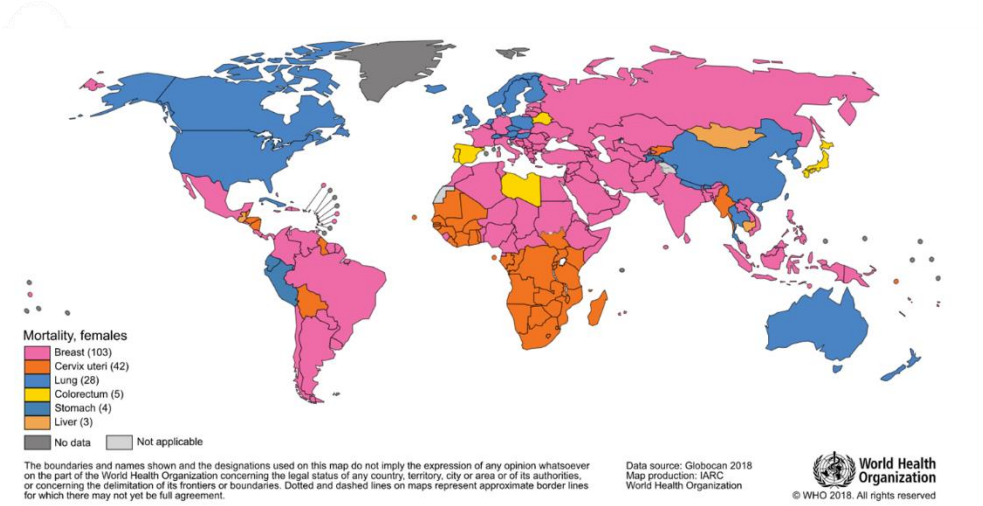
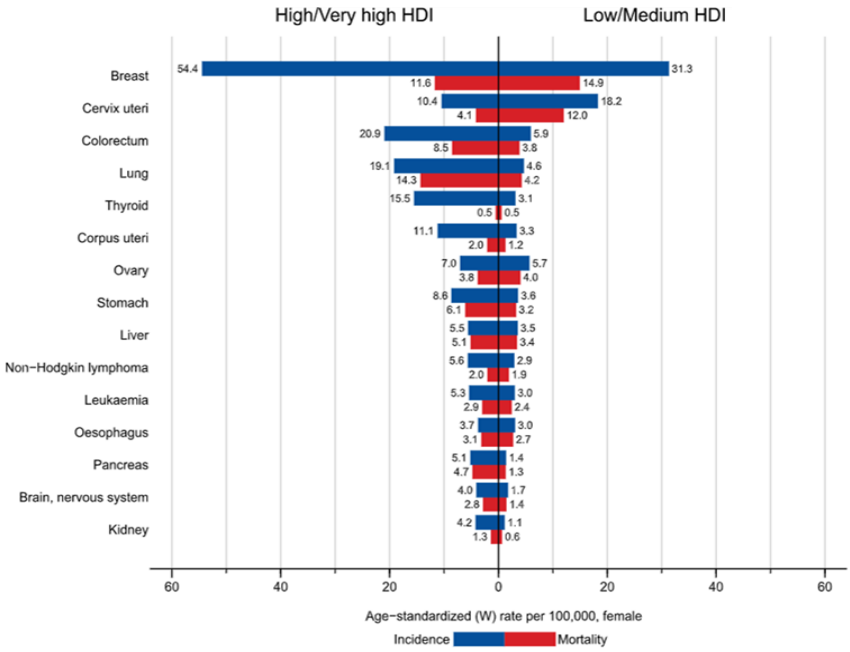


Figure 3: Global map presenting the most common type of cancer mortality by country in 2018 among women. the numbers of countries represented in each ranking group are included in the legend. source: GLOBOCAN 2018.



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1.1.3 Breast cancer epidemiology in Spain

The incidence of breast cancer in Spain is high (75.4 per 100000 habitants in 2018) but it is lower than in the United Kingdom, the Netherlands, Belgium, Germany, France and Switzerland and is similar to the rest of the countries of Mediterranean Europe, Central Europe, Portugal and Ireland **[Figure 5].** In Spain about 30,000 cases are diagnosed per year, which represents almost 30% of all female tumors in our country and the most incident and prevalent tumor among women. Most cases are diagnosed between 35 and 80 years, with a maximum between 45 and 65. It is estimated that 1 out of 8 women will be diagnosed of breast cancer during its lifetime in Spain. As other HDI countries, Spain has a 30% to 40% increase in breast cancer incidence since the 1990s (3). This rise has been related to the spread of environmental and lifestyle risk factors, hormone replacement therapy and to changes in diagnostic patterns. From 2001 and onwards a decline was observed among women aged > or =45 years (4). Despite the high incidence of breast cancer, in 2018 Spain had one of the lowest age standardized mortality rate in the European region and in the world (10.6 per 100000 habitants) **[Figure 6]**. In our country, breast cancer is the 4th leading cause of death (6000 deaths in 2018), behind lung, colorectal, and pancreas. There is some geographical variation in breast cancer mortality that is not fully understood. Although there´s a trend toward equilibrium, some regions of Spain such as west Andalucía and Levante region show higher mortality rate than expected for breast cancer **[Figure 7].**

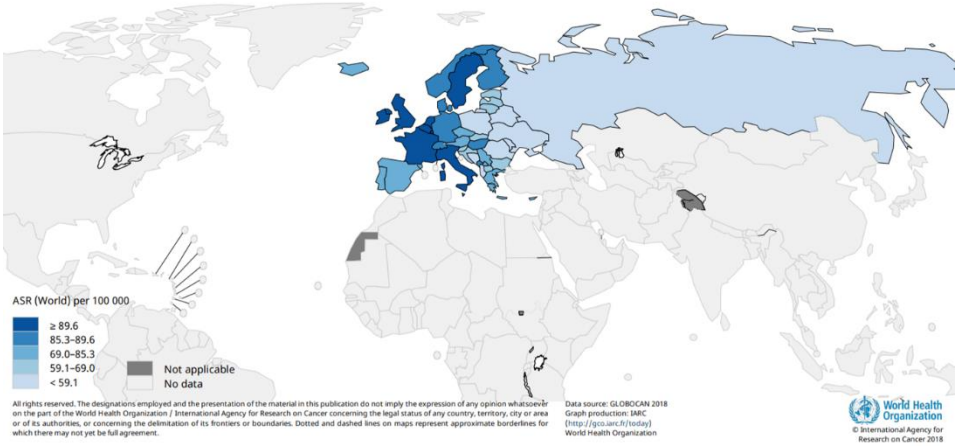
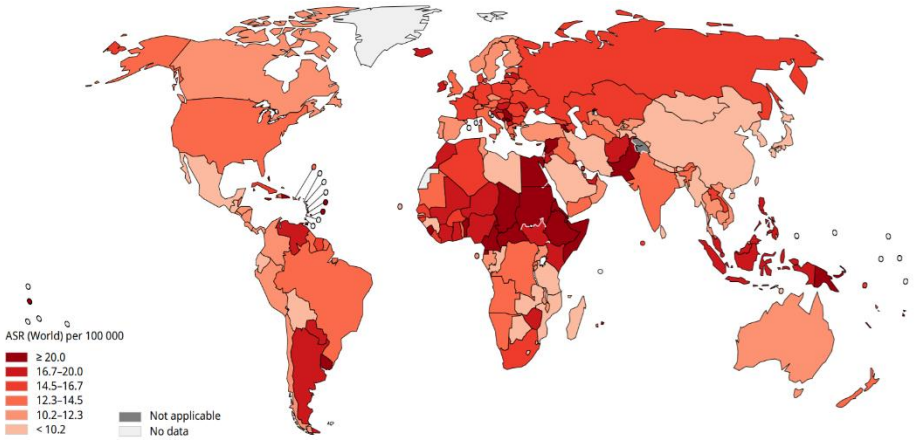


Figure 5: Estimated age-standardized breast cancer incidence rates (Europe) all ages, both sexes combined. Source: GLOBOCAN 2018.



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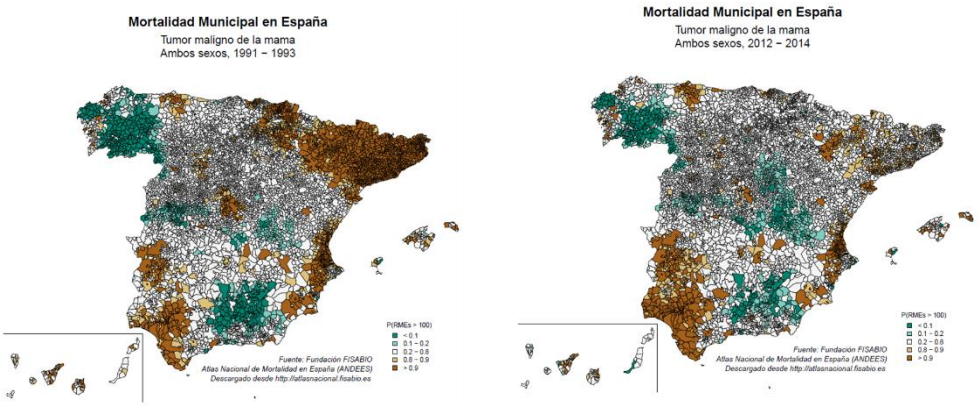

Figure 7: Spain mortality municipality map by breast cancer in two periods (1991-1993) and (2012-2014). The probability that the RMEs of each municipality is greater than 100, called probability of excess risk (Prob (RMEs> 100) is presented. This indicator quantifies how confident we can be that the excess risk that a certain RMEs could point to is really relevant, and not the product of some over-observed death, which could have been taken simply by chance. RMEs: Razón de Mortalidad Estandarizada suavizada. P(RMEs>100): probabilidad de exceso de riesgo. Source: Atlas Nacional de Mortalidad en España (ANDEES).
https://medea3.shinyapps.io/atlas_nacional/

1.1.4 Risk factors for breast cancer

Several risk factors for breast cancer have been well documented. However, for most women presenting with breast cancer it is not possible to identify specific risk factors.

1.1.4.1 Genetic predisposition

Approximately 10% of breast cancers are inherited and associated with a family history. A familial history of breast cancer increases the risk by a factor of two or three, however, in patients with a suggestive personal and/or family history, a specific predisposing gene is identified in less than 30% of cases. Up to 25% of

hereditary cases are due to a mutation in one of the few identified rare, but highly penetrant genes (*BRCA1*, *BRCA2*, *PTEN*, *TP53*, *CDH1*, and *STK11*), which confer up to an 80% lifetime risk of breast cancer. An additional 2%-3% of cases are due to a mutation in a rare, moderate-penetrance gene (e.g. *CHEK2, BRIP1, ATM, and PALB2*), each associated with a twofold increase in risk (5).

1.1.4.2 Lifestyle and other environmental factors

Breast cancer epidemiology pattern differences across countries are attributed to reproductive patterns, lifestyle factors and environment factors that have associations with breast cancer and could be causal. Reproductive factors associated with prolonged exposure to endogenous estrogens, such as early menarche, late menopause, late age at first childbirth are among the most important risk factors for breast cancer. It has been estimated that ~20% of breast cancers worldwide can be attributed to modifiable risk factors, including obesity, physical inactivity and alcohol use (6), offering the potential for reduction in the disease burden by promoting a healthy lifestyle.

1.1.4.3 Reproductive factors

A full-term pregnancy reduces the likelihood of developing breast cancer by 50% compared to nulliparous women (7). Subsequent pregnancies extend the protection against breast cancer by ~10% (8). However, this protective effect of pregnancy is in women who have their first pregnancy between the ages of 30–34 years and is augmented for those whose first pregnancy occurs after age 35 (9,10). Breastfeeding has a protective effect against breast cancer. A review of 32 studies showed that the risk of having breast cancer was 14% lower among parous women who had ever breastfed compared with parous women who never breastfed. The protective effect of breastfeeding persisted regardless of the number of births and was even greater for women who had cumulatively breastfed for 12 months or longer; they had a 28% lower risk of breast cancer (11). Although early pregnancy and breastfeeding

protection is associated with risk reduction for breast cancer, breast cancer diagnosed within the first 5 years after giving birth have a worse clinical prognosis, with increased risk of metastases and mortality compared to nulliparous cases (12,13).

1.1.4.4 Exogenous hormones

Menopausal hormone therapy (MHT) is associated with an increased risk of breast cancer. MHT has been used mostly in western countries (14,15). Use of MHT fell after the reports from the Women's Health Initiative studies linking MHT with breast cancer risk, and this was followed by a decline in the incidence of breast cancer (16). Compared with never users, women who initiate MHT shortly after menopause has a significantly increased risk of invasive breast cancer. The longer women use MHT the greater the risk; MHT is associated with an absolute increase of 2%, or one extra cancer for every 50 users (17).

Hormonal contraception has also been associated with risk of breast cancer. Women who uses or has just stopped using hormonal contraception have an increase of 20% in the risk of being diagnosed of breast cancer compared to women who had never used hormonal contraception. The risk varies between 0-60% among the various estrogen-progestins combinations. The risk is higher for women taking it 5 years or more. The overall absolute increase risk in breast cancer is approximately 1 extra breast cancer for every 7690 women using hormonal contraception for 1 year (18,19).

1.1.4.5 Obesity and physical inactivity

Obesity is the most important modifiable risk factor contributor to breast cancer. The proportion of breast cancer attributable to obesity is higher in HDI countries (27%) than in LDI countries (18%) **[Figure 8].** Obesity defined as a body mass index ≥30 kg/m^2, affects over 600 million adults worldwide, or 13% of the world population. Obesity is increasingly becoming a worldwide epidemic, with global obesity

rates nearly tripling since 1975. In United States 45% of the population is expected to be obese by 2030. In Spain, rates of obesity are below 15%, but are expected to increase and more than 20% of Spanish population will be obese by 2030 (20) **[Figure 9].** Obesity is associated both with a higher risk of developing breast cancer, particularly in postmenopausal women, and with worse disease outcome for women of all ages. Obesity is particularly associated with estrogen receptor (ER) positive breast cancer in postmenopausal women. This is presumably due to accumulation of estrogen in the adipose tissue of the breast and other tissues. Adipose tissue harbors multiple molecular entities that promote carcinogenesis: endocrine molecules/hormones, immunologic factors, inflammatory cytokines, metabolic alterations, and other components of the microenvironment (21). Many studies have shown that physically active women have a lower risk of breast cancer than inactive women. Meta-analysis has shown that active women have a 12–21% lower risk of breast cancer than those least physically active. Physical activity has been associated with similar reductions in risk of breast cancer among both premenopausal and postmenopausal women (22,23).

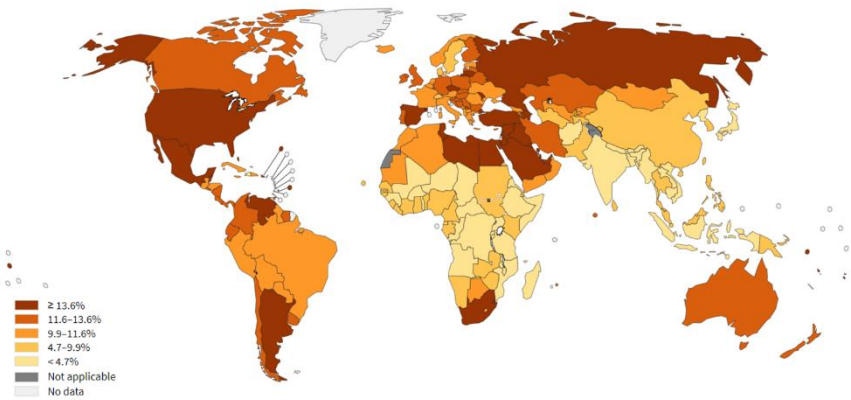

Figure 8: Cancer attributable to obesity: Fraction of all postmenopausal breast cancer among females (worldview) in 2012 attributable to excess body mass index. Source: International Agency for Research on Cancer. Global Cancer Observatory.

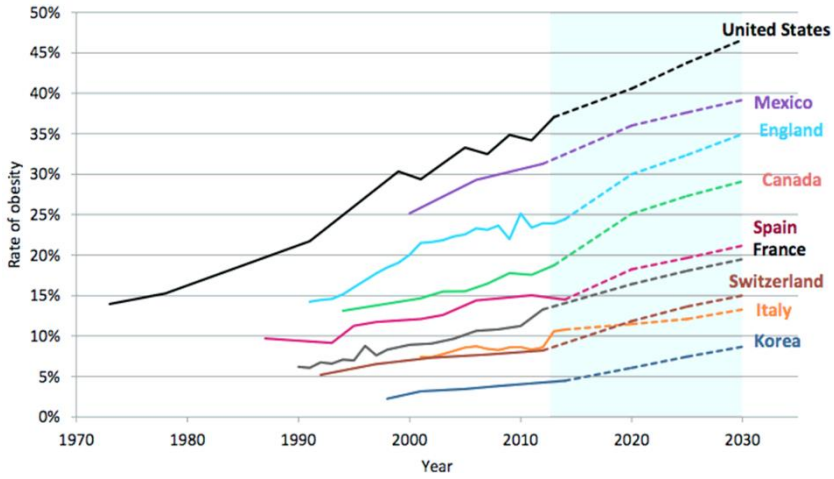

Figure 9: Obesity projection rates in selected countries. Source: Organisation for Economic Co-operation and Development (OECD) analysis of national health survey data.

1.1.4.6 Alcohol

Numerous epidemiologic studies show that moderate alcohol consumption increase the risk of breast cancer. The association of breast cancer risk and alcohol is linear; the relative risk of increase by 30% for those women with intake of 35-44 g/day (approximately 3-4 drinks per day) compared to women who don't drink at all (24). Alcohol may increase breast cancer risk by several mechanisms including mutagenesis by acetaldehyde, perturbation of estrogen metabolism and response, and by inducing oxidative damage (25).

1.2 Breast cancer biology

1.2.1 Breast carcinogenesis and genomic alterations in breast cancer

The exact mechanisms of how a breast cancer begins is unknown. The epithelial cells that line the lobules or ducts are the predominant site for breast cancer initiation. These first detectable lesions are neoplastic growths confined within individual ducts, considered pre-invasive and termed ductal carcinoma *in situ* (DCIS). DCIS is termed a non-obligate precursor to invasive cancer. Some DCIS progress to invasive carcinoma if penetrate the ductal basement membrane and invade the surrounding parenchyma. Genomic profiles of matched DCIS and adjacent invasive breast carcinomas are remarkably similar in terms of their copy number and mutation profile. However, within individual cases there is selection of populations of cancer with specific genetic alterations (26,27). The last stage in the progression of the disease is metastasis of the invasive cells. The current working model is that invasive cancer arises through a series of molecular alterations at the DNA level leading to activation of oncogenes and inactivation of tumor suppressor genes which results in uncontrolled proliferation and immortal features. The most frequently mutated and/or amplified genes in primary breast cancer are *TP53* (41% of tumors), *PIK3CA* (30%), *MYC* (20%), *PTEN* (16%), *CCND1* (16%), *ERBB2* (13%), *FGFR1* (11%) and *GATA3* (10%) (28). Somatic mutations in only three genes (*TP53, PIK3CA and GATA3*) occurred at >10% incidence across all breast cancers, so most breast cancers are caused by multiple, low-incident mutations that act cumulatively (28,29). The majority (~80%) of the driver alterations of the primary breast cancer are conserved in the metastatic sites. However, mutational process and therapeutic pressure can create a subclonal diversification (30) and discrepancies between mutational of primary breast cancer and metastasis and within different metastatic sites in a same patient can occur . Genomic alterations enriched in metastatic tumors include mutations in: *ESR1, FOXA1, NCOR1, AKT1, IGF1R, RB1, CDKN1B, KRAS, NF1, KMT2C, TP53.* Metastatic tumor have also an increase in

mutational genomic signatures such as APOBEC and mutational signatures related to homologous recombination deficiency, and a higher tumor mutation burden (31–33). *ESR1* mutations are a clear example of a mutation selected by therapeutic pressure. *ESR1* mutations are a rare phenomenon in early stage breast cancer, but in metastatic breast cancer treated with aromatase inhibitors (AIs), a drug that prevent conversion from androgens to estrogens, the incidence of *ESR1* mutations increase to 30-40% as a mechanism of resistance to these type of drugs (34). Many mutations acquired during the metastatic process are subclonal, that is, present only in a subset of the cancer population. Subclonal diversification may also explain the discrepancies observed between primary breast cancers and metastatic breast cancer for the expression of estrogen receptor (ER) (~20% discordance), progesterone receptor (PgR) (~33% discordance) and human epidermal growth factor receptor 2 (HER2) (~8% discordance). Thirteen percent of HER2-positive primary tumors generate HER2-negative metastases and 5% of HER2-negative primary tumors generate HER2-positive metastases, "unstable" HER2 status is associated with worse outcome and its implication in treatment strategies are currently unknown (35–37) .

Epigenetic alterations of the genome such as DNA promoter methylation and chromatin remodeling play an important role in breast cancer tumorigenesis. Epigenetic modifications regulate gene expression. Two main DNA methylation patterns are observed in breast cancer; 1) regional hypermethylation of specific genes (leading to gene suppression such as silencing of DNA repair genes) and 2) global hypomethylation, causing activation of oncogenes (38).

1.2.2 Tumor microenvironment in breast cancer

The tumor microenvironment is recognized as a critical element for breast cancer development and progression. The breast cancer microenvironment is composed mainly by cancer associated fibroblast, but also contain lymphocytes, macrophages and myeloid-derived stromal cells which are involved in the immune

response. The immune reaction to breast cancer is initiated by the neoantigens expressed by tumor cells, encoded by altered genes and presented by antigen-presenting cells on major histocompatibility complex class I or II molecules. In the early phase of tumorigenesis, these neoantigens provoke an immune antitumor response through activated lymphocytes (NK cells, CD4 and CD8 T cells). However, breast cancer eventually escapes the immune surveillance and tumor progress to invasive disease. Different mechanisms have been implicated in this immune evasion including loss of expression of immunostimulatory molecules, gain of expression of immunoinhibitory molecules such as PD-L1. PD-L1 interacts with $PD\text{-}1^{+}$ $CD8^{+}$ T cells and induces subsequent anergy/apoptosis, leading to inactivation or exhaustion of lymphocytes in the tumor microenvironment. Thus, the tumor microenvironment becomes immunosuppressive and incapable of stimulating a potent adaptive immune response (39–41). The amount of tumor infiltrating lymphocytes (TILs) reflects the immunogenicity of breast cancer. The level of TILs varies among breast cancer subtypes being higher in triple negative breast (TPN) cancer and HER2 subtype. Levels of TILs have prognostic and predictive significance in TPN and HER2 positive breast cancer, but not in ER+/HER2- breast cancer, suggesting that immune response is probably not a key aspect in ER positive breast cancer biology (42–44).

1.2.3 Breast cancer subtypes and classification

Breast cancer is a group of heterogeneous diseases that show substantial variation in their clinical and molecular characteristics. There are several methods to capture this heterogeneity which provide important prognostic and predictive information.

1.2.3.1 Histological subtypes and tumor grade

Most breast carcinomas (~70–80%) are described as invasive ductal carcinomas not otherwise specified (IDC-NOS) based on architectural patterns and cytological features. In contrast, around 25% of breast cancers are characterized according to 'histological special types' such as lobular, tubular, medullary and metaplastic carcinomas **[Figure 10]** (45). Tumor grade is an assessment of differentiation (tubule formation and nuclear pleomorphism) and proliferative activity (mitotic index), allowing tumors to be further stratified. Grade provides key prognostic information equivalent to that of lymph node status and greater than that of tumor **[Figure 11]** (46).

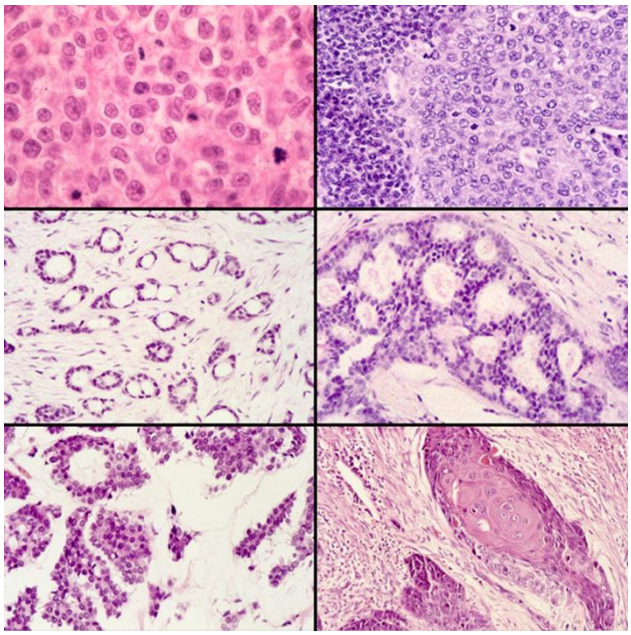

Figure 10: Main breast cancer histological types. From top left to bottom right: ductal NST, medullary, tubular, cribriform, mucinous and (squamous) metaplastic breast cancer. Taken from Atlas of Genetics and Cytogenetics in Oncology and Haematology

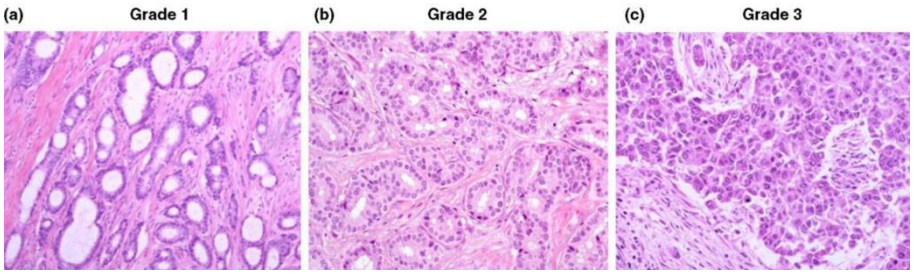

Figure 11: Histological grade of breast cancer as assessed by the Nottingham Grading System. **A)** A well-differentiated tumor (grade 1) that demonstrates high homology to the normal breast terminal duct lobular unit, tubule formation (>75%), a mild degree of nuclear pleomorphism, and low mitotic count. **B)** A moderately differentiated tumor (grade 2). **C)** A poorly differentiated (grade 3) tumor with a marked degree of cellular pleomorphism and frequent mitoses and no tubule formation (<10%). Taken from Atlas of Genetics and Cytogenetics in Oncology and Haematology.

1.2.3.2 Immunohistochemistry characterization of ER, PgR and HER2 status

In conjunction with histopathological assessment, the standard evaluation of breast cancer for clinical purposes involves immunohistochemistry (IHQ) characterization of ER, PgR and HER2 status. The presence or absence of molecular markers for ER or PgR and HER2, categorized breast cancer in 3 main subtypes: 1) hormone receptor (HR) positive/HER2 negative (70% of patients), 2) HER2 positive (15%-20%), and 3) triple-negative (tumors lacking all 3 standard molecular markers; 15%) (47).

1.2.3.3 Tumor, Node, Metastasis (TNM) staging system

The TNM staging system for breast cancer is an internationally accepted system used to determine the disease stage. The TNM staging system correlates important tumor characteristics with survival data to help estimate and follow

outcomes. Traditionally, the TNM staging was based exclusively on extent of cancer as defined by tumor size (T), lymph node status (N), and distant metastasis (M). In the 8th edition of the TNM staging, American Joint Committee on Cancer (AJCC) panel recognized the need to incorporate biologic factors, such as tumor grade, proliferation rate, ER and PgR expression, HER2 expression, and gene expression prognostic panels into the staging system. Several multigene expression signatures (Oncotype DX, EndoPredict, Prediction Analysis of Microarray 50 (PAM50), Breast Cancer Index, and urokinase plasminogen activator and plasminogen activator inhibitor type 1), have been validated to classified early stage breast cancer by its risk of relapse in specific subgroups of breast cancer (48). These gene signatures can guide on to decisions on adjuvant systemic therapy. Low risk scores given by any validated gene signature can be used regardless of the tumor size, to downstage HR positive, HER2 negative and lymph node-negative tumors, placing them into the same prognostic category as T1a-T1b N0 M0 carcinomas. As of this time, no upstaging is recommended based on multigene panel testing (49,50).

1.2.3.4 Breast cancer Intrinsic subtypes

Based on based on PAM50 mRNA gene expression signature, breast cancer can be classified in several distinct entities, known also as the intrinsic subtypes: luminal A (LumA), luminal B (LumB), HER2-enriched and basal-like (51). Intrinsic subtypes show differences in epidemiological factors, prognosis and prediction (52–54). There is a partial overlap between immunohistochemistry and intrinsic subtypes. Approximately 80% of basal like tumors are TPN and 80% of HER2-enriched are HER2 positive tumors. For HR+/HER2 negative tumors, either a high proliferation, Ki67 (≥ 20%) or a low PgR (< 20%) value may be used to distinguish between LumA-like (more endocrine sensitive, indolent, better prognosis) and LumB-like (less endocrine sensitive, more aggressive, worse prognosis) tumors (55,56). LumA–like tumors are ER-positive and HER2-negative with low Ki67 expression (< 14%) or with intermediate Ki67 expression

(14% to 19%) and high PgR levels (> 20%). LumB–like tumors are ER-positive and HER2-negative with intermediate Ki67 expression (14% to 19%) and low PgR levels (< 20%) or with high Ki67 expression (≥ 20%) (57). Luminal A tumors have a high prevalence of *PIK3CA* mutations (49%), whereas a high prevalence of *TP53* mutations is a hallmark of basal-like tumors (84%) **[Figure 12].**

1.2.3.5 Breast cancer Integrative Clusters

METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) employs the strategy of integrating copy number alterations (CNAs) and transcriptomic data in order to identify areas of recurrent alteration associated with deregulated gene expression from the *cis*-associated genes. This clustering analysis revealed 10 novel molecular subgroups (58). The 10 integrative clusters (IntClust 1–10) were each associated with distinct CNAs and gene expression changes. These clusters clearly demonstrated the heterogeneity present within tumors classified according to ER, PgR and HER2 expression, and they divided all of the previously identified intrinsic subtypes into separate groups (59).

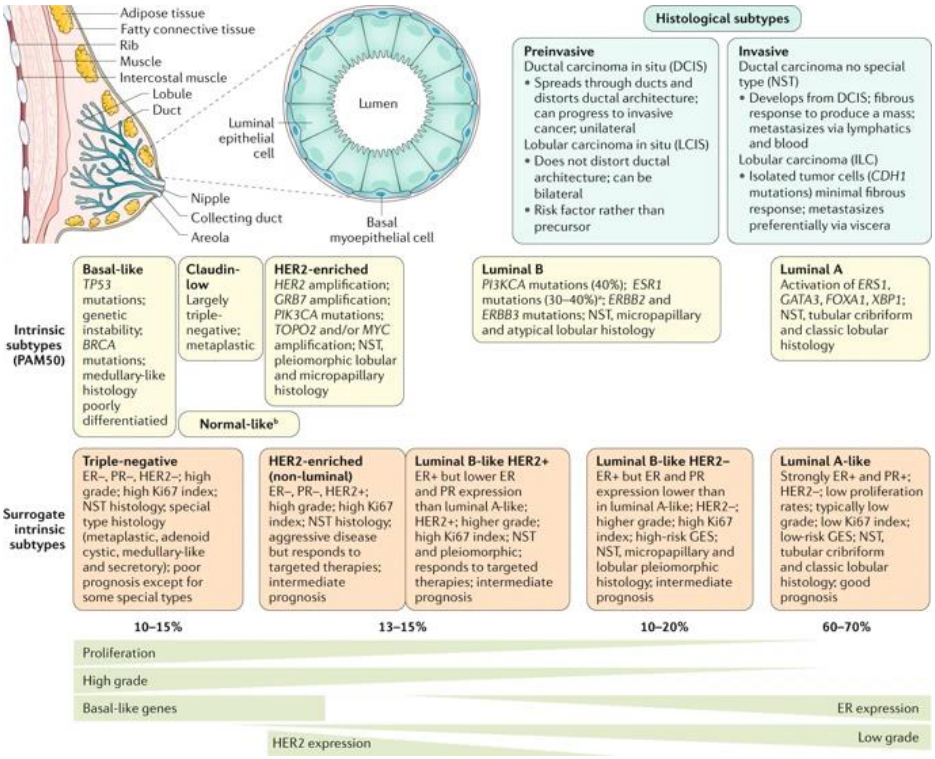

Figure 12: Breast cancer biology overview**.** All breast cancers arise in the terminal duct lobular units (the functional unit of the breast) of the collecting duct. The histological and molecular characteristics have important implications for therapy, and several classifications based on molecular and histological characteristics have been developed. The histological subtypes described here (top right) are the most frequent subtypes of breast cancer; ductal carcinoma (now referred to as 'no special type' (NST)) and lobular carcinoma are the invasive lesions; their preinvasive counterparts are ductal carcinoma in situ and lobular carcinoma in situ (or lobular neoplasia), respectively. The intrinsic subtypes of Perou and Sorlie (51) are based on a 50-gene expression signature (PAM50) (54). The surrogate intrinsic subtypes are typically used clinically and are based on histology and immunohistochemistry expression of key proteins: estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2) and the proliferation marker Ki67. Tumors expressing ER and/or PgR are termed 'hormone receptor-positive'; tumors not expressing ER, PgR and HER2 are called 'triple-negative'. Taken from Harbeck N, et al (60).

1.2.4 Estrogen receptor biology in breast cancer

Approximately two-thirds of breast cancers express ER, which plays a critical role in the growth of estrogen-dependent breast cancer cells. ER is a transcription factor that regulates genes expression programs that eventually trigger cell division and proliferation. As a nuclear receptor, ER is composed mainly of a DNA binding domain (DBD), and a ligand-binding domain (LBD) required for estrogenic ligands and to control responses to anti-estrogen antagonists. In the classical signaling pathway, when ER is not bound to estrogen, it is transcriptionally inactivity due to their association with heat shock proteins (HSP). When ER binds to estrogen, ER dissociate from HSP and change their tertiary structure to promote receptor dimerization and nuclear localization. In the nucleus ER dimers bind through the DBD to ER element (ERE) DNA sequences within the enhancers and promoters of ER target genes. Estrogen binding also induces a conformational change within the LBD domain, allowing coactivator proteins to be recruited. ER and can also bind to non-EREs by attaching to other transcription factors and not with ERE directly ('tethered pathway'). ER can also be activated in an estrogen independent manner via plasma membrane crosstalk with growth factor receptor (GFR) pathways, such as the PI3K/AKT/mTOR pathway signaling, that phosphorylate ER or its coactivators. The crosstalk between ER and GFR signaling, are potential mechanisms of antiestrogen therapy resistance (61–63). ER can activate signal transduction pathways by passing the process of gene transcription, referred as ER non-genomic signaling. This mode of extranuclear ER action typically occurs faster than ER genomic pathway involves trafficking to the plasma membrane, where ER can activate kinase pathways (such as protein kinase C) either directly or indirectly (64) **[Figure 13].**

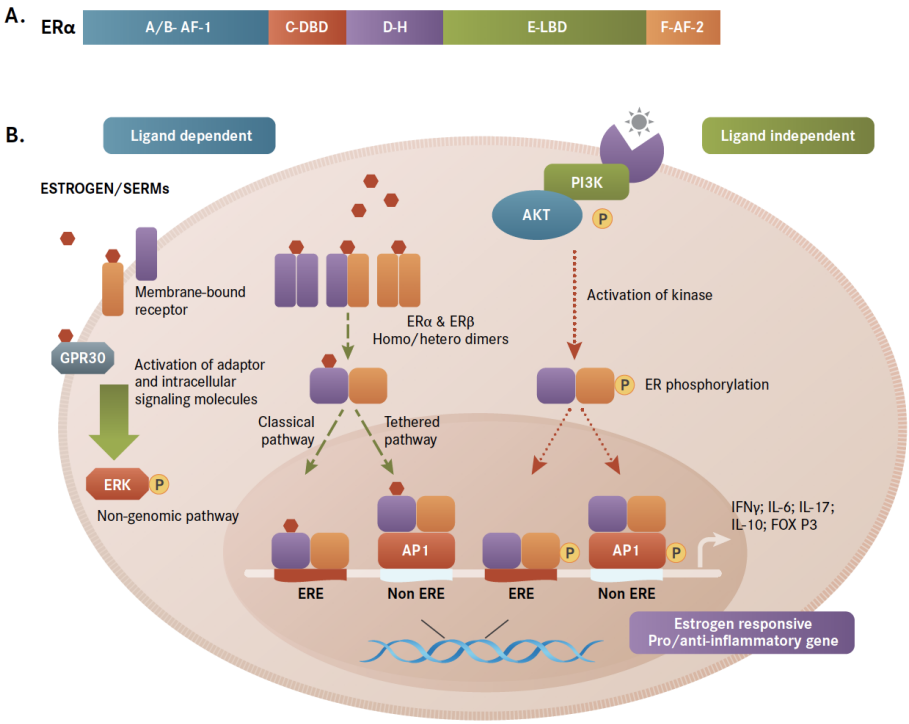

Figure 13: Estrogen Receptor signaling **A)** Shows human estrogen receptor α (595 aa) structure. ER have five distinct structural and functional domains: DNA-binding domain (DBD; C domain), hinge domain (D), ligand-binding domain (LBD; E/F domain), and two transcriptional activation function domains AF-1 (in A/B domain) and AF-2 (in F domain). The binding of ligand (estrogens) to LBD domain results in conformational changes in the receptor (homo/hetero dimerization). The receptor dimer then translocate inside nuclei with the help of DBD domain. This domain is also important for post-translational modifications of receptor by acetylation, lipophilic moieties, and ubiquitination. The DBD domain then recognizes and binds to estrogen-response element (ERE) in DNA. AF-2 region interacts with co-regulatory proteins in ligand-dependent pathway. However, AF-1 region is activated in ligand-independent manner.
B) ER-mediated signaling occurs in a ligand-dependent (green arrows) and ligand-independent (red arrows). The ligand-dependent pathway is triggered by binding of either endogenous hormone or a synthetic compound to the ligand-binding domain of ERs in the cytosol. Different ligands induce unique conformational changes of ERs, and receptor dimerization (homodimers: ERα:ERα or ERβ:ERβ or heterodimer: ERα:ERβ), which then translocate into nuclei and bind to specific EREs (consisting of a 5-bp palindrome with a 3-bp spacer; GGTCAnnnTGACC) in the regulatory regions of estrogen responsive genes. This is also called “classical” signaling pathway. In “tethered” signaling pathway, ligand-activated ERs interact with other transcription factor complexes and bind to non-EREs by attaching to other transcription factors and not with ERE

directly. In third ligand-dependent "non-genomic" pathway, ligand interacts with plasma membrane-bound ERs via palmitoylation on cysteine447, which results in activation of cytoplasmic signaling pathways, such as protein kinase C (PKC). In ligand-independent signaling pathway, there is phosphorylation/activation of ERs by other active signaling cascades in a cell. This activation results in both direct ERE and non-ERE dependent genomic actions. Abbreviation: Akt, protein kinase B; AP-1, activator protein 1; ERE, estrogen-response element; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; FoxP3, fork box 3; GRP30, an orphan G-protein coupled receptor 30; iNOS, inducible nitric oxide synthase; PI3K, phosphatidylinositol-4, 5-bisphosphate 3-kinase; P, indicates phosphorylation. Taken from Khan D, et al (65).

1.2.5 The PI3K/AKT/mTOR pathway signaling in breast cancer

The PI3K/AKT/mTOR pathway plays a central role in cell physiology by transmitting signal transduction events in response to extracellular stimuli. This pathway controls many cellular functions such as proliferation, growth, survival, motility, and metabolism (66). Mutations in this signaling route are frequently found in cancer, being particularly common in breast cancer, where about 60% of tumors harbor genetic alterations that hyperactivate the PI3K/AKT/mTOR pathway. Phosphoinositide 3-kinases (PI3Ks) are a family of three different classes of lipid kinases. Class I PI3K is the most studied and clearly implicated in oncogenic transformation and tumor growth. Class I PI3Ks are heterodimers consisting of a p85 regulatory subunit and a p110 catalytic subunit (p110α, p110β, p110γ, or p110δ). PI3K receives signals from growth factor receptor tyrosine kinases, such as ERBB receptors, FGFR and IGF-1R, and G protein-coupled receptors. Activated receptors phosphorylate adaptor proteins which, in turn, bind the amino-terminal SH2 domain of p85. This binding frees p110 from the inhibitory effect of p85 which then catalyzes the conversion of phosphatidylinositol bisphosphate, PI(4,5)P2, to phosphatidylinositol triphosphate, PI(3,4,5)P3. PIP3 recruits PDK1 and AKT, through their pleckstrin homology domain, to the plasma membrane. PDK1 phosphorylates AKT at Thr308. The mTOR/Rictor (TORC2) complex phosphorylates AKT at Ser473, resulting in full activation of this enzyme (67). PTEN and INPP4B dephosphorylate PIP3 in positions 3 and 5 of the inositol ring, respectively, thereby negatively regulating PI3K signaling output [4, 5]. Activated AKT

phosphorylates and inhibits tuberous sclerosis complex 1 and 2 (TSC1/2) resulting in accumulation of Ras homolog enriched in brain (RHEB) which activates the complex mTOR/Raptor (TORC1). TORC1 phosphorylates ribosomal protein S6 kinase (S6K1) and eIF4E binding protein 1 (4E-BP1) promoting mRNA translation, protein synthesis, and autophagy. AKT also phosphorylates GSK3α, GSK3β, FoxO transcription factors, MDM2, BAD, and p27KIP1 to facilitate survival and cell cycle entry **[Figure 14].**

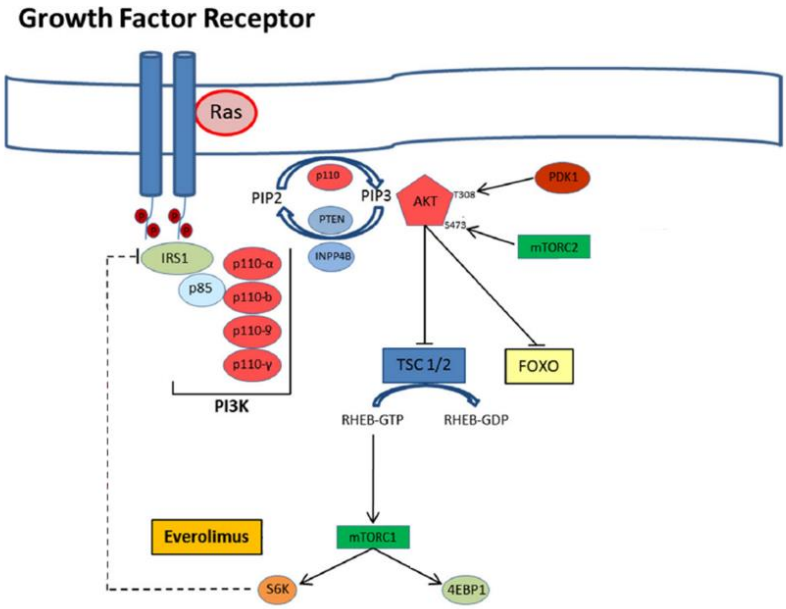

Figure 14: The PI3K/AKT/mTOR pathway signaling. Activated growth factor receptors phosphorylate adaptor proteins like IRS1 which recruit p85/p110 (PI3K) dimers to the plasma membrane. PI3K is a heterodimer composed of a p85 regulatory subunit and a p110 catalytic subunit. p85 binding to IRS1 relieves its inhibitory effect on p110. Activated p110 catalyzes the conversion of PIP2 to PIP3. PTEN and INPP4B dephosphorylate PIP3, thus negatively regulating PI3K. PIP3 recruits PDK1 and AKT to the membrane. Full activation of AKT requires its phosphorylation by PDK1 and mTORC2. Activated AKT inhibits the complex TSC1/2, resulting in RHEB-GTP accumulation which, in turn, activates TORC1. Activated TORC1 phosphorylates ribosomal protein S6 kinase (S6K1) and eIF4E binding protein 1 (4EBP1) promoting mRNA translation, protein synthesis, and autophagy. Small-molecule inhibitors discussed in the text are included in the figure. Dashed lines represent the inhibitory feedback loop relieved upon inhibition of the pathway. Taken from Guerrero-Zotano et al. (68)

Mutations of the *PIK3CA* gene, which encodes p110α, are the most common genetic alteration in breast cancer occurring at frequency of 45% in LumA, 30% in LumB , 39% in HER2-enriched, and 9% in basal-like breast cancer subtypes. More than 80% of the mutations cluster within the helical (E542K and E545K) or the kinase (H1047R) domains of p110α (69). Helical domain mutations increase catalytic activity by reducing the repression of p110α by p85 (70) or facilitating the interaction of p110α with IRS1 (71), whereas kinase domain mutations mainly increase the retention of p110α at the plasma membrane (72). Preclinical data from cell-based studies and genetically engineered mice (GEMs) have clearly shown that these mutations activate PI3K/AKT/mTOR signaling and are oncogenic drivers by promoting cell transformation, tumor initiation, progression, and resistance to apoptosis (73,74). However, data from knock-in GEMs, where the PIK3CA mutant protein is expressed at physiologic levels in the mammary gland, do not show pathway hyperactivation. In this knock-in models, mammary tumors develop after a long latency (75), suggesting that additional genetic alterations are needed to recapitulate a PI3K-induced transformed phenotype . This is consistent with data from primary breast cancers showing a disconnection between PIK3CA mutation and PI3K pathway activation. For example, luminal tumors, despite having the highest incidence of *PIK3CA* mutations, do not exhibit high levels of (activated) p-AKT, p-S6, and p4EBP1 (28). In TPN breast cancer, the PI3K/AKT/mTOR activation appears to be driven mainly by loss of *PTEN* (30%) or *INPP4B* (40%). Around 3% of ER+ tumors harbor *AKT* mutations in the PH domain (E17K), which result in constitutive localization at the plasma membrane and resulting activation of AKT (76). Mutations in *PIK3R1*, the gene encoding the p85 regulatory subunit of PI3K, have also been reported although with a lower frequency (≈2%). Interestingly, *PIK3R1* mutations cluster in the region of the protein that contacts p110, thus reducing the inhibitory effect of p85 on the isozyme (77).

1.2.6 The Cyclin D-CDK4/6-RB1 axis in breast cancer

Dysregulated cell division is one of the key hallmarks of cancer. ER+/HER2 negative breast cancer are dependent on the cyclin D–CDK4/6-RB1 axis to initiate G1 to S transition and initiate cell division. ER+ breast cancer has increased Cyclin D levels, that are controlled by ER and growth factor signaling. Cyclin D complex with and activate CDK4/6 that subsequently phosphorylate RB1. RB1 is a negative regulator of the cell cycle preventing cell division by binding to the E2F transcription factor to inhibit G1 to S transition. Thus, hyperphosphorilaed RB reduces inhibitory control of the E2F transcription factor family initiating the transcription of genes involved in cell cycle such as E-type cyclins, which activates CDK2 and other proteins important for S phase initiation and DNA synthesis.

The pathogenesis of hormone receptor-positive breast cancer is dependent on promoting cyclin D1 expression and CDK4/6 activity, several observations support this: 1) Ablation of cyclin D1 and CDK4 in mouse models prevents breast tumor formation (78,79); 2) ER+ breast cancer is characterized by frequent overexpression of Cyclin D or amplification of *CCND1* (gene encoding for Cyclin D) (80,81); 3) Estrogen induces expression of cyclin D, 23–25 and thereby promotes CDK4/6 activity (82) and 4) Decreasing estrogen signaling reduce cyclin D-CDK4/6 complexing, followed by a subsequent G0 and G1 cell-cycle arrest (83). Apart from ER, other oncogenic signaling such as activation of upstream tyrosine kinase receptors that engage with PI3K or MAPK pathway can lead to activation of cyclin D-CDK4/6 (84,85) **[Figure 15]**. The cyclin D–CDK4/6-RB1 axis is a key mediator of endocrine resistance in breast cancer, and therapeutically targeting with selective CDK4/6 inhibitors are a major milestone in the treatment of metastatic ER+ breast cancer (86), and currently under development in early stage breast cancer.

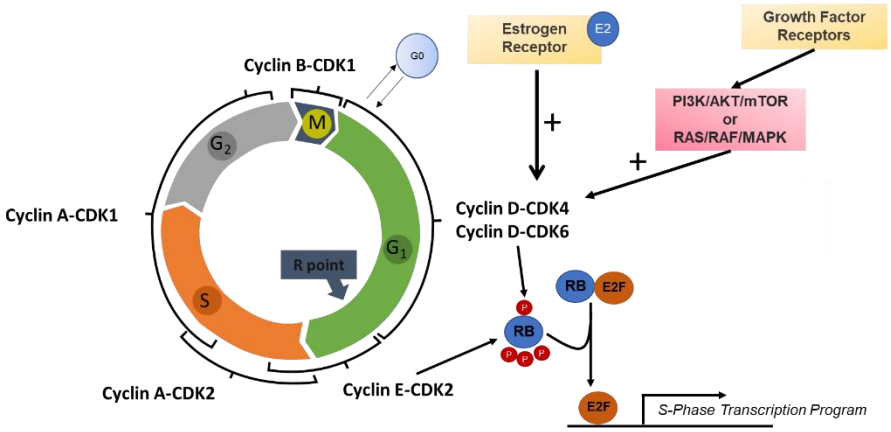

Figure 15: The central role of CyclinD-CDK4/6-RB axis in mediating breast cancer proliferation. The cyclin dependent kinases (CDKs) and cyclins act both downstream of growth factor receptor transduction pathway and estrogen signaling pathways to promote cell cycle progression through up regulation of D-type cyclins and activation of CDK4/6. Adapted from Otto T, et al (87).

1.3 Principles of systemic treatment in HR+/HER2- early stage breast cancer

The vast majority of patients with HR+/HER2- breast cancer are diagnosed at an early stage of their disease which are potentially curable with locoregional treatments such as surgery and radiation (88). However, most women also need some form of systemic therapy. Systemic therapy can be given before surgery (neoadjuvant), in women with large tumors for whom reducing the tumor burden is preferred, or after surgery (adjuvant). After surgery, HR+/HER2- early breast cancer is usually treated with adjuvant endocrine therapy (ET) and, depending on the estimated individual risk of disease relapse, adjuvant chemotherapy (CT) (89). Most validated clinical and pathological features that may indicate a higher risk of disease relapse, and therefore the need for adjuvant CT, include large primary tumor size and involvement of axillary lymph nodes. Others prognostic factors are, histopathological grade, PgR expression,

Ki-67 expression, multi-gene testing recurrence scores, age and comorbidities. Using these factors, early breast cancer can be classified as having low, intermediate/moderate or high risk for recurrence after surgery (90).

Endocrine therapy, which counteracts estrogen-promoted tumor growth, is the primary systemic therapy for HR+/ERBB2− breast cancer. Standard ET consists of oral antiestrogen medication taken daily for 5 years, and options differ according to menopausal status. Tamoxifen is a selective estrogen receptor modulator that competitively inhibits estrogen's binding to ER and is effective in both pre- and postmenopausal women. Aromatase inhibitors (AIs) (anastrozole, exemestane, and letrozole) decrease circulating estrogen levels by inhibiting conversion of androgens to estrogens and are effective only in postmenopausal women (including those who are postmenopausal because of medical ovarian suppression or oophorectomy) (91).

1.3.1 Adjuvant endocrine therapy for premenopausal patients

Tamoxifen alone was considered the standard of care as adjuvant ET for all premenopausal patients with HR-positive breast cancer till data on the role of ovarian function suppression (OFS) in combination with tamoxifen or an AI have become available (92,93) . The addition of OFS to tamoxifen does not provide any benefit in women at low clinical risk of recurrence for whom tamoxifen alone should be still considered standard of care. For high risk patients, those who are normally candidates also to (neo)adjuvant chemotherapy, OFS in combination with endocrine therapy are recommended by major guidelines (89,94,95), the best partner (tamoxifen or an AI) to be combined with OFS remains controversial. The combined analysis of TEXT and SOFT studies demonstrated sustained improvements with exemestane plus OFS versus tamoxifen plus OFS in disease-free survival (DFS), freedom from breast cancer, and freedom from distant recurrence but not from overall survival (OS) (96). Compared to tamoxifen + OFS, AI + OFS seems to reduce DFS events but produce more cancer deaths (97). This discordance between DFS and OS for AI + OFS compared to tamoxifen

+ OFS is intriguing and not fully understand. Some hypotheses are that AI + OFS can produce an incomplete intermittent estrogen suppression which can results in more aggressive recurrent tumors (98) or that AI + OFS could select for *ESR1* mutation which is associated with poor outcome and poor response to ET in the metastatic setting (34,99).

1.3.2 Adjuvant endocrine therapy for postmenopausal patients

According to current clinical guidelines, AIs should form part of adjuvant ET in postmenopausal patients with HR-positive breast cancer. They can be used either as up-front therapy or as sequential treatment after 2–3 or 5 years of tamoxifen (100). The efficacy of AIs depends on the suppression of serum and tissue levels of estradiol and, in contrast to tamoxifen, their therapeutic effect can be abolished in the presence of functional ovaries (101). Despite 5 years of adjuvant ET, HR-positive tumors have a persistent risk of late recurrence; there are more recurrences after 5 years than in the first 5 years after diagnosis (102,103). This clinical situation has led to multiple trials of extended (beyond 5 years) ET (104). Extending to an AI after 5 years of tamoxifen is associated with substantial benefit in preventing late recurrence, however, extending an AI beyond 5 years has little benefit in distant recurrence but can reduce contralateral/local events. Women with greater risk of recurrence based on well-known prognostic factors (nodal involvement, larger tumors, grade, high score in genomic signatures), (105,106) are more likely to get substantial clinical benefits from treatment and thus should receive extended ET up to a total of 10 years of treatment. Women with lower risk of late recurrence, typically stage I disease with lower-risk features, may reasonably stop therapy after 5 years unless there is strong motivation for prevention of late recurrence and/or contralateral/second breast cancers. Expert recommend extended duration with any of the following strategies: AI for up to a total of 10 years; or tamoxifen for 2 to 3 years followed by AI for 7 to 8 years; or tamoxifen for 5 years followed by AI for 5 years; or tamoxifen for 10 years (100).

1.4 Neoadjuvant endocrine treatment for HR-positive breast cancer

When a patient is diagnosed with a localized breast cancer there is an opportunity to administer an oncology drug before surgery with the aim of reducing tumor size and obtaining prognostic information; this is known as preoperative or neoadjuvant treatment. Neoadjuvant therapy can improve surgical outcomes and provide effective systemic therapy. Several studies, have demonstrated that administration of the same chemotherapy in the neoadjuvant versus adjuvant setting is associated with similar outcomes (107,108). Traditionally neoadjuvant chemotherapy (NAC) has been the treatment of choice to downstage locally advanced and unresectable primary breast cancers. However, HR+/HER2- tumors are less likely to respond to NAC than other subtypes (109,110) and neoadjuvant endocrine therapy (NET) is and alternative option, particularly for postmenopausal women and for elderly/frail patients.

1.4.1 Clinical benefits of neoadjuvant endocrine therapy

1.4.1.1 Avoidance of Surgery in Frail/Older Patients

Neoadjuvant endocrine therapy with tamoxifen was first used as an alternative to standard surgery in older women with early breast cancer (111,112) . This approach avoided the inconvenience of surgery, chemotherapy, and/or radiotherapy, resulting in a 60% response rate, and also identified ER as a predictive biomarker of benefit: Nearly 100% of ER-negative tumors were unresponsive compared to a clinical benefit rate of 80% ER+ among tumors, many with long-lasting responses (113,114). These encouraging results triggered several randomized controlled trials comparing tamoxifen versus mastectomy in elderly patients. A metanalysis of these studies reported an increased risk of local failure but similar breast cancer–specific and OS for neoadjuvant tamoxifen versus surgery followed by adjuvant

tamoxifen (115). The efficacy AIs in this context has not been addressed in randomized trials, but indirect comparisons from cohort studies suggest they are superior to tamoxifen, with higher clinical benefit and lower disease progression rates. The high median time to progression ~49 months) and low toxicity make definitive primary endocrine therapy an attractive ER+ treatment choice for patients with low-risk breast cancer and shorter life expectancy (116).

1.4.1.2 Increasing Likelihood of Breast Conserving Surgery

Neoadjuvant endocrine therapy is also used with the aim of reducing tumor size to allow breast-conserving surgery and/or improve breast cosmesis. Third-generation AIs (letrozole, anastrozole, exemestane) have been compared with tamoxifen in several randomized trials, showing superior response rates (76%–37% vs. 40%–36%), and eligibility for breast conservation (45%– 36% vs. 35%–20%)(117).

1.4.1.3 Alternative to Neoadjuvant Chemotherapy

In postmenopausal women (up to 70 years of age) with ER+ breast cancer, adjuvant chemotherapy yields similar risk reduction in mortality compared to postmenopausal women with ER-negative cancer, but a marginal absolute gain in OS compared with adjuvant ET (118). In addition, chemotherapy is associated with toxicities (myelodysplastic syndrome, cardiac dysfunction, permanent neuropathy) of difficult justification in patients with good overall prognosis. Further, prognostic tests, such as Mammaprint or Oncotype Dx, have helped identify those patients with a good prognosis where adjuvant chemotherapy can be safely omitted (119,120). Two randomized phase II trials of NET versus Neoadjuvant chemotherapy (NCT) showed a similar response and rate of breast conservation for both treatment arms, with

substantially less toxicity with endocrine treatment (121,122). The predictive value of the 21-gene signature Oncotype Dx for response to NET has been evaluated in a prospective study where patients were treated with preoperative exemestane for 6 months (123). Patients with a low Oncotype Dx recurrence score (RS) exhibited a clinical response rate of 59% and a breast conservation rate of 91% compared with 20% and 47%, respectively, in patients with a high RS. Thus, for many patients with ER pso low-risk early breast cancer, who want to avoid total mastectomy, NET is a medically reasonable option.

1.4.2 Optimal Duration of Neoadjuvant Endocrine Therapy

Three to four months has been the standard duration of most trials of NET. However, there is consensus that this length of treatment is insufficient to reach maximal tumor response. No studies have formally investigated the optimal duration of NET. More recent nonrandomized studies suggest that some tumors benefit from a longer duration (6–12 months) of anti-ER treatment (124–126).

1.4.3 Neoadjuvant endocrine therapy as a platform for research

1.4.3.1 Biomarkers of response in neoadjuvant endocrine therapy

After neoadjuvant chemotherapy, achievement of a pathologic complete response (pCR) , that is, absence of cancer in the breast and lymph nodes in the surgical specimen, is a surrogate marker of long-term outcome, particularly in HER2-positive and TPN breast cancer, and is been used to support accelerated approval of new drugs (110,127). In patients with early ER+ breast cancer pCR is not an effective surrogate of long-term outcome, because is uncommon both after NAC or NET, and because

failure to achieve a pCR after few months of preoperative treatment does not imply poor patient outcome, since these patients still receive 5 to 10 years of adjuvant ET. NET has been shown to induce downregulation of gene expression signatures of cell-cycle progression, and ER-regulated proteins such as PgR and TTF1, and a reduction in ER phosphorylation at serine 118, a marker of ER transcriptional activity (128–130). However, none of these direct pharmacodynamic biomarkers of antiestrogen action has been sufficiently studied as a measurement of therapeutic efficacy. Because antiestrogen therapy mainly induces cell-cycle arrest, markers of tumor cell proliferation have been used to measure the in-situ action of these drugs. The protein Ki67 detected by immunohistochemistry is currently the most used marker to estimate tumor cell proliferation. Ki67 is expressed in proliferating tissues in all cell-cycle phases is absent in quiescent cells, and correlates well with other markers of proliferation such as the S-phase fraction, mitotic index, and/or in vivo uptake of bromodeoxyuridine (131). A low Ki67 score in response to NET predicts for a good long-term outcome, whereas high levels have been associated with an increased risk of breast cancer recurrence. The main evidence for this comes from three NET studies: the IMPACT trial (132) comparing anastrozole, tamoxifen, or the combination for 12 weeks; the P024 trial (129) comparing letrozole and tamoxifen for 4 months; and the ACOSG Z1031 (133) trial, which compared head to head the performance of letrozole, anastrozole, and exemestane for 4 months. The Ki67 score, measured as a continuous variable after natural log transformation, at 2 weeks in IMPACT, at 16 weeks in P024, and at 2 to 4 weeks in ACOSOG Z1031, was predictive of DFS in multivariate analysis, whereas the pretreatment Ki67 was not (134,135). In the IMPACT trial, when the change in Ki67 was introduced in the multivariable model (instead of the absolute Ki67 score at 2 weeks), the former was not predictive of DFS. Thus, the absolute level of on-treatment Ki67 is a useful biomarker with prognostic and predictive ability because it integrates both the intrinsic proliferative rate and the response to endocrine therapy. A Ki67 >10% after 2 or 4 weeks of endocrine therapy has been suggested as a cutoff for the early identification of non-responders with increased risk of relapse. About 20% of patients

fall into this category after initiation of neoadjuvant AIs (133). In the P024 study, in addition to Ki67, other tumor features such as tumor size, number of axillary lymph nodes, and ER status measured in the surgical specimen after NET were associated with long-term outcome in a multivariate analysis. This analysis served to develop the Preoperative Endocrine Prognostic Index (PEPI) score, a prognostic biomarker that distinguishes between sensitive and resistant disease as a function of the risk of relapse (133,135). Thus, patients with PEPI score 0 [ypT1-2, ypN0, post-Ki67 <2.7%, ER+], have a very low risk of relapse (<4% at 5years) with endocrine therapy alone. There is also a correlation between on-treatment levels of Ki67 and PEPI score: Patients with Ki67 >10% after 2 to 4 weeks of estrogen deprivation with an AI have a probability between 0% and 5% of achieving a PEPI score 0 at surgery (29). Finally, the probability of achieving a PEPI score 0 is greater for patients with Luminal A (27%) than for those with Luminal B tumors (10%) (136) .The Residual Cancer Burden (RCB) index is another biomarker of response to NCT that is increasingly used in NET studies. The RCB index evaluates 5 posttreatment variables: two-dimensional tumor bed, cellularity, percentage of carcinoma in situ, number of metastatic lymph nodes, and the diameter of the largest nodal metastases. It classifies the surgical specimen into four categories: RCB-0 (pCR), RCB-I (minimal residual disease), RCB-II (moderate residual disease), and RCB-III (extensive residual disease). RCB is able to predict risk of relapse after neoadjuvant chemotherapy, which is highest for RCB-III (53.6%) and similar for RCB-0 and RCB-I (2.4% and 5.4%, respectively) (137,138). Interestingly, the incorporation of Ki67 into RCB improved the prognostic ability of either Ki67 and RCB alone (139).

1.4.3.2 Neoadjuvant endocrine therapy predicts results from adjuvant studies

The preoperative therapy setting is an excellent clinical research platform where treatment can be compared and triaged using endpoints that correlate with long-term outcome. There are multiples examples where NET trials results predicts

outcome from adjuvant and metastatic studies (140). An illustrative example supporting the value of the neoadjuvant platform for clinical trial prioritization is provided by IMPACT trial (132) comparing anastrozole, tamoxifen, or the combination for 12 weeks. In IMPACT, following 2 and 12 weeks of treatment, anastrozole suppressed Ki67 by 76% and 82%, respectively, compared with tamoxifen by 59% and 62%, and the combination of both drugs by 64% and 61%. These differences paralleled the outcome of the same three treatment arms in the large adjuvant ATAC trial which enrolled more than 9,000 women (141). After a median follow-up of 30 months, anastrozole significantly improved DFS over tamoxifen and the combination, whereas DFS was similar in the tamoxifen and combination arms. It can be argued that had the results of IMPACT been known before the ATAC trial, these data would have provided a rationale for elimination of the combination arm in ATAC, thus significantly reducing the size, duration, and cost of the adjuvant study.

1.4.3.3 Discovery of mechanisms of resistance to endocrine therapy

Residual drug-resistant disease in the breast after neoadjuvant chemotherapy can be considered a surrogate for drug-resistant micrometastases that ultimately progress to clinically overt metastatic breast cancer. This paradigm has not been explored in breast cancer treated with NET. There are several reasons for this. First, there have been few NET trials with long-term follow-up where the posttreatment specimen has been profiled in any depth. Second, it is unclear whether after a few months of NET the residual cancer in the breast can be considered truly refractory to therapy. Third, it is also unclear if genomic changes induced by endocrine therapy are due to expansion of subpopulations of cells harboring resistant mutations or just fluctuation (“reprograming”) in gene expression of existing cells, as suggested by studies exploring the effects of short-term AIs (described below). Nonetheless, it is reasonable to speculate that a high Ki67 score and/or a high PEPI score after NET may

identify tumors with a high rate of recurrence where mechanisms and/or biomarkers of drug resistance can be interrogated using molecular methods. One of the earliest studies of 18 matched pairs of pre- and post-letrozole biopsies showed an enrichment of cells with tumor-initiating and mesenchymal gene expression signatures in the treated specimens (142), concordant with an enrichment in stem-like cells in triple negative tumors resistant to NAC (143), and the chemoresistance of letrozole-resistant tumors in ACOSOG Z1031B (133). Ellis and colleagues used whole genome sequencing (WGS) to interrogate 77 diagnostic biopsies of postmenopausal patients with breast cancer who received neoadjuvant letrozole for 4 to 6 months (144). This study showed that tumors resistant to letrozole are more complex, with more structural variations and mutation rates than sensitive tumors, which could provide a source for emergence of mechanisms of endocrine resistance. *MAP3K1* mutations were associated with luminal A status, low-grade histology and low proliferation rates, whereas mutant *TP53* was associated with poor prognostic features. Further, mutant *GATA3* correlated with suppression of proliferation upon estrogen deprivation with letrozole. The role of tumor heterogeneity on resistance to estrogen deprivation has been explored in two NET studies. Miller and colleagues (145) used WGS and RNA ER+ sequencing in 22 breast cancers before and after 4 months of treatment with an AI. These authors reported that ER+ breast cancers are clonally heterogeneous, both spatially and temporally, showing that the proportion of some of these clonal subpopulations changes markedly upon treatment. Despite the relatively short treatment, several mutations were selected for or enriched after therapy, including two activating *ESR1* mutations. In a second study, Gellert and colleagues (146) performed whole-exome sequencing on baseline, surgical core-cuts and blood from 40 patients treated with an AI for 2 weeks. In resistant tumors where the Ki67 remained high, there were more somatic mutations than in good responders. Underscoring spatial heterogeneity, 30% of tumors contained subclones that were exclusive to the baseline (pretreatment) or surgical cores (posttreatment), suggesting that core biopsies in this setting provide limited tumor material and thus cannot capture the complete molecular profiles of

heterogeneous cancers. Further, *TP53* mutations and a higher mutational load were associated with highly proliferative tumors that responded poorly to therapy.

1.4.3.4 Limitations of neoadjuvant endocrine therapy as research platform

Although on-treatment Ki67 and PEPI scores have shown utility in clinical investigation and discovery, they are not yet useful for individual patient care decisions. Assuming the framework proposed by Hayes and colleagues (147), a biomarker must demonstrate analytic and clinical validity and clinical utility before it can be used to guide treatment decisions. Analytic validity, that is, the ability of an assay to accurately and reliably measure the analyte of interest, is the first barrier to overcome. Visual interpretation of Ki67 staining has high intraobserver but low interobserver concordance (148). The interobserver concordance is higher for low and high Ki67 values, which manifest the difficulty of establishing a cutoff on the intermediate Ki67 values for making clinical decisions. In order to decrease this variability, the International Ki67 Working Group has conducted several studies to analytically validate and standardize Ki67 evaluation across laboratories (149,150), and recommended that an improved interobserver reproducibility can be achieved on centrally stained tissue sections after training observers on a standardized visual scoring method. Another source of variability, which cannot be diminished through adoption of standard operating procedures, is intratumor heterogeneity of Ki67 levels (151,152). Ki67 expression is usually higher in the tumor periphery than in the center, and some tumors show a diffuse pattern of Ki67 staining whereas some others show "hot" and "cold" spots. It is not clear whether there should be a focus on Ki67 "hotspots" or whether the average Ki67 value is enough. These issues could be more prominent when estimating the proliferation rate in the whole tumor based on core-cut biopsies that usually represent a small fraction of the tumor mass. Because of these limitations, Denkert and colleagues suggested (153) that clinical decisions should not

be based on Ki67 in intermediate cases. These patients would be ideal candidates for the use of gene expression signatures. Despite the interobserver variance and tumor heterogeneity, the clinical validity of the Ki67 and PEPI scores, that is, their ability to distinguish patient populations with different outcomes, is relatively established. Several studies have shown that Ki67 is able to classify tumors as endocrine resistant or sensitive. The fact that different studies show similar results suggests that a certain degree of variability in Ki67 assessment is admissible. The clinical utility of the Ki67 and PEPI scores, that is, their use for individual treatment decisions, is currently being evaluated in several studies. The first study to formally explore the clinical utility of on-treatment Ki67 values was ER+ ACOSOG Z1031B (133). In this study, patients with ER+ (Allred 6–8) breast cancer with a Ki67 >10% after 2 to 4 weeks on an AI were defined as endocrine resistant and switched to any approved neoadjuvant chemotherapy regimen. Notably, these endocrine-resistant tumors were also chemoresistant in that only 2 out of 35 (5.7%) patients achieved a pCR. These results are intriguing and contrast with the reported 20% probability of pCR among highly proliferative tumors with a baseline Ki67 >35% (154). On the other hand, patients in ACOSOG Z1031B whose tumors exhibited a Ki67<=10% at 2 to 4 weeks and remained on an AI for 12 to 14 weeks had a PEPI score 0 rate of 34%, suggesting that early assessment of an on-treatment Ki67 score identifies highly hormonedependent tumors where a recommendation of antiestrogen adjuvant therapy alone might be appropriate.

2 .HYPOTHESIS AND OBJECTIVES

With current standard of care adjuvant therapy, approximately 25-30% of women with high risk early stage HR+/HER2- breast cancer experience relapse (14,15). Consequently, there is a critical need for more optimal adjuvant therapy in patients with early HR positive breast cancer who have a high likelihood of distant recurrence. Neoadjuvant trials with antiestrogens offer an opportunity to interrogate mechanisms of drug resistance. Residual drug-resistant disease in the breast after neoadjuvant endocrine therapy might be enriched in mechanisms of resistance driving cancer relapse and, in turn, could inform the choice of adjuvant therapy.

2.1 Primary objective

To describe genomic alterations at DNA and RNA level in post-treatment HR+/HER2- breast cancer tissue exposed to prolonged neoadjuvant endocrine therapy with the aromatase inhibitor letrozole.

2.2 Secondary Objective

To study functionally genomic alterations enriched in resistant cases and drugs that could revert the resistance.

3 . DESIGN

We performed targeted DNA sequencing and whole transcriptome analysis on whole tumor sections from a cohort of 68 operable ER+ breast cancers treated with the aromatase inhibitor letrozole for a median of 7.2 months before surgery and a with a median follow up of 5 years after surgery. To define endocrine-resistant tumors, we used breast cancer relapse and the PEPI score. As described above, PEPI score is a well validated independent prognostic factor in the setting of neoadjuvant endocrine therapy that evaluates post-treatment ER levels, Ki67 score, tumor size and axillary lymph nodal status (11). By incorporating data from treated surgical specimens rather than core biopsies, the PEPI score is less impacted by spatial intra-tumor heterogeneity (8) and may represent a strong surrogate of multiple drug-tolerant clonal populations.

3.1 Patients and tumor specimens

Tumors were from a cohort of elderly patients with newly diagnosed, operable HR +/HER negative breast cancer treated with neoadjuvant letrozole at the Instituto Valenciano de Oncología in Valencia, Spain (155). Response to neoadjuvant therapy was annotated according to RECIST response criteria (156). Tumor specimens were promptly fixed after acquisition in 10% neutral buffered formalin for 18 to 24 h and embedded in paraffin. Immunohistochemistry (IHQ) was conducted in formalin-fixed, paraffin-embedded (FFPE) tumor blocks from both the diagnostic biopsy and the post-treatment whole surgical specimen. Tumor sections were subjected to IHC using Ki67 (Dako #M7240), ER (Santa Cruz #sc542), PgR (Dako #M3569), and HER2 (Cell Signaling #2242) antibodies according to methods reported elsewhere (157). FFPE tumor sections were scanned at 100x magnification, and the area containing the highest number of Ki67 positive cells was selected. Positive and negative tumor cells were

manually counted at 400x; the percentage of positive cells was calculated with at least 1,000 viable cells. Ki67 IHC was scored by two independent expert breast pathologists.

3.2 DNA and RNA extraction

DNA and RNA were extracted from 4-8 10-µm unstained whole FFPE tumor sections from surgical specimens. Hematoxilin and Eosin-stained FFPE sections were reviewed for assessment of ≥20% tumor cellularity with macrodissection if required. DNA was extracted using the Promega Maxwell® 16 Tissue LEV DNA Kit, following the manufacturer's instructions, and quantified using Qubit HS dsDNA assay (Life Technologies, CA). RNA was extracted using the Promega Maxwell® 16 LEV RNA FFPE Purification Kit and instrument, according to the manufacturer's instructions.

3.3 DNA Targeted Cancer Gene Sequencing

3.3.1 Library preparation and sequencing of DNA

Fifty-five to 1000 ng of dsDNA were fragmented by sonication (Covaris LE220) to obtain fragments with average size distribution of 200 bp. Targeted capture libraries were built following Roche Nimblegen™ SeqCap EZ system protocols, using KAPA reagents. Hybridized DNA was sequenced on a single lane of an Illumina HiSeq2500 to generate paired-end 100bp reads. Data were demultiplexed using CASAVA.

3.3.2 Alignment and quality assessment

Reads were aligned to the reference human genome (hg19) using the Burrows-Wheeler Alignment tool (158). The resulting BAM files were preprocessed using the

Genome Analysis Toolkit (GATK) according to GATK best practices (159), which included using Picard to set read groups and mark duplicates, followed by GATK local re-alignment and base score recalibration. Sequencing quality statistics were obtained using the GATK's DepthOfCoverage tool and Picard's CalculateHsMetrics. Samples were excluded if less than 25% of the targeted bases were covered at a minimum coverage of 50X. We obtained a mean on-target sequence depth of 320-fold (range: 15- to 600-fold). We removed any variant calling with an allele frequency less than 0.1 if supported by less than 10 reads.

3.3.3 Variant calling

To identify all variants in the samples, we used the GATK Haplotype Caller (159) for single nucleotide variant (SNVs) and indels. All reads with a mapping quality less than 70 were removed. Variants were annotated with ANNOVAR (160) using the genes' canonical transcripts as defined by Ensembl. Custom scripts were written to identify variants affecting splice sites using exon coordinates provided by Ensembl. Any spurious variant call with suspicious sequencing artifacts was removed. All SNVs and indels present in ExAC (161) with a population alternate allele frequency >0.1%, that were not present in COSMIC, were considered germline and subsequently removed. We also removed variants with allele frequency between 0.45-0.55, if not present in COSMIC. As a result, we obtained 330 non-synonymous mutations in 153 genes. Mean depth of coverage across all samples was 319X (min: 25, max: 597) (Digital Supplemental Table DS1).

3.3.4 Driver and actionable mutations

To exclude possible passenger mutations we selected all frameshift, nonsense and splice variants, and missense mutations and indels known or predicted to be damaging by at least 2 out of 4 methods [SIFT (162), GERP++ (163), PolyPhen2 (164), OncodriveMUT (165)]. We classified variants as clinically actionable using www.cancergenomeinterpreter.org.

3.4 cDNA library preparation for RNA sequencing

Total RNA was quantified using a Qubit (Life Technologies) and quality was assessed using an Agilent Bioanalyzer. For samples that met quality requirements, 100 ng of was used for library preparation following the manufacturer's protocol for Illumina RNA ACCESS. Briefly, first and second strand cDNA synthesis was performed, universal adapters were ligated, and coding regions were selected by two consecutive hybrid captures followed by PCR enrichment. After enrichment, the libraries were quantified with qPCR using the KAPA Library Quantification Kit for Illumina Sequencing platforms and then pooled equimolarly. Sequencing was performed in an Illumina HiSeq2500 instrument. Paired-end transcriptome reads were aligned to the reference human genome (hg19) using the TopHat (v2.0.9) (166). RNAseq raw count files were generated from BAM files using HTSEQ (167).

3.4.1 RNA sequencing data analysis

Detection of differentially expressed (DE) genes between responders and non-responders cases was performed with DESeq2 package (168), using raw RNA-seq counts as input. Gene Ontology (GO) term enrichment analysis for DE genes were

obtained using the online functional tool GSEA/MSigDB web site v6.1. We generated rlog transformed count data using DESeq2, filtering low expressing genes (<5% tumors with 0 count and mean >4). This resulted in 16730 transcripts that served as input for the following analysis: 1) Single-sample gene set enrichment for 125 previously published breast cancer-related gene expression signatures calculated as previously described (169) and, using a FDR<0.01, for DE signatures among subgroups; 2) PAM50 molecular subtyping using R package genefu, using non scaling option (170); 3) Sample by sample correlation matrix using Pearson distance of differentially expressed transcripts with the highest variance (n=256) with the resulting matrix used to perform hierarchical cluster analysis by ward.D2; and 4) Statistical assessment of transcriptional diversity as described before (171).

3.5 Copy number analysis

Copy number changes were identified using CODEX and Control-FREEC on each bam file. CODEX was modified with a provided script for targeted panels, rather than whole exome. B-allele frequencies from Control-FREEC and copy number changes from CODEX were merged to robustly identify regions of copy number change (172).

3.6 The Cancer Genome Atlas (TCGA), METABRIC and ACOSOG Z1031B data

Somatic mutations, RNA normalized gene expression, and clinical information for the breast invasive breast carcinoma TCGA cohort (29) and METABRIC (58) were downloaded using the cBIO platform. ER+ breast cancers were selected for comparison of somatic mutations (in TCGA) and PAM50 subtypes and survival (in METABRIC). Agilent gene expression arrays (GSE87411) were downloaded and used to compare

E2F4 activation signatures between pre-treatment and 2-4 weeks post-treatment samples from 109 patients' tumors in the ACOSOG Z1031B neoadjuvant trial (173).

3.7 E2F4 activation signature

The E2F4 activation signature was generated by selecting those genes significantly upregulated (log fold change>1, FDR <0.03) in letrozole non-responder vs responder tumors that were also significant downregulated (FDR<0.01) by a 14-day treatment with palbociclib in ER+ tumors in the POP trial (NCT02008734). Eighteen of these genes are predicted to be E2F4 targets: *ANLN, ARHGAP11A, BUB1, CASC5, CDCA5, CDK1, CLSPN, DIAPH3, DTL, FAM111B, HIST1H3B, HIST1H3F, HMMR, KIAA1524, KIF18A, KIF4A, KPNA2, MAD2L1, PRR11, RRM2, STMN1, TICRR, TPX2, ZNF367*. An E2F4 activation z-score was developed by adding values across all genes for each tumor to generate an un-escalated E2F4 score. The un-escalated E2F4 score was then standardized to a z-score by subtracting from each patient's score the mean score in the cohort, and then dividing it by the scores' standard deviation.

3.8 Cell lines

MCF-7 cells (from the American Type Culture Collection, ATCC, authenticated by the STR method) were maintained in Improved modified Eagle's medium (IMEM)/10% FBS (Gibco). Long term estrogen deprived (LTED) cell lines were generated by culturing cells under hormone-depleted conditions [phenol red–free IMEM/10% dextran–charcoal-treated FBS (DCC-FBS, Hyclone; contains <0.0367 pmol/L 17β-estradiol)] as described previously (174). Mycoplasma testing was conducted before use. Experiments were performed less than 3 months after thawing early passage cells.

3.9 Quantitative Reverse Transcription Polymerase Chain Reaction

Cells were harvested, and their RNA was extracted using the RNeasy Mini Kit (QIAGEN Sciences Inc., Germantown, MD). RNA (1 µg) was reverse transcribed to cDNA using the iScript cDNA Synthesis Kit (SuperScript® III First-Strand (Invitrogen, Carlsbad, CA). Real-time PCR reactions were conducted in 96-well plates using the iCycler iQ (Bio-Rad) and primers obtained from SABiosciences (Qiagen). Threshold cycle values were normalized for the housekeeping gene GAPDH. Specific primers for the genes of interest were designed using the tool NCBI/ Primer-BLAST. The sequences of the primers set used for this analysis are:

Gene	**Forward primer** (sequence 5'->3')	**Reverse primer** (sequence 5'->3')
CDK1: NM_001786.4	TGGAAATTGAGCGGAGAGCG	TGGCTACCACTTGACCTGTAG
CDCA5: NM_080668.3	AATCAGGCTCTGAACTCCCG	CCAGGGGGCTCGTTTTCTTT
DTL: NM_016448.3	GTGTTGTGAGAGGCGCAAG	ACCAGTCAGAAGGGATTGAAG
KIF4A: NM_012310.4	TCAGAATGACAGCCAACTGC	CGCTCACTCAACTTGGCTTG
CASC5 (KNL1): NM_144508.4	TCGTCGAGTCAGCTTTGCAG	CCTGTTTCTGTTTCTTCCATTTCTG
RRM2: NM_001165931.1	TGCCTGGCCTCACATTTTCT	TCCGATGGTTTGTGTACCAGG
TPX2: NM_012112.4	GGGAGGCTGTCGGCTAATAA	GGCCTACAGGTCTGACTCAA
PRR11: NM_018304.3	ACTGACTCCAGTGATGACGC	GCAGAGTTGGAGTTGGGCTT
BUB1: NM_001278616.1	ACGCTCTGTCAGCAGACTTC	TGTTGCAGCGAATACCCCAT
ARHGAP11A: NM_014783.5	CGTATCCGGAATGTGGGATCAG	ATGGGGCAGTGCATTAAAAGG
HIST1H3B: NM_003537.3	AGACAGCTCGGAAATCCACC	AACGGTGAGGCTTTTTCACG
KIAA1524 (CIP2A): NM_020890.2	ACAAATCACCTCGACCCCTG	GCCTTTAGCTTTCGCCGC
KIF18A: NM_031217.3	CAGGACACAACATCCCACTGA	GCTGTTTTGTCTTGTTGTCGC
ZNF367: NM_153695.3	ATGGCTGCCTGAGCAGATTC	TCTCTCTTCAGCCTGGCGTA
DIAPH3: NM_001258370.1	GCGGTATGCATTGTAGGGGA	CCGGAGGCCTTCCACAATAC
TICRR (TOBP1): NM_001308025.1	ATGACCCCTACAAAGCAGGC	CCTGCCTGGGTATGTCGTTT
CLSPN: NM_001330490.1	GGGAAGGGAGAAGACTTAGGC	TTTGCACACTCCTCCAGCTA
MAD2L1: NM_002358.3	CGTGCTGCGTCGTTACTTTT	ATGGCCAGGGACACAAACAA
ORC6: NM_014321.3	TCACTTCTGCTGCACTGCTT	CTGTCGACCTGCTGTCCAAT
HIST1H2BJ: NM_021058.3	TTTCCTTTTCGTTGGCGCTTT	TCTTGGAGCCCTTTTTCGGG

3.10 Immunoblot Analysis

Cells were washed in PBS (Phosphate-buffered saline), harvested and lysed in NP-40 buffer [10 mM Tris (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1 mM sodium pyrophosphate, 50 mM NaF, 10 nM beta-glycerophosphate, 5 mM Na_3VO_4, 10% glycerol, 1 mM PMSF, and protease inhibitors] for 10 min on ice. Protein concentrations of the lysates were determined by the BCA assay (Pierce Chemical Co., Rockford, IL). Samples were separated by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) and transferred to PVDF membranes. Membranes were blocked with 3% nonfat dry milk in (Tris Buffered Saline With Tween 20) TBST for 1 h at room temperature and then incubated overnight at 4°C with the appropriate primary antibody. Antibodies specific for RB (#9309; 1:1000), S780 P-RB (#9307; 1:1000), and beta-actin (#4970; 1:1000) were obtained from Cell Signaling Technology (Denver, MA); an ERα (#8002) antibody was from Santa Cruz Technology (Santa Cruz, CA). Following incubation with horseradish peroxidase-conjugated secondary antibodies, proteins were visualized using an enhanced chemiluminescence detection system.

3.11 Methods in POP trial

Immunohistochemistry. Ki67 index (MIB-1, Dako) was independently assessed by two expert breast pathologists following international guidelines (175). Briefly, the average percentage of Ki67 positive cells (nuclear staining irrespective of the intensity) was assessed across the whole tumor section (in at least 3 high power fields representative of the whole section with at least 1000 cells). H-score was performed in FFPE sections stained with a P-Rb antibody (D20B12, Cell Signaling). *DNA and RNA extraction.* Snap-frozen tumor tissue fragments were stored in liquid nitrogen. Nucleic

acid extractions were performed using a combined method associating AllPrep DNA/RNA Mini kit (Qiagen, Courtaboeuf, France), and Trizol LS (Thermo Fisher Scientific, Illkirch, France). Tumor specimens were grinded at first in RLT Plus, and then put on Allprep DNA spin column to separate DNA from RNA and proteins. DNA samples were collected from columns following the manufacturer's instructions. RNA was isolated from eluates using Trizol LS, as described by the Life Technologies method. DNA and RNA concentrations were determined using Qubit (Thermo Fisher Scientific) and Nanodrop (Thermo Fisher Scientific). Quality of RNA-preparations was assessed using Lab-on-a-chip Bioanalyser 2000 technology (Agilent technologies).

Gene Expression Arrays. To characterize gene expression, total RNA was extracted from fresh frozen biopsies. Total RNA concentration and purity were measured using a spectrophotometer (Nanodrop ND8000). RNA integrity was monitored by electrophoresis (Agilent Bioanalyzer, RNA 6000 Nano Assay). Gene expression analysis was conducted according Affymetrix recommendations to hybridize Human Gene ST2.1 arrays. Briefly, 100 ng of total RNA (range of RIN was 2.8-10; average, 7.9) were processed in parallel with an external MAQC A RNA to control robustness and reproducibility of enzymatic steps. Amplified and labelled molecules were monitor in order to hybridize arrays with 2.3 µg of labelled DNA. Raw and normalized data were generated and controlled with Expression console (Affymetrix) at the Institut Curie Genomic facility.

4 . RESULTS

4.1 PEPI score predicts long term outcome after prolonged neoadjuvant letrozole

We treated 68 postmenopausal women with ER+ operable breast cancer with neoadjuvant letrozole followed by surgery. Patients were treated for a median 7.2 months (interquartile range, 5.4-9.2). Median age was 77 years (range, 60-86); 40 (59%) had stage II and 28 (41%) had stage III cancer **[Figure 16, Figure 17, Table 1]**. Twenty-nine patients (42.5%) achieved a complete or partial response as measured by ultrasound; 10 experienced progressive disease within a mean of 5 months and underwent surgery. The median time to achieve a best objective response (complete or partial response) was 6.3 months (range 2-16) **[Figure 18]**. After surgery, patients were classified according to their PEPI score (11) **[Table 2]**. Thirteen (19%) patients had a PEPI score 0, 36 (52%) were PEPI 1-3, and 19 patients (28%) were PEPI ≥4. Adjuvant treatment consisted of endocrine therapy (96%), chemotherapy for 14 patients (20.5%) with high risk features (10 with PEPI≥4, 4 with PEPI 1-3), and radiotherapy (57%) for those patients who underwent breast conserving surgery or mastectomy if the primary tumor was ≥4 cm or had ≥4 axillary lymph nodes involved with cancer [Figure 19]. With a median follow-up of 58 months (range, 50-80), 13 patients (19%: 8 with PEPI≥4, 5 with PEPI 1-3) exhibited a breast cancer recurrence (12 metastatic, 1 loco-regional). The 5-year recurrence free survival rate was 100%, 85% and 61% for PEPI 0, PEPI 1-3 and PEPI ≥4, respectively (Log rank test, p=0.001) **[Figure 20].** Patients with PEPI≥4 continued to exhibit a poor prognosis after adjusting for adjuvant CT (risk of relapse for adjuvant CT, Hazard Ratio (HR): 2.84, p=0.052). The probability of achieving PEPI 0 correlated with a clinical response to neoadjuvant letrozole, with a response rate of 34% for PEPI 0 vs. 5% for PEPI>0 (p=0.002), but not to the length of neoadjuvant treatment **[Table 3]**.

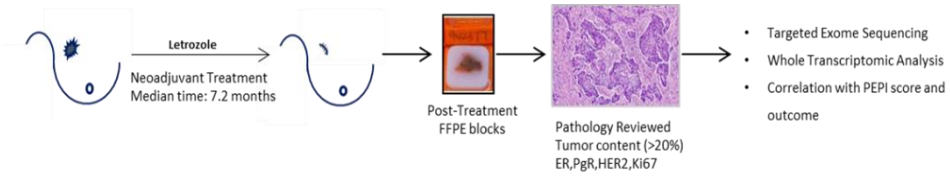

Figure 16: Study Design

	Patients	
Characteristic	**No.**	**%**
Age, years		
60-70	6	8.8
70-80	46	67.6
>80	16	19.5
Tumor Size,cm		
2-5	53	78
>5	15	22
Clinical Stage		
Stage II	40	59
Stage III	28	41
Node status		
Negative	35	51.4
Positive	13	19.1
Unknown	20	29.4
Receptor status		
ER+/PgR+	45	66
ER+/PgR-	19	28
ER-/PgR+	4	6
HER2 status		
Negative	65	95.5
Positive	3	4.5
Ki67 (%)		
0-2	16	23.5
3-7	13	19.1
7-20	3	4.4
>20	27	39.7
Unknown	9	13.2
Histologic subtype		
Ductal Carcinoma	47	69.1
Lobular Carcinoma	13	19.1
Others	8	11.7

Table 1: Patient's Characteristics

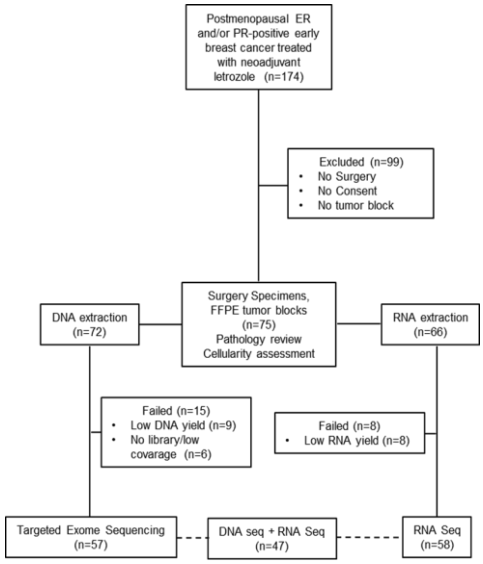

Figure 17: CONSORT diagram

Table 2: The Preoperative Prognostic Index (PEPI) score

Biomarker status	Points
Pathological tumor size	
ypT1/2	0
ypT2/4	3
Node status	
ypN0	0
ypN1-3	3
ER status, Allred score	
0-2	3
3-8	0
Ki67 (%)	
0- 2.7	0
>2.7 – 7.3	1
>7.3 – 19.7	2
>19.7	3

Table 3: Clinical variables associated with the probability of achieving PEPI 0 after neoadjuvant letrozole.

Variable	PEPI 0 (n)	PEPI>0 (n)	Fisher Test (p value)
Baseline Ki67*			
Ki67 >20%	4	23	**1**
Ki67 <20%	5	29	
Time on treatment			
<6 months	8	32	**0.74**
>6 months	4	24	
Best Clinical Response			
CR/PR	11	18	**0. 002**
SD/PD	2	37	
Baseline ER content			
High (+++)	9	25	**0.1**
Low (+/++)	3	31	
Baseline Clinical Stage			
Stage III	3	33	**0. 5**
Stage I-II	8	24	

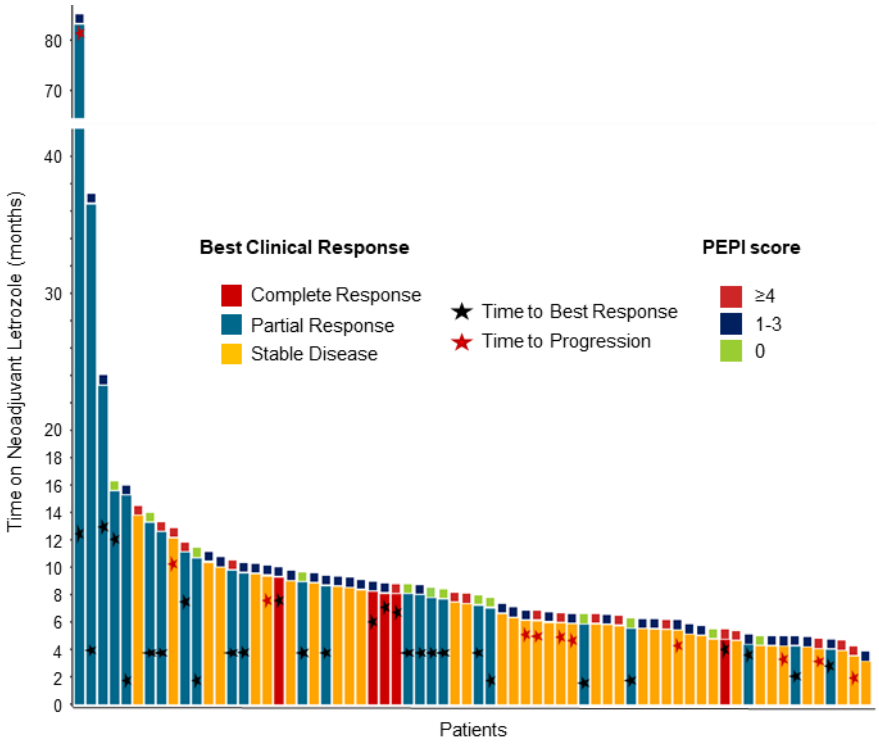

Figure 18: Individual patient response to prolonged neoadjuvant letrozole. Each bar represents a patient. The length of the bar shows duration of therapy; the color of the bar shows the best clinical response observed; stars mark the timing of the response; squares at the end of the bar show the PEPI score achieved.

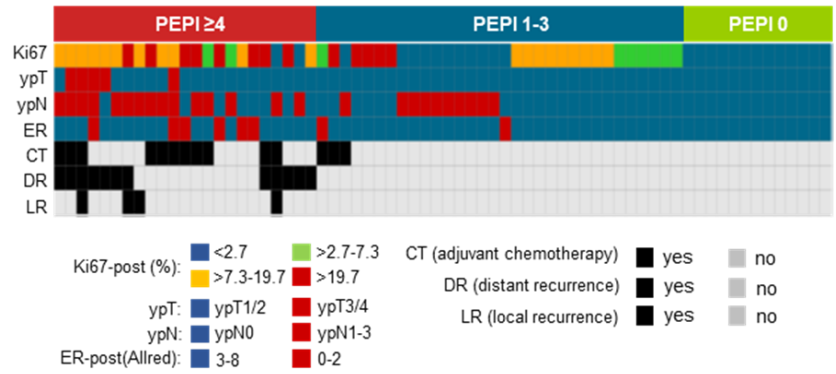

Figure 19: Tile plot representing PEPI score, treatment and outcome. Each column represents a patient and its individual PEPI score assignment, adjuvant systemic treatment and breast cancer events

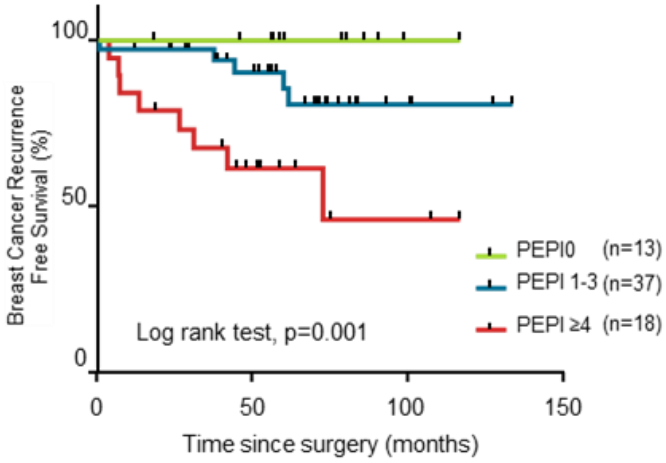

Figure 20: Kaplan-Meir curve for breast cancer recurrence free survival by PEPI score in the study cohort.

4.2 Targeted gene sequencing identifies clinically actionable mutations in endocrine resistant tumors

We performed targeted gene sequencing of 303 cancer related genes, with a median depth of 320X. After applying a filtering algorithm **[Figure 21, Figure 22]**, the median number of mutations per tumor was 4 (range 0-46); in 5 tumors (3 PEPI 0, 2 PEPI 1-3) no somatic mutations were identified.

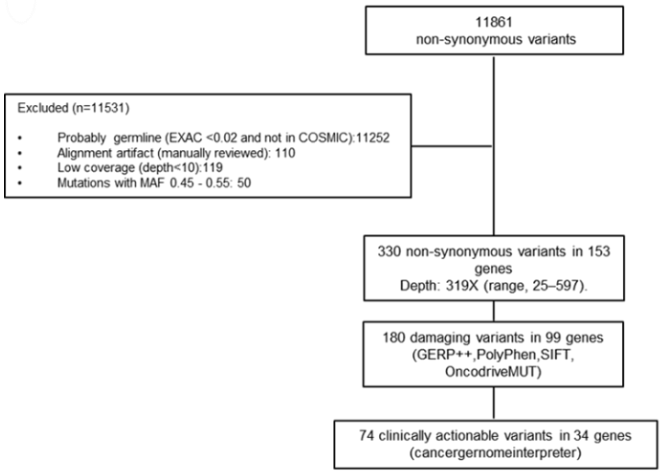

Figure 21: Variant filtering algorithm

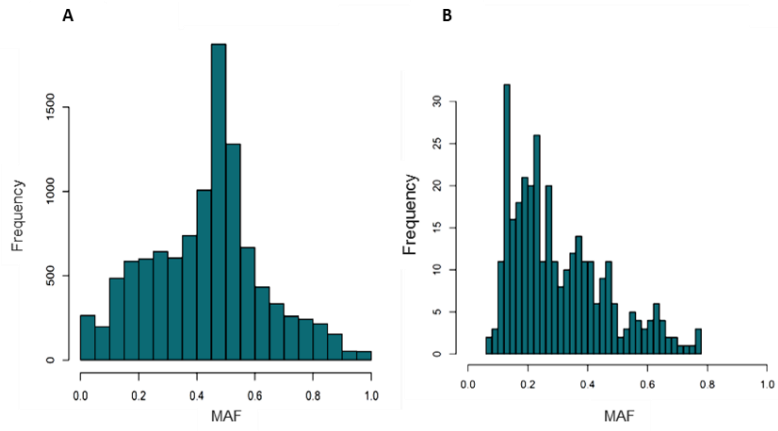

Figure 22: Mutation Allele Frequency (MAF) pre- and post-filtering. A) MAF histogram of 11861 non-synonymous variants. **B)** MAF of 330 non-synonymous variants after filtering out potential germline variants

There were 8 genes mutated in at least 5 patients: *PIK3CA* (40%), *CDH1* (21%), *KMT2C* (16%), *TP53* (14%), *NF1* (9%), *GATA3* (9%), *TBX3* (9%) MAP3K1 (9%) **[Figure 23]**. We detected only 1 *ESR1* ligand binding domain mutation, concordant with their low frequency in patients with progression on adjuvant AIs (12). Using a false discovery rate (FDR)<0.1, 12 genes were more frequently mutated in our cohort of residual tumors after long exposure to letrozole compared to untreated ER+ breast cancer in TCGA. These genes are involved in transcriptional regulation (*MECOM, SETD2, SIN3A, STAG2* and *PRDM1*), DNA repair (*POLE*, *PRKDC*), tumor suppression (*NF1, PHLPP1*), growth factor signaling (*ERBB4, IRS2*) and cytoskeleton remodeling (*EPKK1*) **[Figure 24]**.

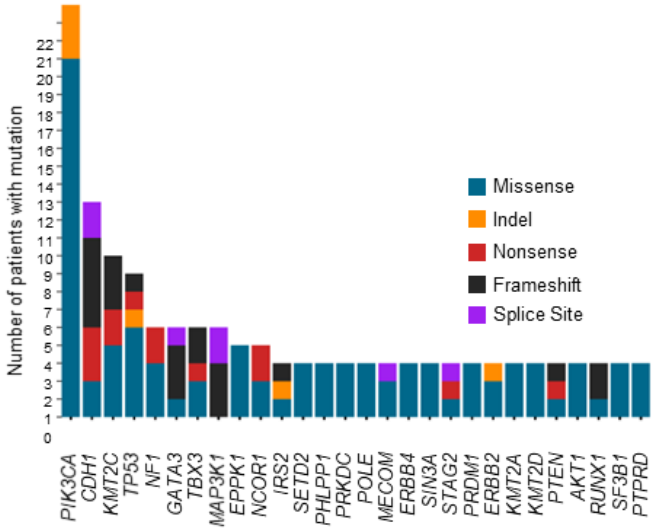

Figure 23: Frequency and type of non-synonymous recurrent gene mutations in 57 tumors from patients treated with neoadjuvant letrozole.

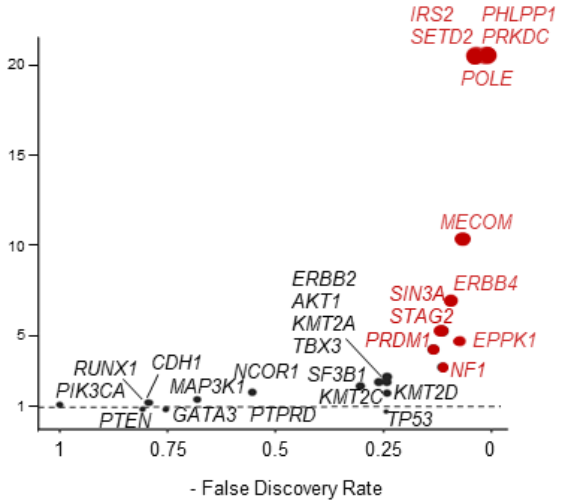

Figure 24: Comparison of mutations detected in this cohort vs. primary untreated ER+ breast cancers in TCGA [Cell 2015, (45)]; in red are genes with Fisher test FDR<0.1.

Using RNA-seq data we assigned PAM50 intrinsic subtypes to each tumor and investigated subtype composition after prolonged estrogen deprivation with letrozole. As presented in Figure 25, the distribution of the intrinsic subtypes varied considerably compared to a cohort of untreated ER+ breast cancers in the METABRIC database (13). There was an increase in cancers with Basal-like and Normal gene expression and a decrease in Luminal A tumors, suggesting treatment with letrozole remodeled the transcriptional landscape of these tumors **[Figure 25]**. Tumors with Basal/HER2-enriched gene expression were enriched among the letrozole resistant tumors (9 of 15, or 56%, exhibited a PEPI score ≥4 and none had a PEPI score of 0).

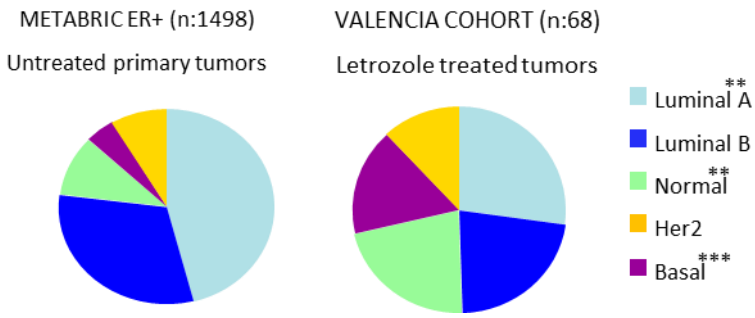

Figure 25: Distribution of PAM50 intrinsic subtypes in the study cohort and in ER+ early breast cancers in METABRIC (p-value by Fisher-test for the comparison among cohorts **<0.001, ***<0.0001)

We found 180 driver mutations in 99 genes (**Digital Suppl. Table DS2**). **Figure 26** shows the distribution of genes with 2 or more driver mutations according to PEPI score, PAM50 subtype and patient outcome. Tumors were classified as non-responder (PEPI ≥4 and/or recurrence) or responder (PEPI <4 and no recurrence). We could not find any statistically significant difference in the frequency of mutations or copy number alterations between the two groups. However, the distribution of these alterations was asymmetrical with some mutations approaching overrepresentation in PEPI ≥4 vs. PEPI 0 (*PIK3CA*: 50% vs 10%, p=.08) and several alterations being absent in tumors with PEPI 0 (i.e., *TP53, AKT1, PTEN, ERBB2*). Other driver mutations, found to be more frequent in the letrozole-treated tumors in this cohort compared to those in TCGA (i.e., *NF1, STAG2, ERBB4, MECOM),* were only detected in tumors with PEPI >0. For CNAs, we focused on allelic imbalances (B-allele frequency>3) in previously reported recurrently altered genomic regions. We detected 159 CNAs in 28 amplicons. These amplicons contained genes such as *CCND1* (16%), *FGFR1* (14%), *MYC* (10%), *ERBB2* (7%) or *ESR1* (5%). Amplicons with copy number loss included genes such as

KMT2C (12%) and *PTEN* (3%). Thirty-nine CNAs events in 9 genes were considered drivers **(Digital Suppl. Table DS3)**.

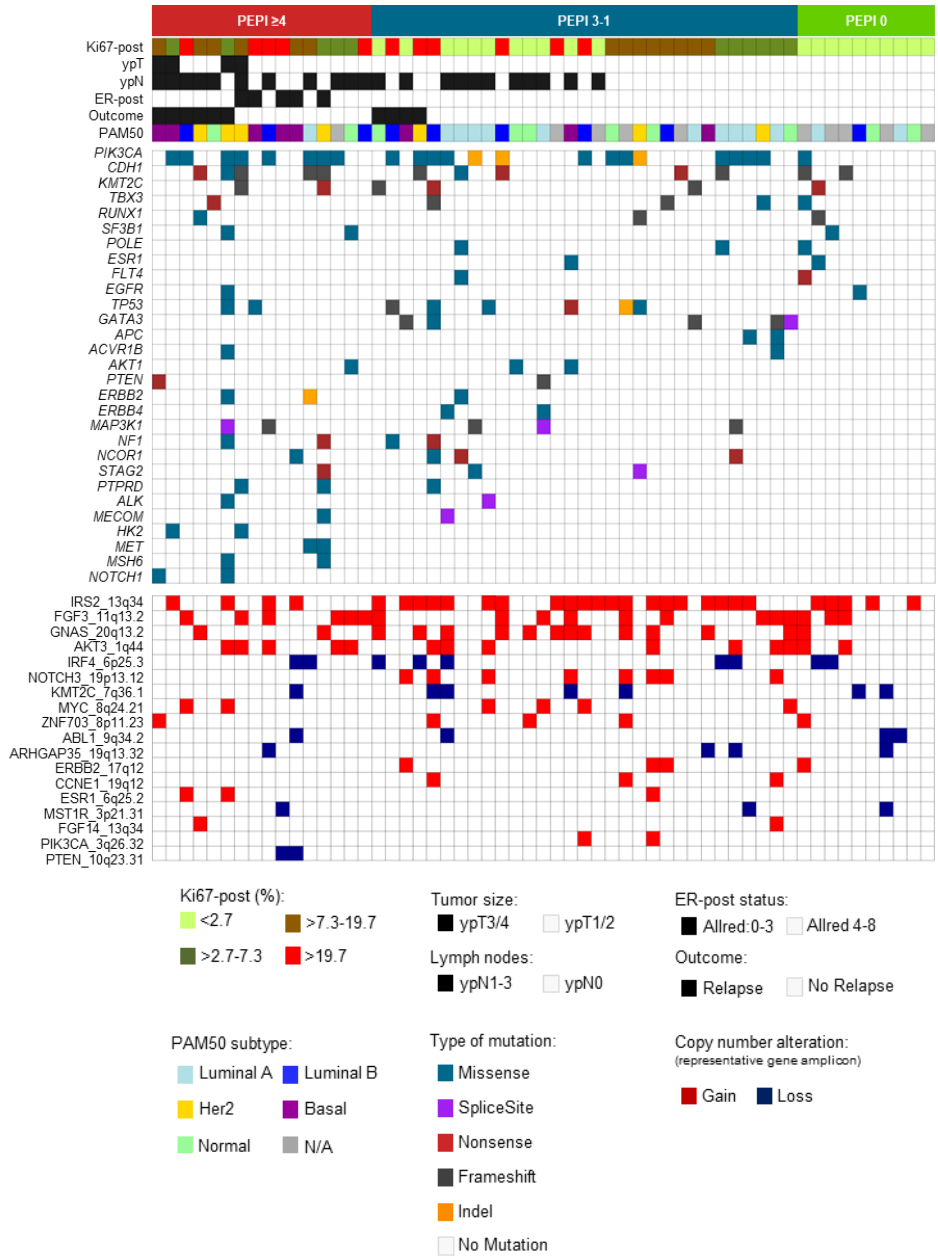

Figure 26: Tile plot showing the distribution of recurrent driver mutations (n≥2) and copy number alterations according to PEPI score and PAM50 subtypes; each column represents a patient.

One hundred two mutations in 48 driver genes and all driver CNAs were classified as clinically actionable. Actionable mutations in the phosphoinositide 3-kinase (PI3K) pathway *(PIK3CA*, *AKT1*, *TSC2,* and/or loss or truncation mutations of *PTEN)* were overrepresented in the PEPI ≥4 group compared to PEPI 0 (70% vs 10%, p=0.003). We also evaluated the association of each actionable mutation or CNA with the expression of a proliferation signature (PCNA), the intrinsic subtype and the PEPI score. This allowed us to identify a subset of druggable somatic alterations (i.e., *NF1* loss, *TP53*, *NOTCH1*, *FGFR4*, *JAK1*, *PTPRD*) associated with multiple poor prognosis features (e.g., high PEPI, high PCNA score, and luminal B/HER2-enriched/basal subtypes), thus supporting the development of drugs targeting these alterations **[Figure 27]. (Digital Supplemental Table DS4 and DS5)**.

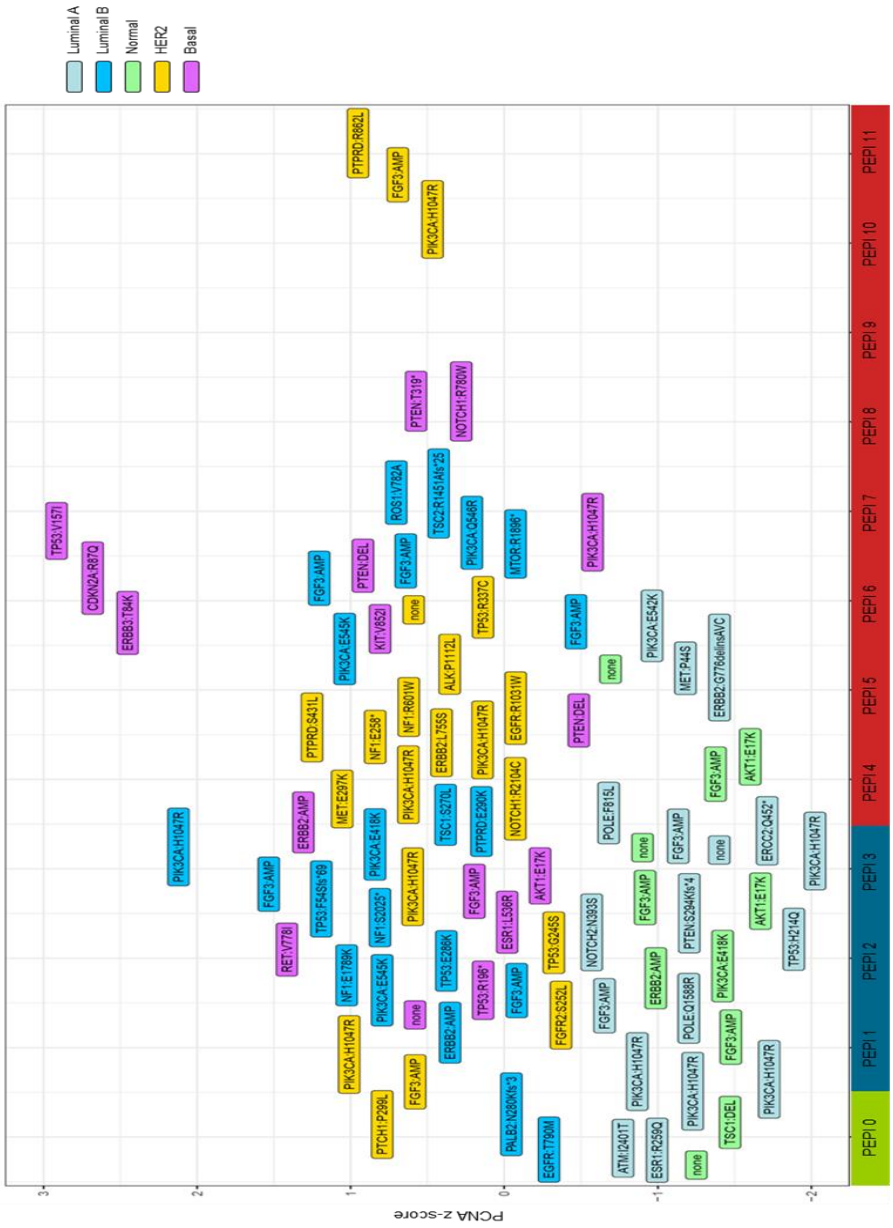

Figure 27: Distribution of clinically actionable mutations according to PEPI score, PCNA proliferation signature and breast cancer subtypes

4.3 Endocrine resistant tumors show enrichment in genes involved in proliferation through heterogeneous transcriptional and mutational profiles

We next performed comparative transcriptional analyses on 58 tumors. Analysis between responders and non-responders showed 566 DE genes with a FDR<0.05, dominated by upregulated genes (n=458) in non-responder vs responder tumors **[Figure 28]**. Gene ontology (GO) enrichment analysis of DE genes showed that non-responding tumors were enriched for cell cycle related genes while no-overlap was found among responders **[Figure 29]**.

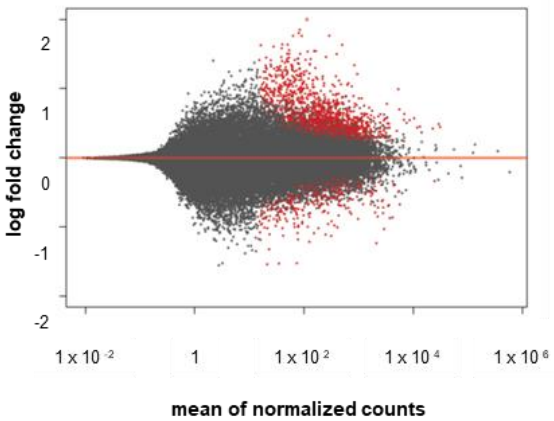

Figure 28: MA plot showing the log2 fold changes from non-responders (PEPI ≥4 and/or breast cancer recurrence) over responder tumors (PEPI <4 and no breast cancer recurrence) of normalized counts (i.e., the average of counts normalized by size factor). Points in red represent normalized counts with an adjusted p-value <0.05

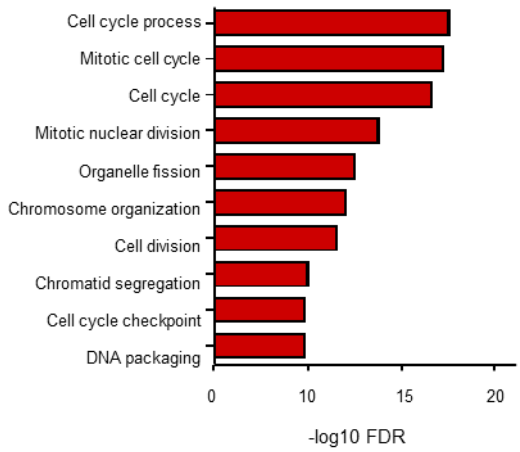

Figure 29: GO enrichment of genes overexpressed in non-responder tumors

To analyze the degree of variability among the tumors in their transcriptional response to estrogen deprivation with letrozole, DE genes between responders and non-responders were used to perform a correlation matrix followed by unsupervised hierarchical clustering. This analysis revealed two main clusters: one relatively homogenous cluster integrating most of the responders and a second heterogeneous cluster enriched with non-responders. Measurement of the transcriptional diversity showed a greater average distance to the median for the non-responder compared to responding tumors (permutation test for homogeneity of multivariate dispersions <0.01). We also observed significant heterogeneity in somatic mutations between the two groups, with a greater mean number of mutations in non-responders vs. responders (12 vs 3, p<0.0001) **[Figure 30]**.

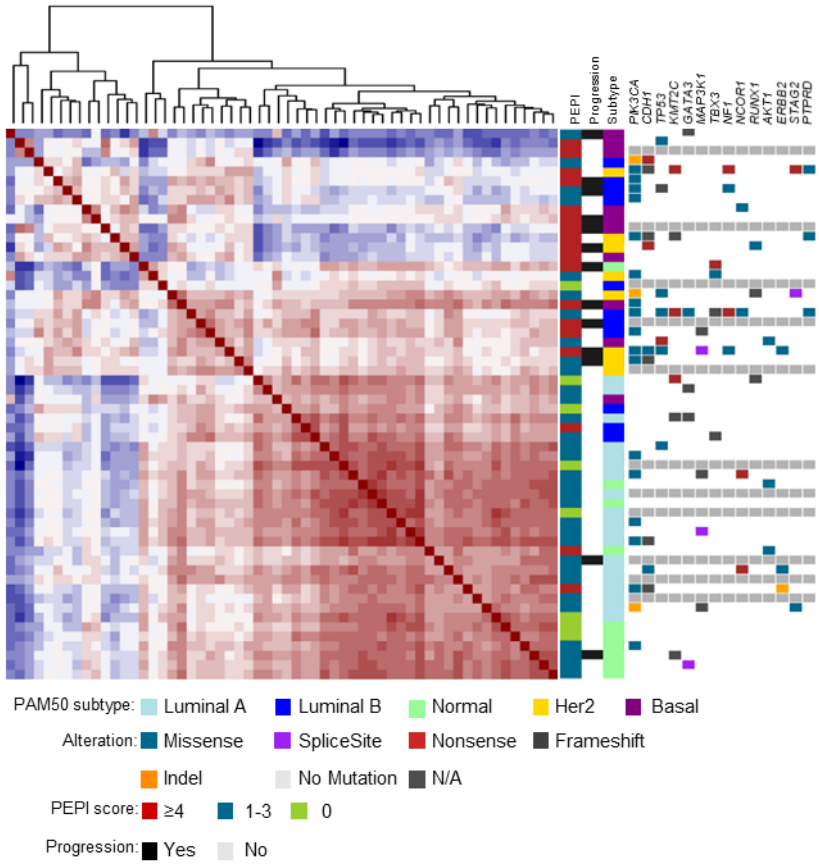

Figure 30: Dendrogram and unsupervised clustered correlation matrix (red positive and blue negative correlation) of 58 breast cancers using Pearson distance. Differentially expressed genes between responders and non-responder tumors were used to compute Pearson distance among the tumors and subsequent hierarchical clustering. Recurrent mutations, PEPI score, patient outcome, and PAM50 subtype are represented in columns in the right panel for each case

To investigate processes that are enriched in responders vs non-responders, we analyzed differential signature enrichment using a set of 125 previously published breast cancer-related gene expression signatures (14). Thirty-six signatures were enriched in non-responders and 4 in the responding tumors (FDR<0.05). Endocrine resistant tumors showed an enrichment of a diverse set of signatures involved cell cycle/proliferation, signaling pathway (EGFR1, PI3K, RAS), DNA repair, breast cancer stemness, ER signaling, and resistance to chemotherapy. Endocrine sensitive tumors were enriched for signatures involved in p53 signaling, genes associated with ER expression, lactic acidosis response and Fos-Jun kinase signaling **[Figure 31]**. These results suggest some ER+ breast cancers adapt to evade estradiol deprivation through different transcriptional programs that ultimately confer the ability of sustain cell cycle progression.

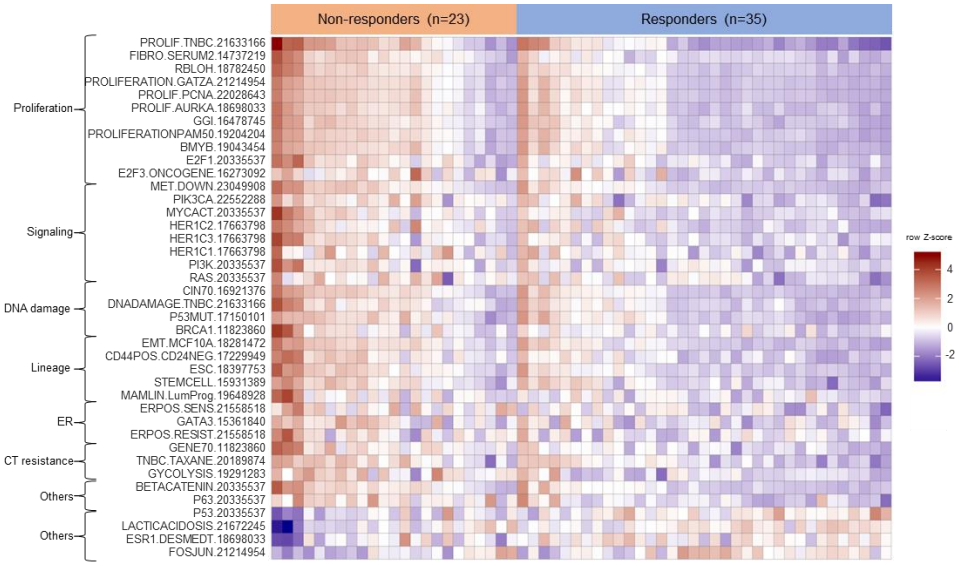

Figure 31: Single sample gene set enrichment analysis using a set of 125 breast cancer related signatures (signature name_# PUBMED ID) shows differentially enrichment (FDR<0.05) of 40 signatures between responder and non-responder tumors

4.4 A CDK4/6 inhibitor-sensitive E2F4 transcriptional program is associated with estrogen-independent proliferation in letrozole-resistant tumors

To identify transcriptional programs with differential activity between sensitive and resistant tumors, we integrated transcription factor binding data from Chromatin Immunoprecipitation (ChIP)-seq studies (CheA 2016 and ENCODE-TF ChIP-seq 2015) with expression of the 47 most upregulated genes in non-responder tumors (log FC>1, FDR<0.03), using the platform Enrichr (15). E2F4 was the transcription factor whose targets demonstrated the most significant overlap with upregulated genes in the resistant list (overlap 20/710, adjusted p=2.56E-15) **[Table 4].**

Derived from ChIP-seq experiment (ENCODE TF and CheA 2016)	Overlap	P-value	Adjusted P-value	Z-score	Combined Score	Genes
E2F4_ENCODE	20/710	3,08E-17	2,56E-15	-1,76978	59,46329	ARHGAP11A;RRM2;HIST1H2BJ; CDCA5;CASC5;TICRR;KIAA1524; TPX2;KIF18A;ORC6;DIAPH3;KIF4A; CDK1;CLSPN;HIST1H3B;PRR11; ZNF367;DTL;BUB1;MAD2L1
SIN3A_ENCODE	18/1131	2,93E-11	1,21E-09	-1,73039	35,52376	RRM2;HIST1H2BJ;CDCA5;HMMR; KIAA1524;ANLN;TPX2;KIF18A;ORC6; CDK1;CLSPN;KPNA2;HIST1H3B;DTL;P RR11;ZNF367;BUB1; MAD2L1
NFYA_ENCODE	18/2250	1,42E-06	3,93E-05	-1,66215	16,86243	RRM2;HIST1H2BJ;HMMR;TICRR; TPX2;KIF18A;ORC6;DIAPH3;CDK1; HIST1H3F;GPC2;CLSPN;KPNA2; HIST1H3B;ZNF367;BUB1;DTL;PRR11
FOXM1_ENCODE	may-95	2,85E-06	4,73E-05	-1,66775	16,60978	TPX2;CDK1;KPNA2;HIST1H3B;PRR11
NFYB_ENCODE	23/3715	2,22E-06	4,61E-05	-1,58108	15,78706	RRM2;HIST1H2BJ;HMMR;TICRR; FAM111B;TPX2;KIF18A;ORC6; DIAPH3;TUBB3;KIF4A;STMN1; HIST1H3F;CDK1;GPC2;PHGDH; CLSPN;KPNA2;HIST1H3B;BUB1; DTL;ZNF367;PRR11
FOS_ENCODE	9/637	1,47E-05	0,000203	-1,6192	13,76335	FAM111B;TPX2;RRM2;HIST1H2BJ; HMMR;KPNA2;HIST1H3B;PRR11; TICRR
IRF3_ENCODE	8/663	0,00014	0,00155	-1,56435	10,12054	FAM111B;TPX2;ORC6;RRM2; HIST1H3B;PRR11;BUB1;TICRR
E2F1_CHEA	9/859	0,000149	0,00155	-1,48242	9,590465	ARHGAP11A;KIF18A;RRM2;CDCA5; HIST1H3F;CASC5;CLSPN;ZNF367; TICRR
NELFE_ENCODE	4/234	0,002196	0,018228	-1,48904	5,963302	RRM2;HIST1H2BJ;STMN1;HIST1H3B
SOX2_CHEA	7/775	0,002078	0,018228	-1,46496	5,866892	HIST1H2BJ;CDCA5;PEG10;STMN1; HMMR;PRR11;TICRR
SP1_ENCODE	6/707	0,005974	0,045074	-1,39384	4,320138	ORC6;HIST1H2BJ;HIST1H3B;PRR11;B UB1; TICRR
PBX3_ENCODE	8/1269	0,008829	0,056369	-1,31184	3,772645	FAM111B;ORC6;DIAPH3;HIST1H2BJ; PADI2; HIST1H3B;PRR11;ZNF367
KLF4_CHEA	7/987	0,007808	0,054007	-1,29042	3,766283	ANLN;TUBB3;STMN1;DTL;PRR11; ZNF367;ASS1
FOSL2_ENCODE	3/196	0,010953	0,061455	-1,25989	3,514413	ANLN;TUBB3;LOXL4
SPI1_CHEA	7/1056	0,011106	0,061455	-1,12911	3,14959	CDCA5;PADI2;ABRACL;HIST1H3B; DTL;MAD2L1;TICRR

Table 4: Overlap between genes upregulated in resistant tumors and transcription factor targets genes according to Chip-seq experiment from ECONDE TF and Chea 2016)

E2F4 is repressed by binding to unphosphorylated Rb. Upon phosphorylation by a cyclinD/CDK4/6 complex, Rb is inactivated and uncoupled from E2F4 which, in turn, can induce transcription of genes associated with progression into S phase of the cell cycle and cell survival (176). Thus, we next tested if the 20 E2F4-regulated genes were overexpressed in ER+ breast cancer cells adapted to long term estrogen deprivation (LTED), and if they could be modulated by treatment with the CDK4/6 inhibitor palbociclib. We found up-regulation of these genes in MCF7/LTED and CAMA1/LTED cells compared to parental MCF7 and CAMA1 cells, respectively **[Figure 32].**

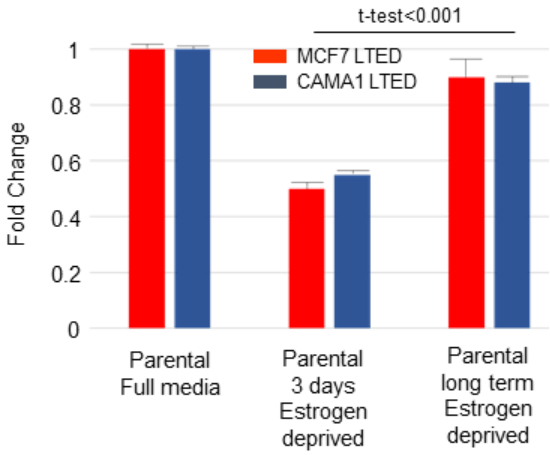

Figure 32: The expression levels of 20 E2F4-regulated genes overexpressed in non-responder tumors were assessed by RT-PCR in MCF7 and CAMA1 long term estrogen deprived (LTED) cells and normalized to their expression in MCF7 and CAMA1 parental cells, respectively. Data are presented as the 20 genes mean fold change +/- SEM

Treatment with palbociclib significantly downregulated the expression of all 20 E2F4-regulated genes (median decrease 78%) with a simultaneous decrease in P-RB levels. Treatment with the ER downregulator fulvestrant or with paclitaxel only

partially suppressed the expression of this set of genes and had no effect on P-RB levels **[Figure 33,Figure 34, Figure 35, Figure 36].**

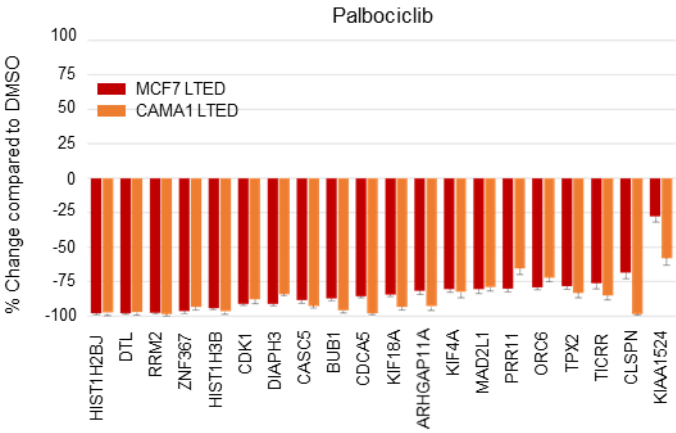

Figure 33: Expression levels of the of 20 E2F4-regulated genes overexpressed in non-responder tumors were assessed by RT-PCR in MCF7/LTED and CAMA1/LTED cells after treatment with palbociclib 1 µM for 24 h.

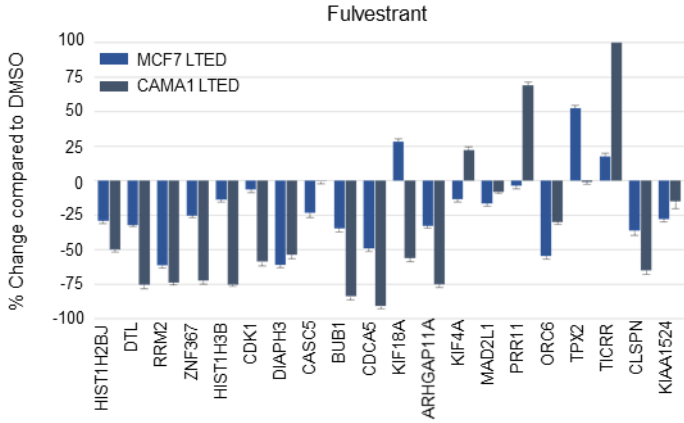

Figure 34: Expression levels of the of 20 E2F4-regulated genes overexpressed in non-responder tumors were assessed by RT-PCR in MCF7/LTED and CAMA1/LTED cells after treatment with Fulvestrant 1 mM for 24 h.

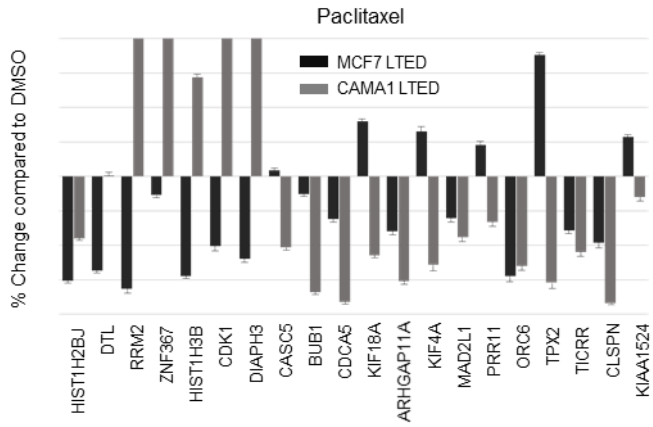

Figure 35: Expression levels of the of 20 E2F4-regulated genes overexpressed in non-responder tumors were assessed by RT-PCR in MCF7/LTED and CAMA1/LTED cells after treatment with Paclitaxel 20 nM for 24 h.

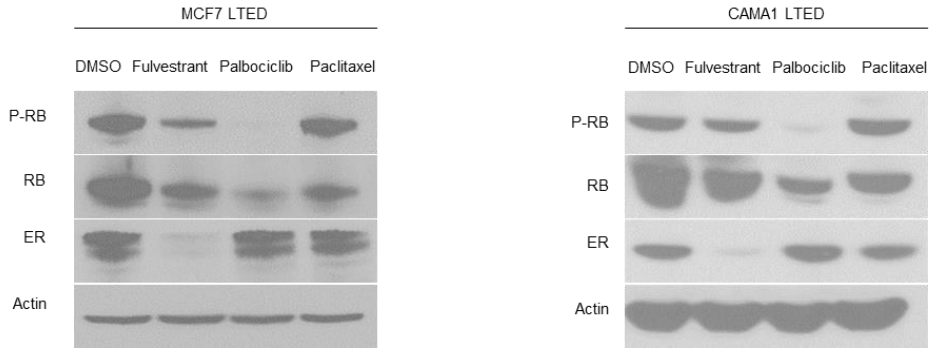

Figure 36: Immunoblots of lysates from MCF7/LTED or f) CAMA1/LTED cells treated with DMSO, fulvestrant 1 µM, palbociclib 1 µM or paclitaxel 20 nM for 24 h.

Next, we investigated if these genes could be modulated in primary breast cancers from patients enrolled in the Pre-Operative Palbociclib (POP) trial (NCT02008734) (177). In this study, patients with newly diagnosed, operable ER+/HER2– breast cancer received palbociclib daily or placebo x14 days leading up to breast cancer surgery. Tumor cell proliferation and CDK4/6 inhibition were assessed by Ki67 IHC and P-RB IHC, respectively, in a pre-treatment biopsy and in the (post-treatment) surgical specimen. Consistent with the inhibition of CDK4/6, treatment with palbociclib induced a significant reduction of P-RB levels and Ki67(177). Next, we used gene expression array data from pre- and post-palbociclib tumors in this trial to assess expression of the 47 most upregulated genes in the tumors resistant to prolonged neoadjuvant letrozole (Fig. 1). Treatment with palbociclib, but not with placebo, significantly decreased expression of 24 of 47 of these resistance-associated genes (FDR<0.01); among these were 18 of the 20 E2F4 target genes **[Figure 37, Table 5)**.

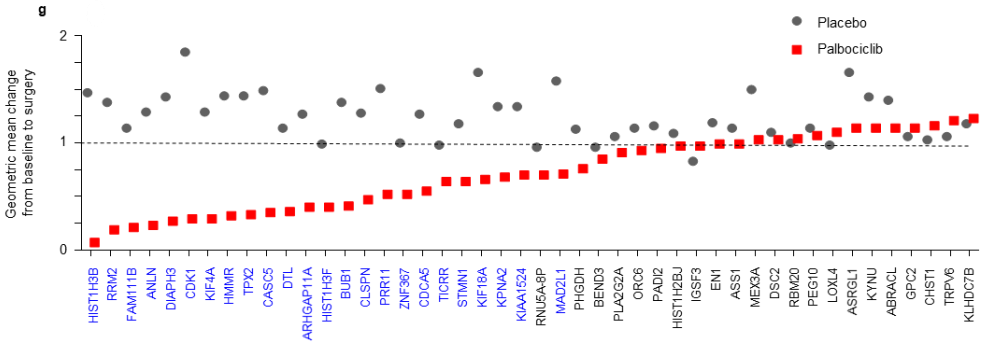

Figure 37: The geometric mean change, between baseline and surgery, for the top 47 genes associated with letrozole resistance in the study cohort, were assessed in tumor samples from 60 ER-positive/HER2-negative primary tumors treated with placebo or palbociclib for 15 days in the POP trial (NCT02008734); in blue text are genes predicted to be E2F4 targets.

Gene.Symbol	Gene description	FDR p-value	Geometric mean change Control	Geometric mean change Palbociclib
CASC5	cancer susceptibility candidate 5	0	1,49	0,35
RRM2	ribonucleotide reductase M2	0	1,38	0,19
TPX2	TPX2, microtubule-associated	0	1,44	0,33
PRR11	proline rich 11	0	1,51	0,52
HMMR	hyaluronan-mediated motility receptor (RHAMM)	0	1,44	0,32
CDCA5	cell division cycle associated 5	0	1,27	0,55
ANLN	anillin, actin binding protein	0	1,29	0,23
BUB1	BUB1 mitotic checkpoint serine/threonine kinase	0	1,38	0,41
ARHGAP11A	Rho GTPase activating protein 11A	0	1,27	0,4
HIST1H3B	histone cluster 1, H3b	0	1,47	0,07
CDK1	cyclin-dependent kinase 1	0	1,85	0,29
FAM111B	family with sequence similarity 111, member B	0	1,14	0,21
KPNA2	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	0,00001	1,34	0,68
KIAA1524	KIAA1524	0,00005	1,34	0,7
STMN1	stathmin 1	0,00005	1,18	0,64
DTL	denticleless E3 ubiquitin protein ligase homolog (Drosophila)	0,00005	1,14	0,36
HIST1H3F	histone cluster 1, H3f	0,00006	0,99	0,4
KIF18A	kinesin family member 18A	0,00013	1,66	0,66
KIF4A	kinesin family member 4A	0,00018	1,29	0,29
ZNF367	zinc finger protein 367	0,00064	1	0,52
MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)	0,00089	1,58	0,71
DIAPH3	diaphanous-related formin 3	0,00134	1,43	0,27
TICRR	TOPBP1-interacting checkpoint and replication regulator	0,00562	0,98	0,64
CLSPN	claspin	0,01516	1,28	0,47
PHGDH	phosphoglycerate dehydrogenase	0,10455	1,13	0,76
ASRGL1	asparaginase like 1	0,18905	1,66	1,14
ORC6	origin recognition complex, subunit 6	0,19573	1,14	0,93
MEX3A	mex-3 RNA binding family member A	0,2111	1,5	1,03
KYNU	kynureninase	0,2111	1,43	1,14
HIST1H2BJ	histone cluster 1, H2bj	0,24137	1,09	0,97
BEND3	BEN domain containing 3	0,26716	0,96	0,85
EN1	engrailed homeobox 1	0,30229	1,19	0,99
ABRACL	ABRA C-terminal like	0,30229	1,4	1,14
LOXL4	lysyl oxidase-like 4	0,30229	0,98	1,1
RNU5A-8P	RNA, U5A small nuclear 8, pseudogene	0,30229	0,96	0,7
IGSF3	immunoglobulin superfamily, member 3	0,32113	0,83	0,97
ASS1	argininosuccinate synthase 1	0,37422	1,14	0,99
PADI2	peptidyl arginine deiminase, type II	0,4693	1,16	0,95
GPC2	glypican 2	0,47575	1,06	1,14
CHST1	carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	0,54653	1,03	1,16
DSC2	desmocollin 2	0,7046	1,1	1,03
PLA2G2A	phospholipase A2, group IIA (platelets, synovial fluid)	0,72682	1,06	0,91
RBM20	RNA binding motif protein 20	0,73747	1	1,04
KLHDC7B	kelch domain containing 7B	0,85831	1,18	1,23
PEG10	paternally expressed 10	0,8636	1,14	1,07
TRPV6	transient receptor potential cation channel, subfamily V, membe	0,88245	1,06	1,21

Table 5: Change from baseline to surgery in gene expressions of the 47 most upregulated genes in the tumors resistant to prolonged neoadjuvant letrozole from 60 HR-positive/HER2-negative breast cancers in POP trial. A decreased gene expression translates into a geometric mean change < 1 . An increased or stable gene expression translates into a geometric mean change ≥ 1.

4.5 An E2F4 target gene signature is associated with resistance to neoadjuvant and adjuvant endocrine therapy.

We generated a signature of E2F4 transcriptional activation, using the genes associated with resistance to neoadjuvant letrozole and relapse in our cohort that were also significantly downregulated by palbociclib treatment in tumors in the POP trial, as compared to the placebo control group. We next assessed the ability of this set of genes to predict breast cancer recurrence in ER+ treated with endocrine therapy. First, we tested the signature in the cohort of patients treated with prolonged neoadjuvant letrozole and showed that tumors within PEPI ≥4 had significantly higher E2F4 activation signature than tumors with PEPI 1-3 or PEPI 0 **[Figure 38 a]**, and was also moderately correlated with post-treatment Ki67 levels **[Figure 38 b]**. Further, the 5-year relapse-free survival was 100%, 79% and 45%, for patients in the low, medium or high tertile of the E2F4 gene expression signature, respectively (log-rank test, p=0.0015) **[Figure 38 c].**

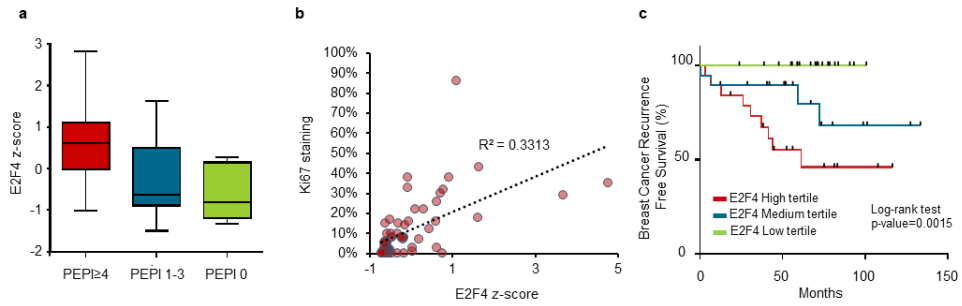

Figure 38: E2F4 signature in the study cohort. **a)** An E2F4 activation signature was enriched in tumors with PEPI≥4, **b)** correlated with post-treatment Ki67 levels and **c)** was associated with increased risk of breast cancer recurrence in the cohort of patients treated with prolonged neoadjuvant letrozole.

To externally validate the performance of the signature, we used gene expression data from patients treated with neoadjuvant AIs in the ACOSOG Z1031B study (n=110) (173). In this trial, tumors that failed to achieve a complete cell cycle arrest (CCCA), defined as an on-treatment, 2-week Ki67 ≤2.7%, exhibited a high E2F4 signature score **[Figure 39]**.

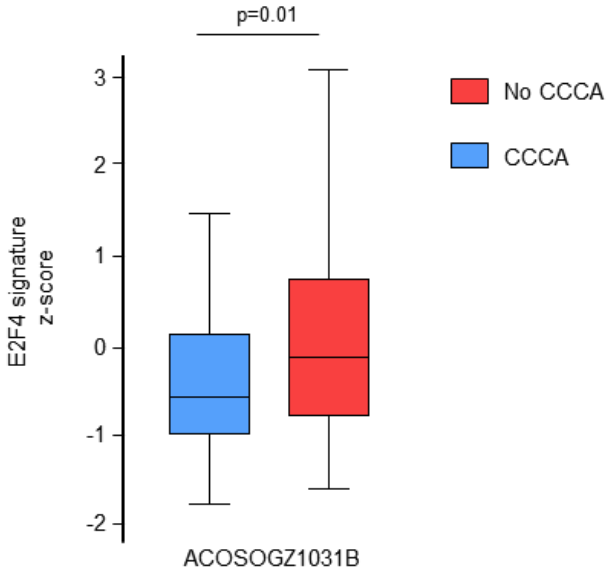

Figure 39: Box plots comparing the E2F4 signature score in ER+/HER2– tumors from patients in the ACOSOG Z1031B study (n=110, NCT01953588) after treatment with an aromatase inhibitor (AI). According to the 2-week Ki67 score, tumors were classified as achieving complete cell arrest (CCCA, Ki67 ≤2.7%) or no-CCCA (Ki67 >2.7%), p-value for t-test.

Also, in ACOSOG Z1031B, tumors with a high E2F4 score at baseline had a higher baseline Ki67 score and a worse response to AIs compared to patients with a low E2F4 signature score. CCCA rate was 18% vs 50% for high and low baseline E2F4 scores, respectively (p<0.001) **[Figure 40].** Of note, up to 40% of tumors with a high baseline E2F4 score switched to a low E2F4 score after 2-week treatment with an AI **[Figure 41].**

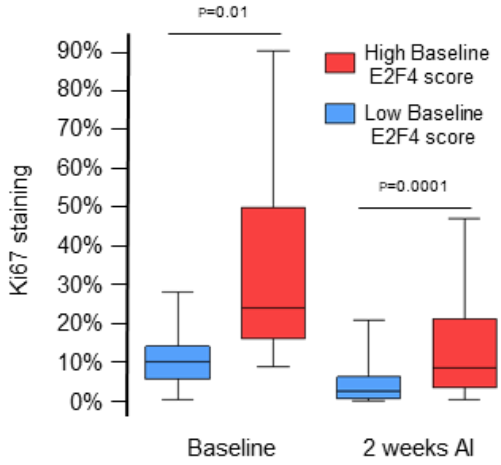

Figure 40: Box plot correlating high vs. low E2F4 signature score with the Ki67 score at baseline and after 2-weeks of treatment with an AI in tumors from the ACOSOG Z1031B study. Tumors with a high E2F4 score at baseline exhibited a lower rate of CCCA upon treatment compared to tumors with a low EF4 score (18% vs. 50%), p-value for t-test.

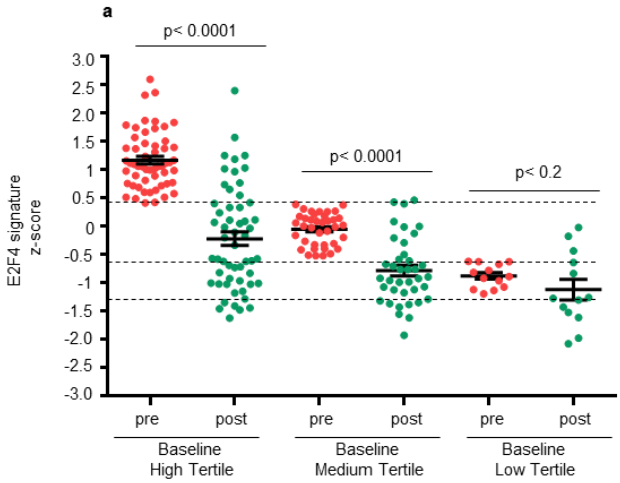

Figure 41: E2F4 gene signature expression at baseline (pre) and after 2-weeks of aromatase inhibitor treatment (post), data from ACOSOGZ1031b study. Tumors were divided according to the E2F4 score at baseline in high, medium or low tertiles. After two-weeks of aromatase inhibitor treatment, there was a decrease in the E2F4 score in all groups. However, the

proportion of tumors still expressing a high post-treatment E2F4 score was greater in patients with a high E2F4 score at baseline (60%) than the other groups

To assess the predictive value of the E2F4 activation signature in the adjuvant setting, we selected patients with ER+ breast cancer treated with adjuvant endocrine therapy in the METABRIC cohort (n=1408). Patients with E2F4 scores in the higher tertile showed an increased risk of relapse (HR: 2.96, 95% CI: 2.176 - 3.670) and death (HR: 1.59, 95% CI: 1.32-1.94) compared to those in the lower tertile **[Figure 42].**

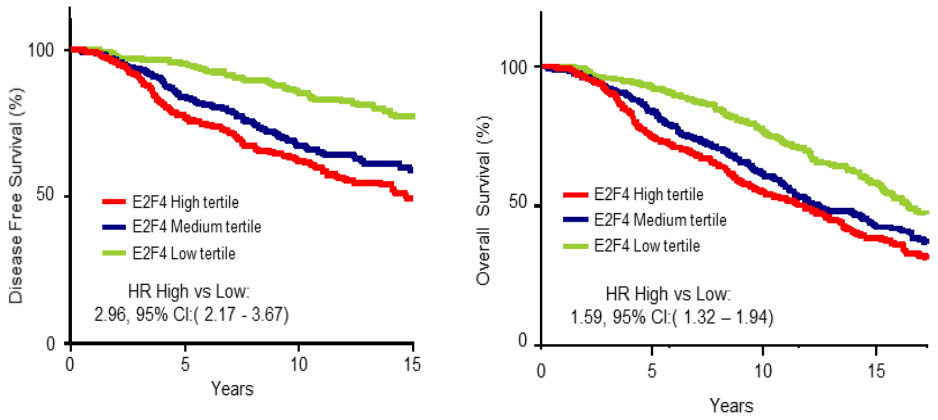

Figure 42: Disease free survival and overall survival in patients with ER+ breast cancer treated with adjuvant endocrine therapy in the METABRIC database (n=1498) according to E2F4 signature score tertiles.

In addition, we evaluated luminal PAM50 subtypes and noted a significant association the E2F4 signature score with survival in both Luminal A and Luminal B breast cancer subtypes **[Figure 43].** Principal component analysis according to E2F4 gene signatures revealed a cleared separation of tumors according to levels of E2F4 gene signature activation **[Figure 44].**

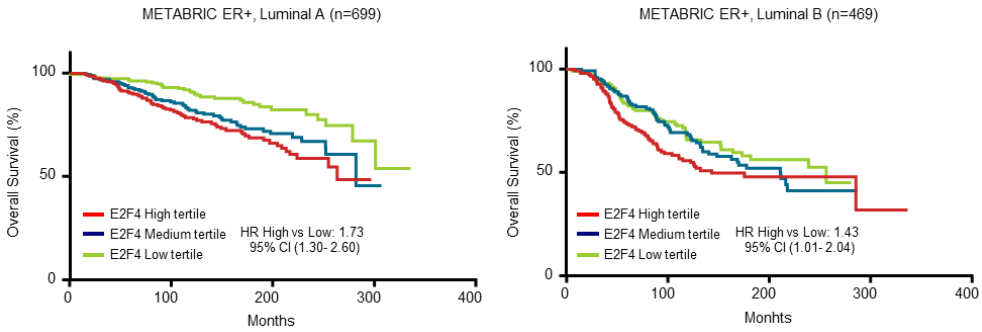

Figure 43: Overall survival in patients with ER+ tumors from METABRIC according to E2F4 score tertiles in Luminal A and Luminal B tumors.

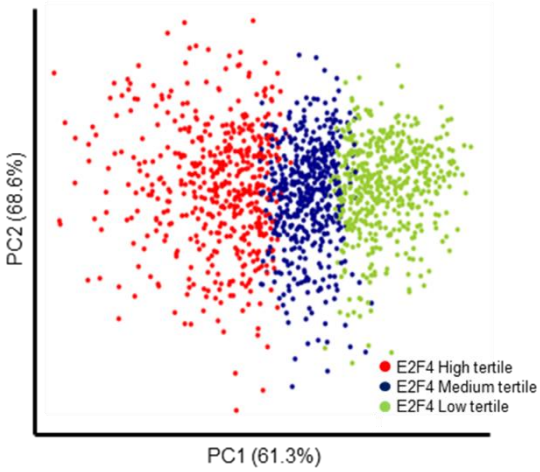

Figure 44: Clustering with principal component (PC) analysis of METABRIC: ER+ Breast Cancer Treated with adjuvant HT (n=1498) according to E2F4 gene signature activation.

Next, we assessed the efficacy of palbociclib in tumors from patients in the POP trial. Treatment for 2 weeks with palbociclib downregulated the expression of all the genes composing the signature, together with suppression of Ki67 and P-RB levels **[Figure 45].**

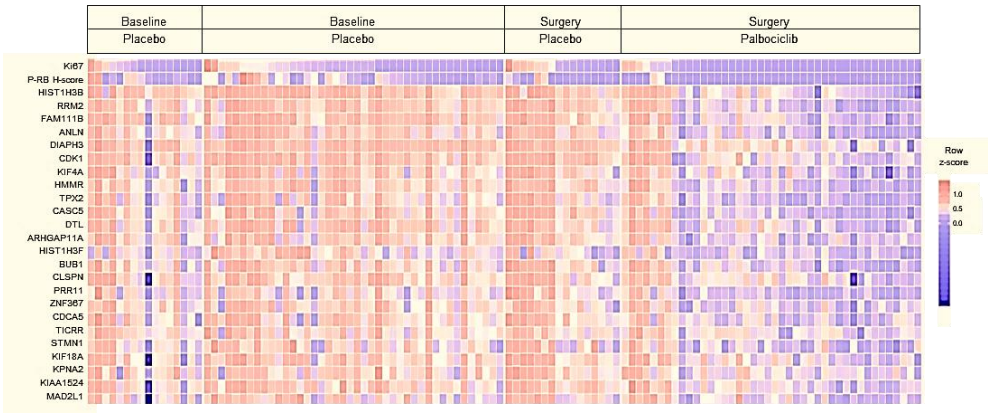

Figure 45: Tile plot showing baseline and surgery gene expression values for each of the components of the E2F4 gene signature, Ki67 and P-RB score from 60 ER+/HER2- tumors treated in the POP trial with either placebo or two weeks of palbociclib.

In the group of tumors expressing levels of E2F4 signature activity above the median (n=30), treatment with the CDK4/6 inhibitor was able to suppress P-RB by 90% but Ki67 by only 67% **[Figure 46]**. We speculate that the partial suppression of Ki67 despite almost complete inhibition of P-RB could be accounted for by the lack of simultaneous antiestrogen therapy.

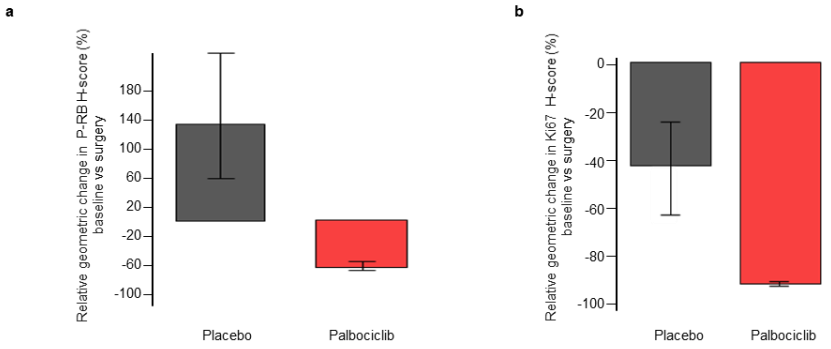

Figure 46: Geometric mean change (± SD) in P-RB H-score (h) and Ki67 score (i) in 30 ER+/HER2– tumor pairs before and after a two-week treatment with placebo or palbociclib. Tumors are those with a high baseline E2F4 score.

In sum, we have identified a CDK4/6 inhibitor-sensitive E2F4 activation signature that defines ER+ breast cancers with poor prognostic features. This signature is of potential use for the identification of patients with ER+ breast cancer candidates for adjuvant therapy with CDK4/6 inhibitors in combination with antiestrogens.

4.6 PRR11 is associated with poor clinical outcome of ER + breast cancers treated with endocrine therapy

In the study cohort PRR11 (Proline rich 11) was one of the most significantly upregulated genes in resistant tumors **[Figure 47a]** and patients with a high PRR11 mRNA level showed an increased risk of relapse (HR=4.1) **[Figure 47b]**.

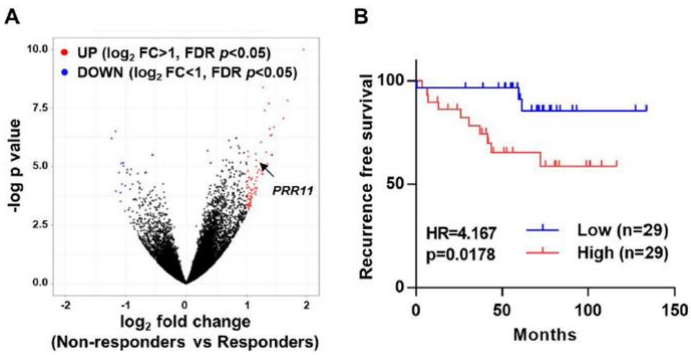

Figure 40: PRR11 is overexpressed in resistant tumors. A) Volcano plot is shown for genes differentially expressed in non-responding tumors compared to responding tumors. Non-responding tumors were defined by cancer relapse after a median follow up of 5 years and/or a preoperative endocrine prognostic index (PEPI) ≥4. B) Recurrence-free survival was plotted against *PRR11* mRNA level in ER +/HER2 negative breast cancers following long-term neoadjuvant letrozole treatment (Log-rank).

The prognostic value of PRR11 was also confirmed in two independent meta-clinic database, KM plotter and METABRIC, where ER+/HER2- tumors with mRNA expression levels of PRR11 above the median show an increase risk of relapse after adjuvant ET (178) **[Figure 48]** .

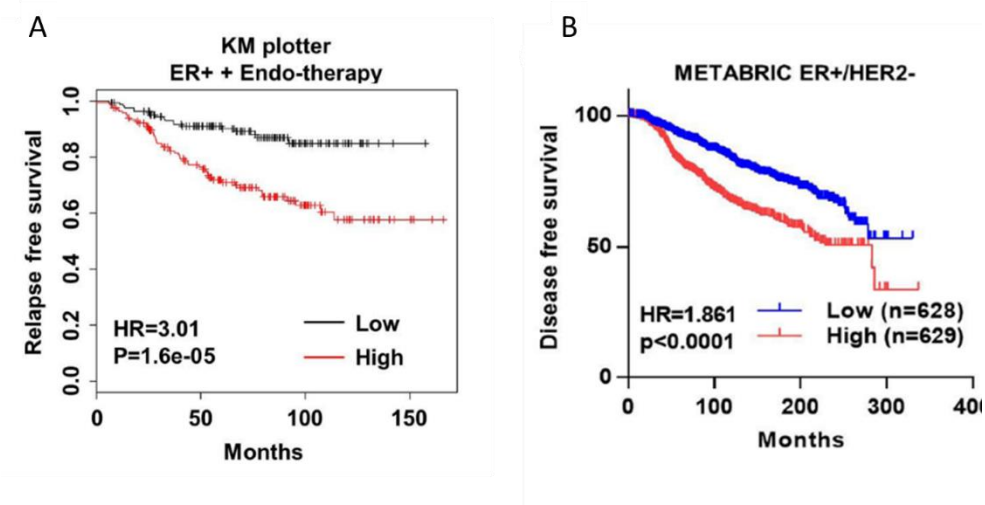

Figure 48: Prognostic value of PRR11 in external cohorts. A) Relapse free survival by *PRR11* mRNA level in KM plotter $ER^{+}/HER2^{-}$ breast cancers treated with adjuvant endocrine therapy. B) Disease free survival by PRR11 mRNA levels in METABRIC ER+/HER2- breast cancer treated with adjuvant endocrine therapy.

Next, we analyzed expression of breast cancer gene signatures in our cohort treated with long term letrozole (169); 10 of 13 proliferation-associated signatures were significantly enriched in tumors with high *PRR11* mRNA expression (FDR<0.01), **[Figure 49]** suggesting that high *PRR11* is associated with poor clinical outcome in ER+ breast cancers and may contribute to breast cancer aggressiveness.

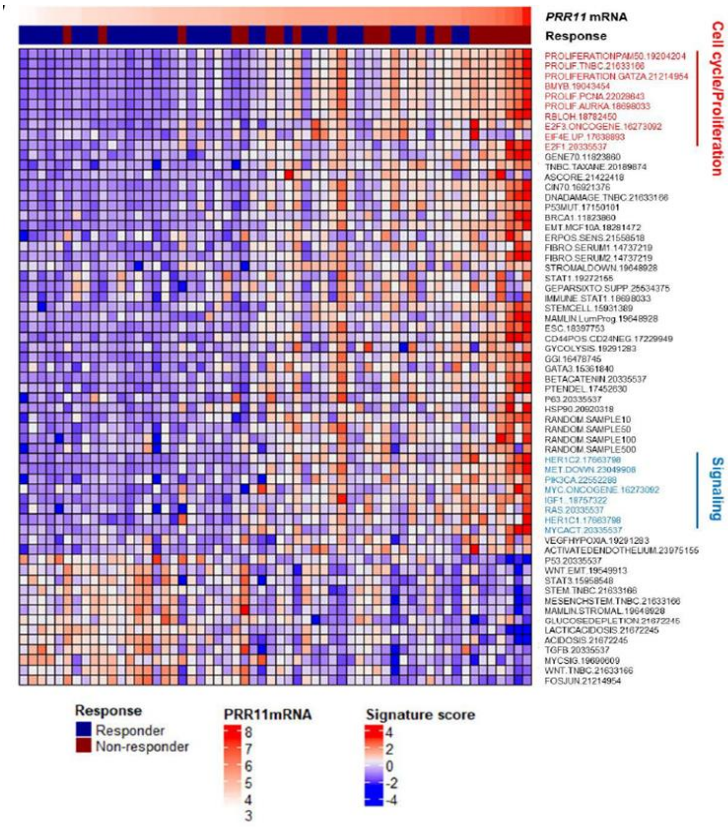

Figure 49: Single sample gene set analysis was performed using a set of 125 breast cancer-related signatures. Gene sets that were differentially enriched (FDR<0.01) between *PRR11* high and low tumors are shown

4.7 *PRR11* amplification is associated with proliferative gene signatures in ER + breast cancer

PRR11 (Proline rich 11), is a protein-coding gene located in chromosome 17q21-23. About 65% of breast tumors harboring 17q21-23 amplification are classified in the

luminal B subtype, which typically display a poor outcome compared to luminal A cancers (59). Several genes in the 17q21-23 amplicon have been suggested to be associated with poor outcome in breast cancer (59,179). To investigate which gene(s) in this amplicon may play a critical role in breast cancer cell survival, we interrogated genome-scale RNAi screening data of MCF7 cells in Project Achilles (v2.4.3) (180). These cells harbor 17q23 amplification (181). Short hairpin RNA (shRNA) scores were derived by calculating the median from individual shRNA $\log_2$ fold change (FC) scores passing quality control. Based on the Atlas of Genetics and Cytogenetics (182), we identified 527 genes located in the 17q21-23 locus; shRNAs targeting 241 of these 527 genes were evaluated in Project Achilles. Of these 241 genes, the *PRR11* shRNA's score was the 4th lowest, thus implying a strong anti-survival effect as a result of *PRR11* suppression **[Figure 50; lower panel]**. In addition, among those genes in 17q21-23 assessed by Project Achilles, *PRR11* predicted the highest relative risk of relapse in the meta-clinic cohort of ER$^+$ breast cancers treated with endocrine therapy **[Figure 50; upper panel]**. These data suggest that *PRR11* is a key gene in the 17q21-23 amplicon that promotes cell proliferation/survival and endocrine resistance of ER$^+$ breast cancers and warrants further investigation to understand its mechanisms of action.

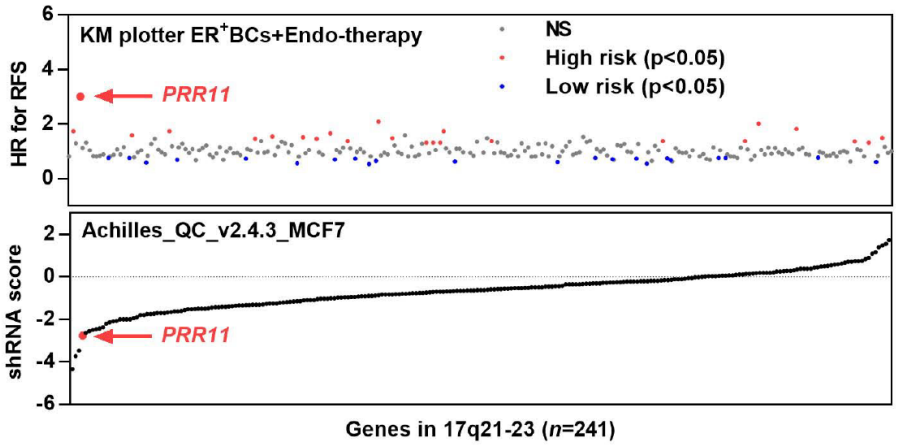

Figure 50: PRR11 is a putative driver in 17q21-23 locus. Hazard ratio for relapse free survival of genes in 17q21-23 was calculated by Kaplan-Meier Plotter. Red, blue, and grey dots represent genes exhibiting significantly high, low, and non-significant hazard ratio, respectively (Log-rank $p<0.05$; upper panel). shRNA score of genes in 17q21-23 from Project Achilles (lower panel) were plotted in the same order as the upper panel.

In sum, gene expression profiling of resistant tumors to long term estradiol deprivation in the neoadjuvant setting, identifies PRR11, a gene located in 17q21_23 locus as a putative driver of endocrine resistance.

5 . DISCUSSION

Neoadjuvant endocrine therapy offers an opportunity to discover functional genomic alterations associated with drug resistance that may inform post-operative adjuvant treatment. Most of the clinical trials exploiting this concept have been restricted to short-term presurgical exposure to AI where patients were treated for 2 to 4 weeks before surgery (146,173,183). Although informative, these studies do not address the long-term effect of AI therapy that may be necessary to evaluate the full impact of AI-induced phenotypic/genotypic alterations and to capture acquired mechanisms of resistance. In this study, we performed targeted DNA and whole transcriptome sequencing in residual ER+ breast cancers treated with letrozole for a median of 7.2 months. In addition, the long term follow up of our cohort (58 months) allowed us to integer relapse and PEPI score to define two clear patients' groups (sensitive and resistance to endocrine therapy). This phenotypic difference at the clinical level was also apparent at the genomic level. We were able to find mutations predominantly present in resistance tumors, and a highly different transcriptional profile of resistance cases compared to sensitive.

We found a higher number of mutations in patients with a poor response to estrogen deprivation with letrozole, confirming other studies (184),(185), and also suggesting a source of genetic diversity that may identify cancers that recur after adjuvant endocrine therapy. In agreement with other studies (186), we detected a different composition of intrinsic molecular subtypes to what would be expected in a cohort of untreated ER+/HER2 negative postmenopausal breast cancers. The increase in tumors with a Normal subtype and reduction in Luminal A tumors suggest a change induced by treatment, while the increase in tumors of the Basal-like subtype suggests a loss of luminal expression and the outgrowth of endocrine resistant cancer cell sub-populations.

We did not detect recurrent mutations or copy number alterations significantly enriched in tumors resistant to letrozole. However, there was a numerical increase in few clinically actionable mutations, such as *NF1* loss and in genes like *JAK1, NOTCH1*,

FGFR4 and *PTPRD*, whose role in endocrine resistance has not yet been elucidated. We found a greater number of mutations in the PI3K pathway (*PIK3CA*, *AKT1*, *TSC2*, and/or loss or truncation mutations of *PTEN*) associated with poor response to letrozole, but only those PI3K pathway mutations in Luminal B/HER2-enriched/Basal tumors were associated with poor features (high PEPI score, high proliferation, and breast cancer relapse). The PI3K-mTOR pathway is the most frequently altered pathway in ER positive breast cancer and plays a critical role in endocrine resistance (68). In the metastatic setting, HR+/HER2 negative breast cancer patients with acquired resistance to endocrine therapy benefit from targeting the PI3K-mTOR pathway with everolimus and in patients with *PIK3CA*-mutant, addition of alpelisib (PIK3 alfa inhibitor) has shown to improve progression-free survival . However, in early-stage breast cancer presence of *PIK3CA* mutations does not affect the risk of recurrence of early ER+ breast cancer treated with adjuvant ET (187), and *PIK3CA* mutation neither preclude a response to neoadjuvant AI (188) nor correlate with neoadjuvant PI3K inhibition efficacy (189,190). Thus, it follows from these studies that early-stage *PIK3CA*-mutant breast cancers may be less dependent on PI3K signaling compared with recurrent or metastatic disease.

However, our data shows a subset of Luminal B/highly proliferative/resistant tumors harboring PI3K pathway mutation together with PI3K-mTOR pathway gene expression activation, suggesting a reliance of the tumor on the PI3K pathway for growth and survival. This dependence PI3K signaling of early stage breast cancer for growth might vary by breast cancer subtype. Luminal A tumors show a disconnect between PIK3CA mutation an biomarkers of pathway activation, while Luminal B tumors show an hyperactive PI3K pathway signaling (191). A window of opportunity study has shown that adding the PI3K inhibitor pictilisib to anastrozole significantly suppress proliferation in Luminal B but not in Luminal A tumors (192). So, our data suggest a context dependent role of PI3K mutations and merits further investigation to clarify if PI3K inhibitors might have a role in the adjuvant treatment of breast cancer that evade estrogen deprivation through increase PI3K signaling activation.

While *ESR1* mutations are very rare (<1%) in untreated ER+/HER2- early breast cancer, in the metastatic setting, acquired *ESR1* mutations are a stablished mechanism of endocrine resistance exclusively to long term exposure to AI. *ESR1* mutations are found exclusively in ER+ breast cancer patients previously exposed to AI and its associated shorter progression-free survival on subsequent AI-based therapy. *ESR1* mutation prevalence in ctDNA differs between patients exposed first to AI during the adjuvant setting (6%) and metastatic settings (40%) (193,194). Due to the extended duration of letrozole in our study (median 7.5 months), we were expecting to detect an enrichment for *ESR1* mutations, however, the incidence was just 1.5%. This contrast with results from a similar neoadjuvant study with letrozole for up to 6 months that report tissue *ESR1* mutations in 7% of cases after treatment. One explanation for this difference is that in the latter study they used digital droplet PCR (ddPCR) that allow identification of rare genomics events with a MAF as low as 0.2% while we used WES to a median covered of 320X which might have a decrease sensitivity for very low MAF events (195).

By applying a comprehensive set of breast cancer related gene signatures, we showed that multiple pathways are involved in evading estrogen deprivation, including gene signatures related to growth factor receptor, RAS and PI3K signaling, and cancer cell stemness. Different to some prior reports (196), we did not observe an enrichment in immune related gene expression signatures.

Integration of the 47 most upregulated genes in letrozole-resistant tumors with transcription binding data identified a set of genes controlled by the E2F4 transcription factor. Consistent with activation of E2F4 by cyclin D/CDK4/6 complexes, treatment with the CDK4/6 inhibitor palbociclib downregulated this set of genes in primary ER+ breast cancers simultaneous with a reduction in P-RB levels and tumor cell proliferation measured by Ki67 IHC. The prognostic ability of this set of genes was independent of adjuvant chemotherapy, suggesting they may also be causal to chemotherapy resistance. In line with this hypothesis, paclitaxel was not able to suppress this set of

genes in vivo. We found a marked upregulation of the E2F4 gene expression signature in AI-resistant tumors from patients in the ACOSOG Z1031B study. Of note, the endocrine resistant tumors in ACOSOG Z1031B were also resistant to neoadjuvant chemotherapy and several of the genes that compose the signature (i.e., *KIF4A, KIF18A, DIAPH3, TPX2*) have been causally associated with resistance to chemotherapy (198,199).

The role of E2F in endocrine resistance has been previously documented by (200) (201). Our results agree with studies that have shown the prognostic value of an E2F4 signature in ER+ breast cancer (202). This signature, based on 199 E2F4 target genes, identified by in vitro ChiP-seq experiments, remains a significant prognostic factor in the adjuvant setting even after adjusting for clinic-pathological variables and adjuvant therapy (endocrine and/or chemotherapy). The finding that current adjuvant treatments cannot improve the prognosis of patients exhibiting high expression of this E2F4 signature also agrees with our results that only CDK4/6 inhibition, and not chemotherapy nor fulvestrant, is able to suppress completely E2F4 target gene expression. However, the same authors reported that high levels of the 199-gene E2F4 signature are predictive of pCR to neoadjuvant chemotherapy in ER+ breast cancer (203). Although this might seem contradictory, transcriptional activity of E2F4 is a marker of highly proliferative tumors, which is a recognized predictive factor of an initial response to chemotherapy. However, if a tumor does not respond to preoperative endocrine therapy or chemotherapy, it is also known that high proliferation (measured by Ki67) is a marker of poor prognosis (153). Thus, we believe that a pharmacodynamic assessment of proliferation, particularly in ER+ tumors, can clearly unmask highly proliferative tumors with a poor prognosis. Our E2F4 signature differs from the E2F4 signature mentioned above mainly on the biological and clinical contexts from which it is derived. Instead of in vitro data, we used on-treatment primary tumor data from a cohort of patients with ER+ breast cancer treated preoperatively with standard of care letrozole. The prognostic ability of our E2F4 signature has been validated externally in a presurgical clinical trial (POETIC), showing

that high levels of E2F4 activation is associated with poor anti proliferation response after 2 weeks of AI (183). All this suggest the presence of an ER-independent E2F4 gene expression program in tumors resistant to estradiol deprivation that can be blocked by inhibition of CDK4/6 and not by chemotherapy. Thus, CDK4/6 inhibitors would be an excellent therapeutic strategy against ER+ breast cancers where antiestrogens do not inhibit tumor cell proliferation, and/or other pharmacodynamic surrogates like the E2F4 score described herein.

Several studies have explored the efficacy of CDK4/6 inhibitors (CDK4/6i) and ET over ET alone as preoperative treatment. In all of them, the combination induced a more potent cell cycle arrest than endocrine therapy alone (204–207). This high activity of CDK4/6i in the neoadjuvant setting supports several ongoing phase III adjuvant trials evaluating the potential for CDK4/6i to enhanced adjuvant ET. If adjuvant studies with CDK4/6i mirrors results from preoperative studies, then CDK4/6i will obtain the indication in the adjuvant setting for ER+/HER2- breast cancer. Due to inclusion criteria within ongoing adjuvant studies evaluating CDK4/6i, these drugs are being explored mostly in a post-chemotherapy setting. So, one question adjuvant studies with CDK4/6i won´t answer is if CDK4/6i could avoid the need of adjuvant CT.

To test this hypothesis, we have designed the CARABELA trial within the Grupo Español de Investigación en Cáncer de Mama (GEICAM) group. CARABELA is an international, multicenter, open-label, randomized phase II study in the neoadjuvant setting for high-intermediate risk ER+/HER2- breast cancer patients, defined by TNM and Ki67. Pre- and postmenopausal patients will be treated with prolonged neoadjuvant abemaciclib + letrozole (+/- aLHRH) for up to 1 year or standard neoadjuvant chemotherapy. Main inclusion criteria are:

1. HR+/HER2- breast cancer
2. Centralized Ki67 >20%; T2 (> 2cm) – T3, T4b, N0 – N2, M0 (stages IIA, IIB, IIIA or IIIB). Subpopulation with tumors T2 N0 M0 will include high risk

patients based on Ki67 index > 30% or Ki67 index between 20-30% and PgR negative with or without histological grade 3.

Patients will be stratified by tumor stage (II vs III) and by Ki67 levels (>30% vs <30%). The primary end-point of the study is RCB 0-1 in both treatment arms. A Bayesian design has been used to define the most appropriate sample size (n=200) to detect a RCB0-1 rate of 20% for the abemaciclib arm. Although the primary end point of the study is RCB, it offers an excellent opportunity to explore additional biomarkers of efficacy that might capture better the action of CDK4/6i such as our E2F4 signature. Another biomarker we are planning to study is the performance of OncotypeDx® RS in each treatment arm. OncotypeDx RS is a widely use commercial 21 gene expression signature that quantifies the risk of relapse in ER+/HER2-negative early stage breast cancer (208) and predict chemotherapy benefit (209,210). Results from TAILOR-Rx (120) trial shows that adjuvant chemotherapy can be safely spared in patients with low or intermedium risk of recurrence. Benefit of adjuvant chemotherapy in patients with high-RS (> 30) is in part because those patients harbor tumors that are resistant to standard ET. The margin of benefit of CT in high-RS ER+/HER2-negative breast cancer could be lower in the context of a more potent ET regimen, such as the combination of ET+CDK4/6i. Our study CARABELA provides an ideal scenario to test the predictive and prognostic ability of OncotypeDx RS in the context of CDK4/6I and chemotherapy. We are planning to study the predictive ability of baseline RS (as a continuous and categorical variable) for several end-points of efficacy such as:

a. RCB 0-1
b. PEPI score 0
c. Ki67 <2.7% at surgery and after 2-3 weeks of treatment
d. Conversion rates from mastectomy to breast conservative surgery
e. ctDNA negativity after surgery
f. Event free survival

We would also assess rate of OncotypeDx RS downstaging (baseline to surgery) from high-intermediate to low. And since the study will follow patients up to 10 years after surgery, we will study the association of post-treament OncotypeDx RS with DFS.

Two neoadjuvant studies have already compared CT vs CDK4/6i + ET in the neoadjuvant setting of ER+/HER2-negative breast cancer :

1) NeoPAL study (211): randomized, parallel, non-comparative, phase II study. Patients with ER+/HER2-negative, Prosigna®- defined luminal B, or luminal A and nodal status (N) positive, stage II–IIIA breast cancer were assigned to either letrozole and palbociclib during 19 weeks, or to CT (FEC100 followed by docetaxel). The primary end point was RCB 0–1, and the study was designed to detect a 20% RCB0-1 rate for letrozole/palbociclib. One hundred and six randomized patients had a median Prosigna® score of 71, thus leading ~85% of tumors to be classified as 'high-risk'. The study was considered negative for the hypothesis of superiority of letrozole/palbociclib (RCB 0-1 CT: 15%, letrozole/palbociclib: 7%). pCR were 5.9% and 3.8% for CT and letrozole/palbociclib, respectively. Clinical response (75%) and BCS rates (69%) were similar in both arms. Interestingly, final median Ki67 (3% vs 8%) and PEPI score 0 rate (17.6% vs 8.0%) favoured letrozole/palbociclib vs. CT.

2) CORALLEEN study (212): a parallel, randomized phase II clinical trial for 106 postmenopausal, stages I-IIIA, luminal B according to PAM50 intrinsic subtype breast cancer patients. This study compared neoadjuvant CT (adryamicin/doxorubicin and cyclophosphamide 4 cycles followed by weekly paclitaxel during 12 weeks) vs. neoadjuvant letrozole/ribociclib for 24 weeks. Primary endpoint was rate of the score risk-of-recurrence (ROR)-low according to the Prosigna test. At baseline, of the 106 patients, 92 (87%) patients had high-ROR disease. At surgery, 23 (47%) of 49 patients in the ribociclib/ letrozole group and 24 (46%) of 52 patients in the chemotherapy

group were low-ROR. Same as NeoPal, RCB 0-1 rates were higher for CT over letrzozole/ribociclib (11.1% vs 6.8%), but PEPI score favored letrozole/ribociclib over CT (PEPI score 0: 22.4% vs 17.3%).

Both studies, NeoPal and Coraleen, suggest that ET+CDK4/6i might not be inferior to CT encouraging studies to de-escalate CT from the (neo)adjuvant treatment of selected ER+/HER2-negative breast cancer. However, is uncertain if PEPI score or ROR dowsnatging would be a validate surrogate marker of long-term outcome in the context of CDK4/6i. Although PEPI scores have shown preliminary validity as surrogate markers of long-term outcome, it should be noted that this has been based on studies that used the same endocrine therapy in the neoadjuvant and adjuvant parts of the trial. Regarding, prognostic gene expression signatures such as ROR, is unknown the value of a posttreatment score and how to integrate it with the prognostic information of residual tumor and lymph node. So, it remains to be seen whether the enhanced antiproliferative differences observed with CDK4/6 inhibitors trial will translate into an effect on time to recurrence in ongoing adjuvant studies. An interesting observation is that antiproliferative effects of CDK4/6i, measured by Ki67, are reversible after patients stopped CDK4/6 inhibitor treatment but continued ET until surgery (205), suggesting the need for continuous treatment with CDK4/6 inhibitors to maintain cell cycle arrest and questioning if a few months of CDK4/6i therapy could have any effect on eradicating micrometastases . Some key differences among CARABELA and NeoPal and Coralleen studies are:

1) Study design: CARABELA is a randomized comparative study. A Bayesian design has been used to define the most appropriate sample size (n=200) .The major advantage of this Bayesian approach is to allow us to evaluate how similar RCB 0-1 rates between both treatment arms are, without using a very large non-inferiority study. On the contrary, NeoPal and Coraleen are non-comparative

studies, so conclusions on the non-inferiority of CDK4/6 inhibitors over chemotherapy cannot be claimed.

2) Treatment duration: Current preoperative studies in HR+/HER2 negative are optimized regarding the CT duration (4-6 months), but not for CDK4/6i/ET duration. The optimal length of CDK4/6i/ET treatment in the neoadjuvant setting is unknown. NeoPal and Coraleen administered CDK4/6i for 4-6 months. However, previous experience of NET with AIs monotherapy suggest that some patients might benefit from longer NET duration (125,155). So we hypothesize that a longer treatment with CDK4/6 inhibitors is needed to better capture mechanism of action of CDK4/6 inhibitors ,beyond cell cycle arrest, such as induction of senescence, immune cell recruitment and apoptosis (213). In fact, in the metastatic setting abemaciclib/ET achieves, in patients with measurable disease, an impressive 60% overall response rate, in the upper range of CT (214). Interestingly, the response rate increases with treatment duration (45% tumor reduction at 6 months vs. 64% at 12 months).

3) Abemaciclib treatment. Although in the metastatic setting the three CDK4/6 inhibitors (palbociclib, ribociclib and abemaciclib) are considered equivalent, abemaciclib has some particularities and advantages over palbociclib and ribocilib. Transcriptional and proteomic changes induced by the three drugs differ significantly; abemaciclib has unique cellular activities including induction of cell death (even in pRb-deficient cells), arrest in the G2 phase of the cell cycle, and reduced drug adaptation. These activities appear to arise from inhibition of kinases other than CDK4/6 including CDK2/Cyclin A/E and CDK1/Cyclin B (215). Based on this enhanced activity of abemaciclib and longer treatment duration of up to one year in CARABELA study, we expect to detect and RCB 0-1 rate of 20%, similar to what is achieved by chemotherapy in HR+/HER2-negative breast cancer and superior to RCB 0-1 rate reported with ribociclib (6%) and palbociclib (7%) in Coraleen and NeoPAL studies.

4) Novel surrogate biomarkers of response. During CARABELA trial we will track circulating tumor DNA (ctDNA), both in the preoperative and postoperative part of the study. ctDNA is an emerging biomarker in early stage breast cancer employed to measure minimal residual disease. Several studies have shown that detection of plasma ctDNA, following surgery and adjuvant therapy, is associated with increased risk of relapse and anticipates clinical or radiologic metastatic relapse. ctDNA could be of value to measure the impact of CDK4/6i on micrometastatic disease and might complement other biomarkers of response to NET.

Other ongoing studies comparing CT with CDK4/6i in the neoadjuvant setting are:

- NEOLBC study (NCT03283384): an ongoing randomized, multicenter, open-label, phase II clinical trial in n=100 postmenopausal patients with HR+/HER2-negative, stages II/III BC. Based on Ki67 levels after two weeks of initial letrozole treatment, patients are advised to continue ET with letrozole (if Ki67 level <1%) or to be randomized between standard CT vs. letrozole in combination with ribociclib (if Ki67 ≥1%). The primary endpoint is to measure the difference in CCCA defined as Ki67 <1% determined by IHC between letrozole plus ribociclib vs. CT in the surgical specimen (around seven months after starting the initial treatment with letrozole) and to determine if letrozole plus ribociclib is associated to a ≥100% improvement in CCCA as compared to CT in the surgical specimen.

- PREDIX LumB study (NCT02603679): an ongoing randomized phase II clinical trial in n=200 luminal A/B BC patients with regional lymph node metastases, comparing weekly paclitaxel vs. standard ET plus palbociclib for 12 weeks; after 12 weeks treatment is switched crossover. During the 24-

weekly treatment period, clinical and radiological evaluations are performed repeatedly. Switch between the treatment groups is allowed in case of lack of response or toxicity. Postoperatively, patients receive 3-weekly courses of CT with a combination of epirubicin and cyclophosphamide (EC). The primary endpoint is radiological objective response rate after completion of the first 12-week treatment period.

Comparative genomic hybridization (CGH) studies have reported that gain or amplification of 17q21-23 occurs in approximately 20% of breast cancers (216,217). The 17q23 locus is amplified in ≈7% of breast cancers and defines the cluster 1 (IntClust1) in the METABRIC classification (58). IntClust1 is predominantly composed of highly proliferative ER+ luminal B breast cancers, exhibits an intermediate prognosis and relatively high level of genomic instability. IntClust1 is one of four ER+ IntClust associated with a high risk of late recurrence (218).The cytogenetic band of PRR11 is designated 17q22 or 17q23.2 (Ensembl or HGNC, respectively), located at the terminal region of 17q22 close to the 17q23 region. Initial studies aimed at identifying potential oncogenes drivers in 17q23 focused on genes that are both amplified and overexpressed. The mTOR effector *RPS6KB1* (S6K1) and *TBX2* were first proposed as putative candidates following extensive mapping of the amplicon in breast tumors and breast cancer cell lines (219). Subsequent comprehensive analysis of copy number and gene expression predicted *MUL*, *APPBP2*, and *TRAP240* as potential oncogenes (220). However, functional studies of these alterations have been incomplete. In our analysis, integrating the genome-scale shRNA screening data with survival data of patients with ER+ breast cancer suggests that PRR11 is more strongly associated with breast cancer progression than the genes proposed previously. The functional consequences of PRR11 overexpression in models of ER+/HER2-negative breast cancer and the mechanism of action to drive endocrine resistance is currently under study, and has been recently communicated in San Antonio Breast Cancer Symposium (221).

6 . CONCLUSIONS

In conclusion, we have identified genomic alterations and transcriptional phenotypes in a cohort of ER+/HER2– breast cancers resistant to prolonged estrogen deprivation.

ER+/HER2- breast cancer evades estrogen deprivation through an heterogenous transcriptional and mutational profile that ultimately converge on sustained cell proliferation.

This enhance proliferation of resistant tumors is driven by an ER-independent E2F4 gene expression program. We identified a gene expression signature of E2F4 target activation that identifies HR+/HER2-negative breast cancer with poor outcome in the neoadjuvant and adjuvant setting.

CDK4/6 inhibition suppressed E2F4 target gene expression in estrogen-deprived ER+ breast cancer cells and in patients' ER+ tumors. This suggests a potential benefit of adjuvant CDK4/6 inhibitors in patients with ER+ breast cancer who fail to respond to preoperative estrogen deprivation.

We also, identified PRR11, a gene in the 17q21-23 amplicon, as a novel putative driver of endocrine resistance in ER +/HER2- breast cancer. Integrative analyses including clinical data from the study cohort and genome-scale shRNA screening data strongly suggested a role for PRR11 in endocrine resistance.

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