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Investigating the molecular diversity of luminal breast cancer arising in young women

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ABSTRACT

Breast cancer (BC) is the most common malignancy in women and a highly heterogeneous disease. In young women (<40y), BC is uncommon (approximately 7% of all BC) but clinically significant. Even after adjusting for tumor stage, grade and subtype, young patients exhibit worse response to standard therapies, higher risk of relapse and lower overall survival compared to older patients particularly evident in the Luminal subtype (LBC). However, young LBC biology remains poorly understood and models that accurately reflect the molecular characteristics of this pathology are still lacking.

To capture LBC heterogeneity and to investigate the molecular mechanisms driving cancer progression and drug resistance in young patients we collected a large cohort of samples from young pre-menopausal (<45y, Y) and non-Y post-menopausal (>55y, nY) LBC patients and we performed a comprehensive mutational profiling analysis using Next Generation Sequencing (NGS) approaches. Samples were sequenced first using a small custom-made amplicon-based panel, targeting four of the most frequently mutated genes in LBC (PIK3CA, AKT1, TP53, GATA3), then by whole exome sequencing (WES).

Targeted NGS analyses revealed that the Y group exhibited a higher number of mutated (81% vs 62%, Y vs nY) and co-mutated (30% vs 18%, Y vs nY) patients, in these four considered genes. In detail, mutations in PIK3CA/AKT1 and GATA3 were significantly enriched in Y patients (57% and 34%, respectively, vs 36% and 14%, Y vs nY). Interestingly, we found that Y women harbor also higher frequency of PIK3CA/AKT1 co-mutations with GATA3 (16% vs 7%, Y vs nY) or TP53 (17% vs 12%, Y vs nY), especially in the luminal B subtype.

WES analyses confirmed the enrichment of PIK3CA (40% vs 32%, Y vs nY) and GATA3 (19% vs 5%, Y vs nY) mutations in Y patients, although it was evident that many subclonal ones were lost. Additionally, WES data revealed other genes mostly mutated in Y (ERBB2, FAT3, CBFB and MAP3K1) and in nY (LRP1B, EPHA3 and CSMD3) patients. Copy number variation (CNV) analysis revealed that Y patients displayed TP53 deletions (55% vs 44%, Y vs nY) and GATA3 amplification (45% vs 25%, Y vs nY) at higher frequency compared to nY ones.

Despite the well-known relevance of PIK3CA and TP53 pathway in oncogenesis, the role and crosstalk of PIK3CA/TP53 co-mutations (PIK3CA/TP53^{co-mut}) is still unclear, especially in the context of LBC. Therefore, we decided to investigate the biological impact of PIK3CA/TP53^{co-mut} on mammary tumorigenesis, using primary luminal breast epithelial (BPE) cells carrying PIK3CA^{E545K}, PIK3CA^{H1047H}, TP53^{R175H} mutants, either alone or in combination. Co-occurring mutations of PIK3CA and TP53 altered the growth of these normal cells, leading to a transformed phenotype, much

more than each of the single mutant alone. Moreover, PIK3CA/TP53^{co-mut} cells displayed lower response to endocrine therapy, chemotherapy and targeted therapy, such as CDK4/6 and PI3K inhibitors, that are commonly used to treat LBC patients.

It has been recently demonstrated that occurrence of PIK3CA and TP53 mutations may be responsible of epigenetic dependency of cancer cells, increasing their sensitivity to epigenetic therapy approaches. However, whether this is true also in LBC is unknown. In search for new therapeutic vulnerabilities, we thus performed an epigenetic drug screening evaluating the response of BPE co-mutated clones to a broad spectrum of epigenetic regulators inhibitors. We discovered that inhibitors of EZH2 methyl-transferase consistently reduced the viability of PIK3CA/TP53^{co-mut} cells, suggesting that they might act in a synthetic lethal manner.

Overall, our results provide new insights into the molecular fingerprint of LBC patients supporting the hypothesis that BC arise in Y women may represent a unique biological entity. Moreover, we shed new light on the biological impact of PIK3CA/TP53^{co-mut} on tumorigenesis and drug resistance in LBC context. PIK3CA and TP53 co-altered pathways might represent novel predictive biomarkers for epigenetic therapies, such as EZH2i, not just in Y patients but across LBC patients harboring these mutations

1. INTRODUCTION

1.1 Breast Cancer

1.1.1 Demographics, incidence and mortality

Breast cancer (BC) is the predominant malignancy among women with an estimated 2.3 million new cases (11.7% of all cancer) and the foremost cause of cancer-related deaths globally with 685,000 deaths (6.9% of total deaths) ranking as the fifth leading cause of cancer mortality worldwide¹. The global cancer burden in women is increasing in countries regardless of income level, due to population growth and aging. However, large geographic incidence variations are observed across countries with different Human Development Index (HDI) levels. Age-Standardized Incidence Rates (ASIR) of BC are strongly and positively associated with the HDI. Incidence rates are 88% higher in countries with high HDI (Australia/New Zealand, Western Europe, Northern America, and Northern Europe) than in those with low HDI (Melanesia, Western Africa, Micronesia/Polynesia, and the Caribbean), at 75.6 per 100,000 and 36.1 per 100,000, respectively².

Although incidence rates are higher in developed regions, Asia and Africa account for 63% of total deaths in 2020 with a 17% higher mortality rate than those in high-income regions³. Higher incidence rates in high HDI regions reflect changes in the prevalence of risk factors and the widespread availability of mammographic screening during the 1980s and 1990s⁴. In these countries, BC is often diagnosed at an early stage, and the prognosis is usually good. In contrast, in low HDI regions the BC diagnosis occurs at an advanced stage and there is limited access to treatment⁵. This disparity is primarily due to limited access to early detection diagnostic services like mammographic screening and the lack of effective treatment options or appropriate healthcare infrastructures in low HDI countries⁶. In low-income countries, BC incidence is expected to increase further due to the westernization of lifestyles driven by economic development⁷. Therefore, improving BC awareness through the promotion of early detection via clinical breast examinations, followed by timely and appropriate treatment, is crucial to increasing survival rates in these areas⁸.

Several studies have shown that BC patients in low HDI countries are diagnosed around 10 years earlier than those in developed nations⁹. The proportion of young patients (under 35) is about 10% in high HDI regions but can reach up to 25% in developing Asian countries¹⁰. Tumor biology also varies by ethnicity influencing mortality rates. This pattern is multifactorial, involving genetic predisposition, lifestyle and other environmental factors¹¹.

1.1.2 Risk Factors

Risk factors of BC are both modifiable and non-modifiable

Non-modifiable risk factors

Aging. Currently, approximately 80% of BC patients are over 50 years old. The probability of developing BC rise with age, from a 1,5% risk at 40 years to 3% at age 50 and surpasses 4% at 70 years¹². This may be due to prolonged exposure to endocrine stimuli, accumulation of DNA damage and epigenetic changes, along with aberrant expression of genes related to the cell cycle and apoptosis¹³.

Family history and genetic predisposition. Hereditary Breast Cancer (HBC) is primarily due to mutations occurring in BC-related genes. These predisposing mutations have been classified based on their Relative Risk (RR) of developing specific types of cancer. High-penetrant mutations ($RR > 5$), moderate-penetrant mutations ($RR = 1.5-5$) and low-penetrant mutations ($RR < 1.5$) exhibit varying risks across populations and age groups¹⁴. It is estimated that around 5-10% of BC cases are hereditary, with approximately 50% attributed to deleterious mutations in high or moderate penetrance genes¹⁵. The most common high-penetrance genes are BRCA1 and BRCA2 that contribute to approximately 20-25% of HBC¹⁵. These genes are involved in DNA repair and recombination, cell cycle regulation and transcription processes. Mutations in BRCA1 and BRCA2 can impair the DNA repair machinery, accelerating the rate of random mutations thus leading to the clonal outgrowth of tumor cells with advantageous traits¹⁶. Other high and moderate –penetrance genes are CDH1, PTEN, TP53 and STK11, along with several DNA repair genes that can interact with BRCA such as CHEK2, ATM, PALB2 and BRIP1¹⁷.

Reproductive history and hormonal changes. Hormonal changes during pregnancy, breastfeeding, first menstruation and menopause, along with their durations and the associated hormonal imbalances, can affect mammary epithelial cell proliferation. These factors are crucial in potentially inducing carcinogenic events in the breast microenvironment¹⁷. An early first full-term pregnancy (particularly in the early twenties), a greater number of births and a longer breastfeeding are associated with a reduced risk of developing both Estrogen Receptor/Progesterone Receptor-positive (ER+/PR+) and ER/PR-negative (ER-/PR-) BC¹⁸. Early menopause, whether natural or surgical, is another protective factor that decreases the risk of BC onset, while early menarche, late menopause, late age at first pregnancy and low parity can increase this risk¹⁹.

Other risk factors include race/ethnicity, breast tissue density, history of benign breast diseases and previous radiation therapy¹⁷.

Modifiable risk factors

Modifiable risk factors are closely linked to the individual's lifestyle: smoking, alcohol consuming and high-fat diet can increase the risk of BC onset via an higher release of estrogen into the bloodstream, thus triggering the estrogen signaling pathway¹⁷. Obesity, implying an excess of adipose tissue that produces estrogen, can lead an high hormonal stimulation of the mammary glands²⁰. Conversely, regular physical activity represents a protective factor against BC possibly reducing exposure to sex hormones, altering immune responses or affecting insulin-like growth factor-1 levels²¹. Additional risk factors include exposure to artificial light, chemicals, and drugs used in clinics as some antibiotics (tetracyclines)²², antidepressants (paroxetine, selective serotonin reuptake inhibitors)²³, antihypertensive drugs (calcium channel blockers, angiotensin II-converting enzyme inhibitors) and Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) such as aspirin and ibuprofen used in the long term²⁴. However, further investigation is required to corroborate these findings.

1.1.3 Breast Cancer classification

BC is a heterogeneous disease, consisting of multiple distinct entities that exhibit different morphological appearances, molecular features and clinical behaviors²⁵. Due to the great heterogeneity of BC, highly detailed morphological and molecular classifications have been purposed to predict prognosis and to develop more targeted therapies.

Although most BCs originate within the same segment of the mammary gland, the so called Terminal Duct Lobular Unit (TDLU)²⁶, they can be classified into biological and clinical subgroups based on the growth pattern and morphological appearance of the tumors^{27,28}. They are classified as Ductal or Lobular based on the site of origin or on differences in tumor cell biology such as the expression of immunohistochemical markers like E-Cadherin or Cytocheratin-8. Additionally, tumors that do not invade the myoepithelial cell layer, remaining within the duct wall, are considered “In Situ”, whereas those characterized by malignant proliferation of neoplastic cells that penetrate the stroma are “Invasive”²⁹.

The evaluation of tumor grade and stage is essential for determining the prognosis and guiding the treatment strategy for BC patients³⁰. The histologic grade assesses how much the tumor differs from normal breast tissue providing insight into the cancer's aggressiveness factors like tumor growth and spread rate. The Nottingham grading system, also known as the Scarff-Bloom-Richardson (SBR) is most used system for BC grading. This method evaluates three key morphological features of breast tumors: tubule formation, nuclear pleomorphism and mitotic count. Each feature is scored, and the

total score determines the overall tumoral grade: Grade 1 (score of 3-5), Grade 2 (score of 6-7) and Grade 3 (score of 8-9)³¹.

Tumor Nodes Metastasis (TNM) staging system is one of most widely classification system used to assess the disease's extent and to guide treatment decisions. Developed by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC), the TNM staging system categorizes the size and extent of the primary tumor, the extent of regional lymph node involvement and the presence of metastasis in distant parts of the body³².

Based on these definitions, the histological classification of BC is the following:

Ductal Carcinoma *In Situ* (DCIS). DCIS is a neoplastic proliferation of epithelial cells within the ductal system¹⁹. It features cellular and nuclear atypia and can potentially progress to invasive BC. Typically, DCIS is positive for E-cadherin and β -catenin, while shows reduced or absent High Molecular Weight (HMW) keratin²⁹. It is the most prevalent form of BC, representing 70-80% of all pre-invasive lesions and generally has a favorable outcome³³.

Lobular Carcinoma *In Situ* (LCIS). LCIS originates from adenomatous cells within the milk-producing lobules of the breast²⁹. It is characterized by the absence of E-cadherin and β -catenin expression and it is positive for HMW keratin. LCIS represent 20-30% of pre-invasive BCs³³.

Invasive Ductal Carcinoma (IDC). IDC involves ductal proliferation with stromal invasion and eventually the spreading to lymph nodes and other organs²⁸. IDC is the most common form of invasive BC, representing 70-80% of all cases³³ and it is classified into numerous histological subtypes based on cell type (apocrine carcinoma), secretion characteristics (mucinous carcinoma), architectural features (papillary, tubular, and micro papillary carcinoma) and immunohistochemical profile (neuroendocrine carcinoma)³⁴. Approximately 75% of IDC do not show distinct morphological features and are classified as IDC "No Special Type" (NST).

Invasive Lobular Carcinoma (ILC). ILC originates in the lobules, occurs in both breasts in approximately 20% of cases and it is more challenging to detect on mammograms. There are five distinct histological variants of ILC based on the tumors' cytoarchitectural features: classic type, pleomorphic lobular carcinoma, histiocytoid carcinoma, signet ring carcinoma, and tubule lobular carcinoma²⁹. ILC accounts for 5%–15% of invasive breast carcinomas and typically affects older women who are also commonly diagnosed with conventional IDC³⁵. The incidence of ILC is increasing, particularly among postmenopausal women, possibly due to hormone replacement therapy³⁶.

Special types of breast tumors. This category includes rare and histologically or clinically distinct BCs such as inflammatory breast cancer, Paget's disease of the breast nipple, angiosarcoma and Phyllodes tumor³⁷.

Histological classification alone cannot fully capture the great molecular heterogeneity of BC. Over the past 20 years, high-throughput gene expression profiling has been extensively used to study BC, revealing the molecular basis of metastatic potential, therapy resistance and histological grade²⁵. This has led to the enhancement of traditional pathology-driven classification through molecular classification, resulting in the sub-classification of BCs into distinct molecular entities associated with different prognosis and treatment response. In 2000, Perou and colleagues analyzed microarray gene expression data from 38 BCs showing that, despite their phenotypic diversity, human BCs could be grouped into distinct categories based on intrinsic gene expression patterns. Further analyses refined this classification, identifying four distinct subtypes of breast carcinoma: Luminal A, Luminal B, erythroblastic oncogene B (ERBB2) positive and basal-like^{38,39}. In 2007, Herschkowitz and colleagues discovered an additional intrinsic subtype called “claudin-low” BC⁴⁰. In the Cancer Genome Atlas Project (TCGA) more than 300 primary tumors were extensively profiled using six distinct technological platforms, focusing on abnormalities in DNA methylation, DNA copy number analysis, RNA and protein expression. This comprehensive analysis confirmed that BCs comprise several distinct disease entities, commonly known as the intrinsic subtypes⁴¹. Gene expression profiling has provided valuable insights into the complexity of breast tumors, offering prognostic and predictive information beyond traditional pathological staging, histologic grade and standard clinical assessments. Consequently, several multigene assays have been developed to assign subtypes based on gene panel's evaluation using different technologies such as qRT-PCR and microarrays²¹. Among these, in 2009, Parker et al. developed the PAM50, a 50-gene signature capable of classifying BC into the main intrinsic subtypes with 93% of accuracy⁴².

Due to the high costs associated with commercially available multigene assays, surrogate markers identified through Immunohistochemistry (IHC) are also used to classify BC into surrogate intrinsic subtypes. These markers include Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2) and the proliferation marker Ki67. Tumors expressing ER and/or PR are termed “hormone receptor-positive” (HR+), with Luminal A and Luminal B subtypes distinguished by the expression level of Ki67^{43,44}. Tumors overexpressing HER2 are defined “HER2-positive” and can be either HR+ (Lum HER2) or HR- (Basal HER2 or HER2 enriched), and those that do not express ER, PR, and HER2 are termed “triple-negative”.

The molecular classification of BC is the following (Fig.1):

❖ **Luminal Breast Cancer (LBC).** LBC is the most common subtype, comprising over 70% of all diagnosed breast tumors⁴⁵. These tumors are termed "luminal" due to the high expression of breast luminal epithelium genes. They are characterized by the expression of ER, PR, keratins 8 and 18, GATA3, FOXA1 and XBP1^{46,47}. Consequently, these tumors are considered Hormone Receptor Positive (HR+). IHC surrogate markers for proliferation-related and luminal-regulated pathways are used to classify LBC into two distinct entities with different clinical outcomes: Luminal A (Lum A) and Luminal B (Lum B) subtypes³⁸. Lum A subtypes are the most common, accounting for approximately 50% of all BC⁴⁵. In clinical, they are defined as ER+, PR+, HER2- and Ki67 with fewer than 20% positive cells⁴⁸. These subtypes are typically low-grade, slow-growing, with better DSF and OS than other BC subtypes⁴⁹. The Lum B subtype is less common than Lum A BC, accounting for approximately 20% of LBC cases⁴⁵. These tumors are ER+, PR positive or negative (PR+/-) and HER2 positive or negative (HER2+/-) with a Ki67 index higher than 20%⁴⁸. Typically, Lum B tumors show lower expression of genes specific of luminal epithelium such as ER, PR and FOXA1, while exhibiting increased expression of genes related to proliferation and cell cycle including Ki67, MKI67 and AURKA⁵⁰. Accordingly, Lum B tumors tend to have a higher histological grade and worse prognosis compared to the Lum A ones. They respond to hormone therapy and, to a greater extent, chemotherapy, but have a higher risk of relapse than Lum A BC⁴³.

PIK3CA is the most frequently mutated gene in Lum A, with point mutations occurring in approximately 45-50% of cases⁴¹. It is followed by GATA3 (14%), MAP3K1 (13%) TP53 (12%), CDH1 (9%), KMT2C (8%)⁴¹. Amplification of CCND1 (20%) and FGFR1 (15%) represent the most common CNVs in LUM A BC⁵¹.

In Lum B BC TP53 is the most frequently mutated gene (29%) followed by PIK3CA (29%), GATA3 (15%), KMT2C (6%), MAP3K1 (5%) and CDH1 (5%)⁴¹. Common Lum B CNVs include amplification of ERBB2 (20%) and MYC (15%)⁵¹. BRCA1 mutations are very rare while BRCA2 mutations are more frequent compared to Lum A BC (approximately 5-10%) contributing to the higher genomic instability of this subtype⁵².

Lum A and Lum B BC subtypes have a more favorable prognosis compared to other subtypes, partly due to their dependence on estrogen signaling. This signaling can be targeted through Endocrine Therapy (ET), which works by preventing estrogen stimulation of BC cells or by reducing estrogen levels⁴⁹. Drugs targeting estrogen receptors (ERs) include Selective Estrogen Receptor Modulators (SERMs) such as Tamoxifen and Toremifene, and Selective Estrogen Receptor Degradors (SERDs) such as Fulvestrant. Both SERMs and SERDs inhibit estrogen activity binding its receptor⁵³.

Additionally, Aromatase Inhibitors (AIs) such as Letrozole, Anastrozole and Exemestane are used to reduce estrogen levels inhibiting its production⁵³. ET can be applied as neoadjuvant or adjuvant

therapy and is effective in recurrent or metastatic LBC, often in combination with other treatments⁴⁹. The discovery of non-hormonal molecular targets promoting BC progression, such as Cyclin-Dependent Kinase 4/6 (CDK4/6) and the Phosphatidylinositol 3-Kinase (PI3K) / Mammalian Target of Rapamycin (mTOR) pathway, together with the development of corresponding therapeutic agents, has further enhanced patient outcomes. Three CDK4/6 inhibitors are currently approved for clinical use in metastatic BC: Palbociclib (PD-0332991), Ribociclib (LEE-011) and Abemaciclib (LY-2835219)⁵⁴. The Food and Drug Administration (FDA)-PI3K/mTOR pathway-approved drugs for BC treatment are Everolimus (mTOR inhibitor) and Alpelisib (PI3K inhibitor), while other PI3K/mTOR targeted drugs are currently in clinical trials^{55,56}. However, advanced HR+ BC remains incurable due to other mechanisms involved that contribute to recurrence, therapeutic resistance and metastasis⁵⁷.

❖ **HER2-Positive Breast Cancer (HER2+ BC).** HER2+ BC accounts for 10-20% of all BC and it is characterized by the amplification of the ERBB2/HER2 gene and/or the overexpression of its related kinase receptor protein⁵⁸. These tumors usually exhibit a high grade and a high Ki67 index indicating rapid cell proliferation. In 2012, a TCGA project identified two HER2-related subtypes: the Luminal B-like subtype, which features ER and/or PR expression (approximately 40-50% of tumors) and the HER2-enriched (HER2-e) subtype, which lacks expression of both hormone receptors⁵⁹.

HER2+ tumors generally grow faster than LBC and historically had a poor prognosis with reduced Disease-Free Survival (DFS) and Overall Survival (OS)⁶⁰. Somatic mutation analysis revealed that, besides ERBB2 amplification, HER2+ tumors are characterized by a broad range of rare mutations (<10%), in ERBB2, PTEN and PIK3R1⁵⁹. Instead, the most frequent mutations were found in PIK3CA and TP53 (31% and 40% in Luminal B-like HER2+ tumors, and 39% and 72% in the HER2-e subtype respectively)⁶⁰. The development of HER2-targeted therapies based on the administration of humanized anti-HER2 monoclonal antibodies (Trastuzumab, Pertuzumab), tyrosine-kinase inhibitors (Lapatinib, Neratinib) and antibody-drug conjugates (Trastuzumab-Emtansine, Trastuzumab-Deruxtecan) has significantly improved the prognosis for HER2+ patients. Currently, survival rates exceed 90% for those with HER2+ early BC treated with chemotherapy and dual antibody therapy. However, metastatic HER2+ BC develop resistance to therapies and progress⁶¹.

❖ **Basal Like/ Triple Negative Breast Cancer (TNBC).** TNBC is characterized by the absence of ER, PR and HER2 expression, high grade and high Ki67 index⁶². These tumors express genes characteristic of basal epithelial cells (cytokeratins 5, 6, 14, 17, vimentin and P-cadherin) and overexpress genes involved in cellular proliferation (MEK, ERK, PI3K, AKT, NF- κ B, cyclin E1, c-kit and EGFR), suppression of apoptosis and cell migration or invasion (TGF- β 2, etc.) that contribute

to an aggressive phenotype⁶³. Epidemiological data indicate that TNBC predominantly affects premenopausal women under the age of 40 years, representing approximately 15-20% of all BCs⁶⁴. TNBC patients have a shorter survival time with a mortality rate of 40% within the first five years following diagnosis and approximately 46% of patients develop distant metastases⁶⁵.

Over 85% of BC with germline BRCA mutations exhibit a triple-negative phenotype and approximately 15% of TNBC patients carries BRCA1 germline or somatic mutations⁶⁶. In 2011, Lehmann and colleague, through gene expression analysis of TNBC samples from 587 patients, categorized TNBC into six subtypes: basal-like 1 (BL1), basal-like 2 (BL2), mesenchymal (M), mesenchymal stem-like (MSL), immunomodulatory (IM) and luminal androgen receptor (LAR). However, the clinical relevance of these subtypes remains uncertain and further researches are needed to understand their implications for TNBC therapeutic strategies⁶⁷. TNBC samples revealed abnormal expression of genes regulating cell cycle and DNA repair. TP53 is the most frequently mutated gene (80%) followed by PIK3CA (9%)⁶⁸. Other genetic alterations, detected at very low frequencies (<5%) occur in PTEN, KMT2C and RB1⁶⁸. The most frequent copy number alterations in TNBCs include gains of 1q, 8q and 10q; losses of 5q and 8p; amplifications of EGFR and FGFR2 along with PTEN loss⁶⁹.

Due to its distinct molecular phenotype, TNBC does not respond to endocrine or targeted molecular therapies. Therefore, chemotherapy remains the primary systemic treatment although still ineffective⁷⁰. However, to date, new therapeutic strategies, including PARP inhibitors, immunotherapy (such as CAR-T, anti-PD1 therapy and anti-CTLA4 therapy) and antibody-drug conjugates, are under investigation^{70,71}.

❖ **Claudin-Low Breast Cancer (CLBC).** The incidence of CLBC is between 7% and 14% of all invasive BC⁷². These tumors are associated with a poor prognosis, exhibiting worse DFS compared to other subtypes⁷³, an higher local recurrence rate⁷⁴ and a greater incidence of lymph node metastasis. The predominant histological type is invasive ductal carcinoma, often associated with metaplastic carcinoma and medullary carcinoma⁷⁵.

Despite CLBCs respond to chemotherapy, their overall prognosis remains poor⁷⁶. The aggressiveness of these tumors is due to low expression of genes involved in cell-cell adhesion, such as claudins 3, 4, and 7, occludin, E-cadherin, combined with the high expression of Epithelial-Mesenchymal Transition (EMT) genes (Vimentin, SNAIL1, SNAIL2, TWIST1, TWIST2 and ZEB1, ZEB2) and stem cell-like gene expression patterns (LDH1 and CD44hi/CD24-/low) conferring motility, invasiveness and self-renewal ability. Additionally, CLBC is characterized by immune and stromal cell infiltration due to the high expression of lymphocyte or endothelial cell-related markers (CD14

and CD79a)⁷⁷. Since, usually, it is HR- and HER2-, it has been classified within the category of TNBC⁷⁸. Moreover, CLBC shows a low genetic instability and low frequency of TP53 and PIK3CA mutations⁷⁹

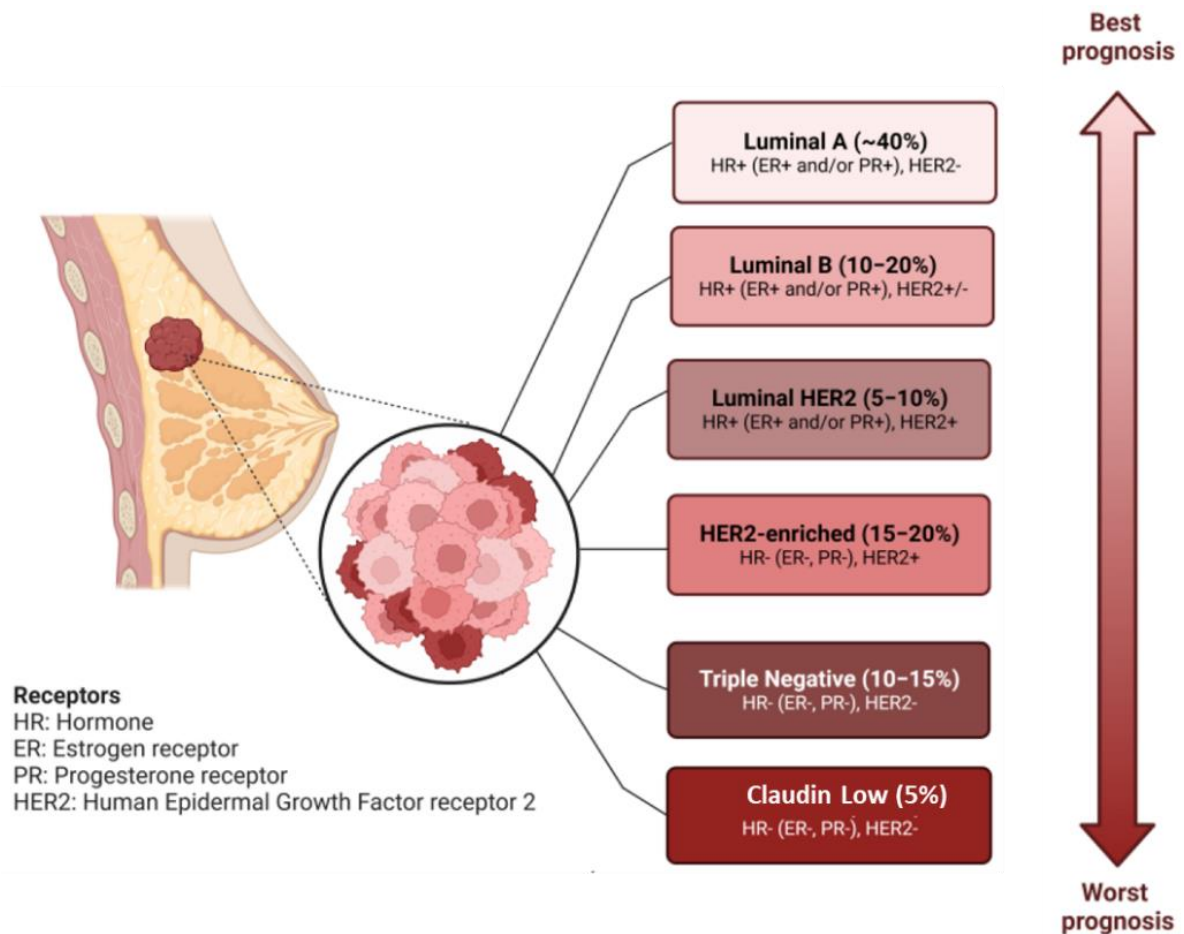


Fig. 1: Breast cancer molecular subtype classification. Breast cancer molecular subtypes are categorized into Luminal A, Luminal B, Luminal HER2, HER2-Enriched, Triple-Negative and Claudin-low. The incidence rates, receptor expressions and prognoses are indicated for each subtype. Figure has been adapted from “*Molecular, Cellular and Technical Aspects of Breast Cancer Cell Lines as a Foundational Tool in Cancer Research*”, Witt BL et al.⁸⁰

1.1.4 Mutational landscape of BC

The cancer genome is highly dynamic, with each cancer cell clone arising from the accumulation of Single Nucleotide Variations (SNVs), Copy Number Alterations (CNAs), Structural Variants (SVs) and epigenetic modifications⁸¹. Over the past decade, high throughput sequencing technologies such as Whole-Genome Sequencing (WGS), Whole-Exome Sequencing (WES) and RNA Sequencing (RNA-seq) approaches provided in-depth examinations of cancer genetic alterations, gene expression profiles and epigenetic modifications revealing new insights into the molecular heterogeneity of BC that drives tumor onset and progression.

Literature data indicate that the well-known BC most mutated driver genes, regardless the molecular subtype, are PIK3CA (~40%) and TP53 (~35%), followed by GATA3 (~11%), MAP3K1 (~8%),

CDH1 ($\approx 7\%$) and KMT2C ($\approx 7\%$)^{41,82}. Other driver genes, with mutation rates below 5%, are involved in chromatin remodeling, transcription (NCOR1, ARID1A, CTCF, TBX3, FOXA1, FOXO3, RUNX1, CBFB), splicing (SF3B1) and cell cycle regulation (RB1, CDKN1B). Similarly, different proliferation and survival pathways, such as the PIK3CA/mTOR pathway (AKT1, PIK3R1, PTEN), MAPK signaling (MAP2K3, NF1) and others (ERBB2, PTPN22, PTPRD), can be altered by mutations^{41,83}. The most frequently amplified oncogenes in BC are ERBB2 ($\approx 10\text{-}15\%$ in HR+ BC; $\approx 15\text{-}20\%$ in HR- BC)^{82,84}, CCND1 ($\approx 20\text{-}30\%$ in HR+ BC; $\approx 5\text{-}10\%$ in HR- BC)^{85,86} and MYC ($\approx 15\text{-}20\%$ in HR+ BC; $\approx 30\text{-}40\%$ in HR- BC)^{85,87}, followed by FGFR1, PIK3CA and MCL1⁸². In contrast, tumor suppressor genes commonly deleted in BC include TP53 ($\approx 15\text{-}20\%$ in HR+ BC; $\approx 50\text{-}75\%$ in HR- BC), CDH1 ($\approx 10\%$ in HR+ BC; $\approx 5\%$ in HR- BC) and PTEN ($\approx 5\%$ in HR+ BC; $\approx 10\%$ in HR- BC) followed by BRCA1/2, MAP2K4 and RB1^{51,82}.

Epigenetic changes, including DNA methylation, histone modifications and non-coding RNAs (ncRNAs)-associated regulation, together with genetic mutations, also contribute to cancer development, significantly affecting cell's global gene expression and genomic stability⁸⁸.

BC is associated with several epigenetic changes: CpG promoters hyper-methylation of oncosuppressors (BRCA1, CDH1, PTEN, ESR1, RUNX3, CDKN2A etc.) and global DNA hypomethylation at CpG-poor regions (repetitive regions, transposable elements) promoting structural rearrangement⁸⁹. Moreover, changes in histone modifications (methylation, acetylation, ubiquitylation, sumoylation and phosphorylation) alters the chromatin structure due to the dysregulation of epigenetic modulators such as histone acetyl-transferases (HATs), histone deacetylases (HDACs) and histone modifying complexes (COMPASS, SWI/SNF, etc.)⁹⁰.

Even the ncRNAs, particularly miRNAs, are involved in different steps of BC progression. miRNA profile data have shown that tumor suppressor miRNAs, such as miR-34a, are often mutated or decreased in BC⁹¹, while oncogenic miRNAs, such as miR-214, related to the PI3K/Akt/mTOR pathway, are often upregulated⁹².

TP53 and PIK3CA are the most frequently mutated genes in BC, which have been extensively studied concerning prognosis and drug resistance. Co-mutations of TP53 and PIK3CA are not uncommon, however, their impact on the clinic pathological characteristics of BC has yet to be fully explored. Scientific evidences indicate that TP53 and PIK3CA co-mutations are associated with more aggressive BC phenotypes, including HR- status, high Ki-67 expression, advanced TNM stage⁹³ and, in residual disease after neoadjuvant chemotherapy, they are associated with high-grade tumors and poorer survival rates⁹⁴. *In vitro* and *in vivo* studies have shown that TP53 and PIK3CA co-mutated cells exhibit enhanced cancerous behaviors; moreover, BC patients carrying variants in these two

gene experience less favorable responses to neoadjuvant systemic therapy with worse survival rates compared to those with only TP53 mutations⁹³.

In detail, it was demonstrated that PIK3CA alterations in TP53-mutant cells reduces the efficacy of therapies: PIK3CA-mutated BC is in fact less sensitive to chemotherapy, endocrine therapy or anti HER2 therapy, while TP53 mutations alone have been linked to better responses to Neoadjuvant Systemic Therapy (NST), including Docetaxel⁹³. However, TP53 mutations co-occurring in PIK3CA-mutated BC do not improve chemotherapy sensitivity. Thus, it was hypothesized that the PIK3CA mutations primarily determine the therapeutic efficacy of NST in BC with PIK3CA/TP53 co-mutations⁹³.

1.2 Breast Cancer in Young Women

1.2.1 Clinical and molecular insights of BC in young women

BC is a chronic disease influenced by endocrine factors, becoming clinically detectable in post-menopausal period. Consequently, BC screening programs usually exclude women under 40 years unless they carry known genetic mutations highly associated with the risk of BC onset⁹⁵. Nevertheless, BC is the most prevalent cancer among women under 40 years worldwide, with an increasing incidence each year. Nowadays, it represents the second leading cause of cancer-related deaths in women aged 0-39 globally, resulting in approximately 44,800 deaths annually⁹⁶. Recent guidelines from the European School of Oncology and the European Society of Medical Oncology (ESMO) categorize “young women” as those diagnosed with BC before age 40 and “very young women” as those diagnosed at 35 or younger⁹⁷.

Young women with BC constitute a distinct group of patients with unique features in tumor pathology, diagnosis, prognosis, treatment options and survivorship (Fig. 2). Unlike older patients younger women often have more aggressive ER-negative, triple-negative and HER2+ tumors⁹⁸. Nonetheless, in young patients, LBC remains the most representative cancer (over 60%)⁹⁹, especially the Lum B subtype¹⁰⁰. Interestingly, the risk of relapse in Lum A and Lum B patients under 40 years is significantly higher compared to their older counterparts, although this difference is less in HER2+ and triple-negative subtypes¹⁰¹. Furthermore, BC in young women is poorly differentiated, with high Ki67 expression and greater lymph node involvement, resulting in a worse prognosis compared to women older than 50 years⁹⁷. Scientific evidences reveal that young women face worse clinical outcomes and more long-term treatment complications than the older counterpart¹⁰².

Breast Cancer in Young Women (BCYW) Prognosis

Overview of clinicopathological characteristics, survival and risk of metastasis or recurrence

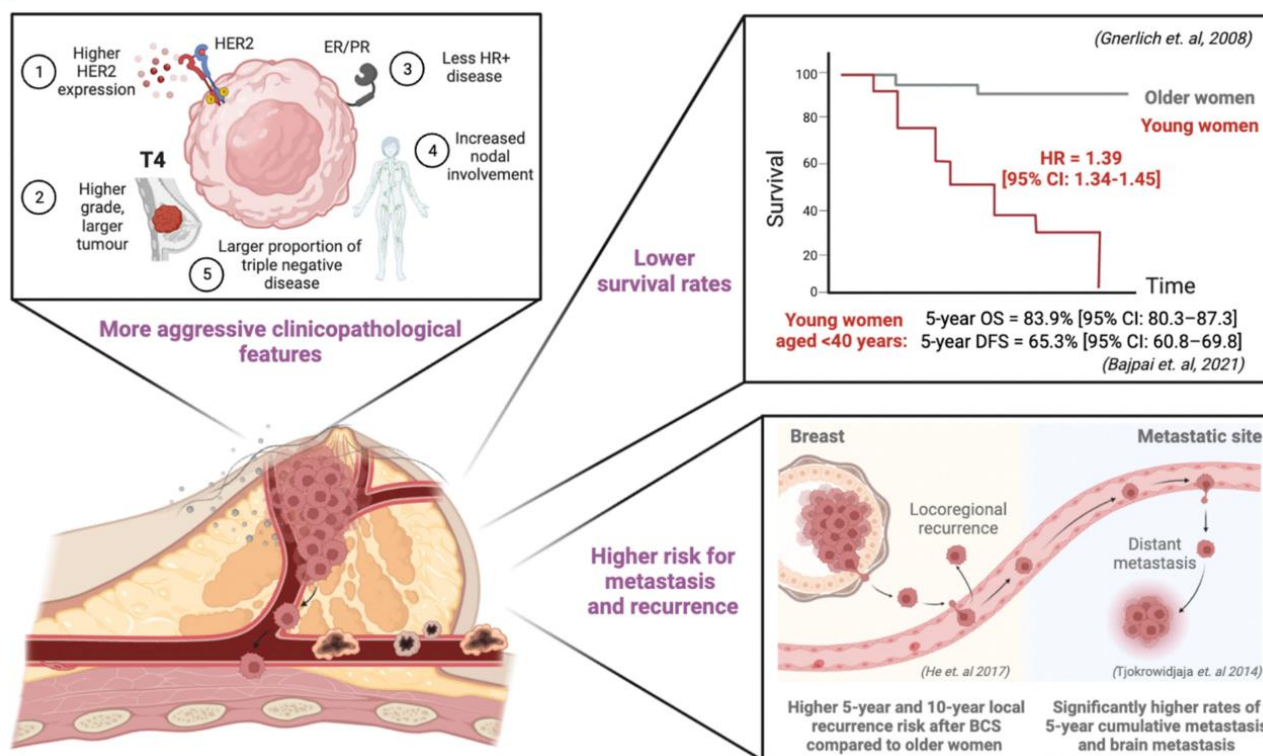


Fig. 2. Overview of clinical-pathological characteristics of BC in young women. Major differences of BC in young patients Compared to older women, including more aggressive clinic-pathological features, lower survival rates, increased risk of metastasis and mortality. Figure has been adapted from “What Is Known about Breast Cancer in Young Women?”, Zhu JW et al.⁹⁷

1.2.2 Genetics of BC in young women

The biological reasons why BC in young women is highly aggressive with poor prognosis is still unclear.

The Cancer Genome Atlas (TCGA)-Breast dataset indicated higher gene expression signatures linked to proliferation, stem cell traits and endocrine resistance in young women compared to older ones¹⁰³. Furthermore, in 2023, Luen et al. investigated the genomic characteristics of a cohort of 1276 premenopausal women diagnosed with HR+/HER2- early BC, providing insights into the age-related differences in prognosis and clinical features among these patients. Comparing oncogenic driver mutational frequencies between younger premenopausal patients (<40 years) and older premenopausal ones ($\geq 40 \leq 45$ years), they found that younger patients had notably lower frequencies of PIK3CA mutations (32% vs 47%), CDH1 (3% vs 9%) and MAP3K1 (7% vs 12%) and higher frequency of TP53 mutations (7% vs 3%). Compared to postmenopausal women (>55 years) from the BIG 1-98 and TCGA-Breast cohorts, women under 40 years once again exhibited a similar

mutational trend, characterized by lower mutation frequencies in PIK3CA, CDH1, and MAP3K1, alongside higher frequencies of TP53, BRCA2, and GATA3 mutations¹⁰⁴. Luen and colleague's study also revealed that young premenopausal patients displayed significantly higher frequencies of CNAs compared to older premenopausal ones (47% vs 26%) especially in <35 years old women who showed around 57% of CNA and low PIK3CA mutation frequency (24%)¹⁰⁴. PIK3CA mutations uniquely exhibited different prognostic associations in women <40 years (HR 1.78, 95% CI 1.08–2.92) vs ≥40 years ones (HR 0.58, 95% CI 0.33–0.99).

Scientific evidences indicated that double or multiple PIK3CA mutations were associated to hyperactivated PI3K signaling and increased proliferation¹⁰⁵ but did not correlate with higher Ki-67 levels, poorer outcomes or increased prevalence in <40 years patients. On the other hand, PIK3CA mutations with concurrent CNAs, although rare, were more frequent in <40 patients respect to the older ones (31% vs 15%)¹⁰⁴. These young patients showed significantly higher Ki-67 levels and worse disease outcomes compared to patients PIK3CA mutated without CNAs¹⁰⁶.

Moreover, a significantly higher frequency of Homologous Recombination Deficiency (HRD) was observed in <40 years women compared to the ≥40 years ones (27% vs 21%). Interestingly, this frequency rise to 36% in <35 years patients showing that HRD phenomenon is notably enriched with decreasing age in young premenopausal women with HR+/HER2- early BC¹⁰⁴. Overall, several key genomic features in young HR+/HER2- early BC patients are linked with distinct prognostic profiles which may help to identify potential therapeutic targets for clinical trials and to develop new treatment strategies.

1.2.3 Therapies for BC in young women

Mastectomy or breast-conserving surgery with radiation therapy are the standard treatment options also for BC in young women¹⁰³. However, local recurrence is more common in young women treated with breast-conserving surgery respect to older patients¹⁰⁷.

The adjuvant and neoadjuvant systemic approaches for young patients are also generally similar to those used for older patients; however, randomized trials have highlighted a relationship between women's age and the chemotherapy benefits. Preliminary results from the TAILORx¹⁰⁸, MINDACT¹⁰⁹ and RxPONDER¹¹⁰ trials showed that chemotherapy is more effective for younger patients with a low or low-intermediate genomic tumor risk score.

Interestingly, three phase III clinical studies have examined the effects of temporary Ovarian Function Suppression (OFS), induced by Luteinizing Hormone-Releasing Hormone agonists (LHRHa), when combined with adjuvant or neoadjuvant chemotherapy to preserve women fertility. These studies have shown that combining Goserelin¹¹¹ or Triptorelin¹¹² with chemotherapy increases the rate of post-

treatment pregnancies in the groups receiving OFS treatment compared to those receiving chemotherapy alone. In particular, the OFS-treated groups had a 5-year cumulative incidence of pregnancy of 23.1% versus 12.2%¹¹¹ and a 10-year cumulative incidence of pregnancy of 6.5% versus 3.2%¹¹². Moreover, the administration of LHRHa alongside chemotherapy did not affect disease-free survival. Therefore, it may preserve premenopausal women's fertility without compromising treatment efficacy. Regardless of menopausal status, tamoxifen is routinely used as adjuvant therapy for patients with HR+ malignancies. Aromatase inhibitors (AIs) were considered to be ineffective for premenopausal women because they increase ovarian estrogen production mediated by gonadotropins¹¹³. However, recent studies have shown that using AIs or Tamoxifen combined to OFS, improves treatment outcomes. Tamoxifen combined with LHRHa was the most used treatment for BC in young women without prior chemotherapy and for women with high body mass index¹¹⁴. However, a recent meta-analysis found that the use of AIs was more effective in decreasing the recurrence of BC compared to Tamoxifen with OFS in pre-menopausal women (median age: < 45 years)¹¹⁵. Despite these scientific evidence and improvements, further studies are needed to optimize therapy for young BC patients.

1.3 Role of PI3K/AKT/mTOR pathway in BC

1.3.1 PI3K/AKT/mTOR pathway

The PI3K/AKT/mTOR (PAM) pathway is a crucial and highly conserved signaling network present in all eukaryotic cells playing a central role in regulating numerous cellular functions in response to external signals, including cell cycle progression, transcription, differentiation, survival, cell motility and metabolism¹¹⁶. The two major functional proteins in this signaling pathway are PI3K and AKT. The Phosphatidyl-Inositol 3-Kinase (PI3K) family includes three classes of plasma-membrane-bound kinases (I, II and III) distinguished by their structural and biochemical properties and substrate specificity. There are three class II PI3Ks (PI3K-C2 α , β , γ) and a single class III PI3K (hVPS34)¹¹⁷. Class I PI3Ks, divided in IA and IB PI3Ks, are the most numerous groups, associated with extracellular signals such as growth factors, hormones and cytokines. They are heterodimeric proteins having a catalytic p110 subunit complexed with a regulatory subunit. There are four different catalytic p110 subunit (α , β , γ and δ) encoded by different genes (PIK3CA, PIK3CB, PIK3CG, and PIK3CD respectively), whereas the regulatory subunit consists in three alternative splicing isoforms p85, p55 and p50 (p85 α , p55 α , p50 α , encoded by PIK3R1; p85 β encoded by PIK3R2; p55 γ encoded by PIK3R3). The catalytic subunits share a common domain structure: an amino-terminal Adaptor-binding domain (ABD) that provides the interaction surface with the regulatory subunit, a Ras-

binding domain (RBD) that mediates the interaction between p110 and Ras-GTP, a C2 (protein-kinase-C homology-2) domain with affinity for lipid membranes, an helical domain acting as a scaffold for other p110 domains and a carboxyl-terminal kinase domain¹¹⁸ (Fig. 3).

The regulatory subunits consist of five structurally and functionally defined domains. Starting from the C-terminal, they contain two SH2 domains (N-SH2, C-SH2) flanking a coiled-coil region known as the inter-SH2 (iSH2) domain, a Rho-GAP domain also referred to as breakpoint-cluster region homology (BH) domain and an SH3 domain at the N-terminal (Fig. 3). The regulatory subunits interact with various proteins involved in different cellular signaling pathways¹¹⁹. Specifically, the N-SH2 domain binds to the helical domain of the catalytic subunit p110, exerting an inhibitory effect. Other interactions between domains of the two subunits help keep the enzyme in a low-activity state under normal conditions preventing p110's kinase domain from interacting with the membrane¹¹⁸.

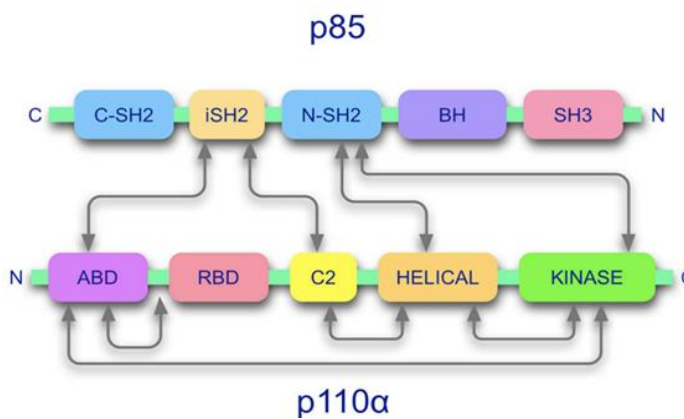
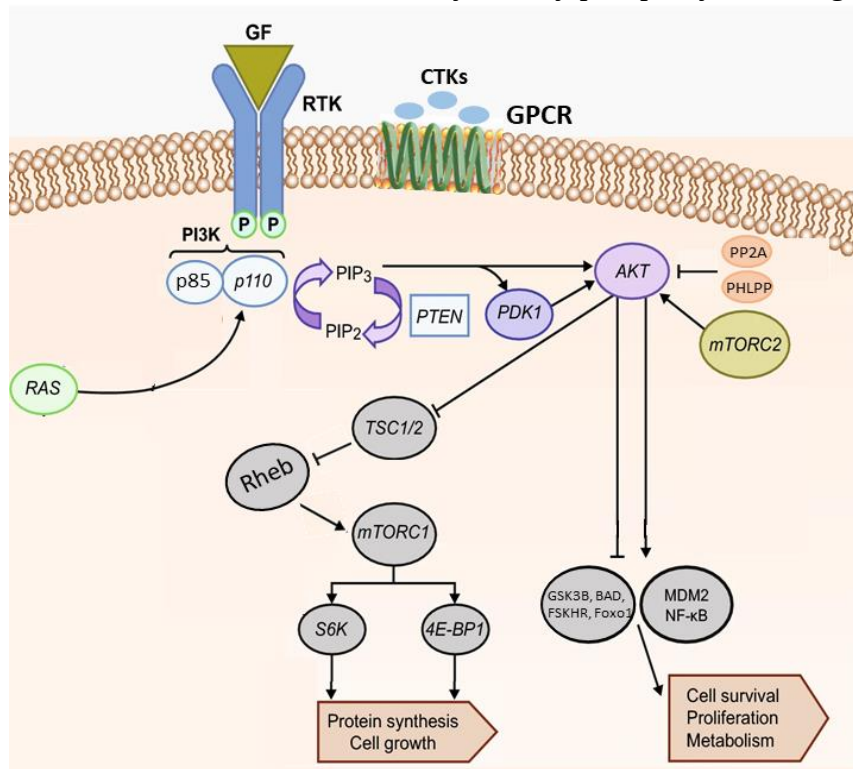


Fig. 3: PI3K domains' structure and interactions. The figure shows the domains' structure and domain interaction map of the standard regulatory subunit, p85 and the catalytic subunit, p110 α of PI3K. Figure has been adapted from "Class I PI3K in oncogenic cellular transformation", Zhao L et al.¹¹⁸

The activation of Class I PI3Ks occurs through multiple upstream pathways that couple a broad range of cell surface receptors responding to extracellular signals: G protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs) and small Ras-related GTPases¹²⁰. When PI3K is activated, it catalyzes the conversion of the lipid phosphatidylinositol 4,5-bisphosphate (PIP₂) into phosphatidylinositol 3,4,5-triphosphate (PIP₃). Accumulation of PIP₃ at the cell membrane leads to the activation of AKT¹²¹ which is crucial for the regulation of glucose metabolism, cell survival, growth and proliferation by interacting with over hundred different proteins across various pathways (mTORC1, GSK3 β , FoxO1, BAD, FSKHR, MDM2, NF κ B etc).¹²² Phosphorylation of FoxO1 promoted by AKT1 leads to its ubiquitination and degradation thereby promoting cell survival and proliferation as well as when AKT phosphorylates and inhibits GSK3 β . Usually, GSK3 β -mediated phosphorylation of β -catenin leads to its degradation by the proteasome. However, when GSK3 β is inhibited by AKT, β -catenin translocates to the nucleus, where it acts as a transcription factor promoting cell proliferation and invasion¹²³. Moreover AKT enhances cell survival and growth by inhibiting the p53 response through MDM2 phosphorylation and by suppressing the anti-proliferative function of p21 and p27¹¹⁶.

The phosphorylation of the tuberous sclerosis complex 1/2 (TSC1/2) leads to the mTORC1 activation. The serine/threonine kinase mammalian Target of Rapamycin Complex (mTOR) exists as two distinct multiprotein complexes: mTORC1 and mTORC2 both involved in the PAM pathway. mTORC1 coordinates anabolic metabolism and cell mass accumulation with growth factor receptor stimulation and nutrient availability¹²⁴. Key phosphorylation targets of mTORC1 include S6 Kinase



(S6K) and Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). When S6K is phosphorylated, it becomes active and subsequently phosphorylates ribosomal protein S6, leading to increased ribosome production. In contrast, the phosphorylation of 4E-BP1 inhibits its function as a suppressor of the translation initiation factor eIF4E, enhancing protein synthesis¹²⁵ (Fig. 4)

Fig. 4: PI3K/AKT1/mTOR pathway. PI3K is activated by the binding of extracellular ligands to RTKs (growth factors or hormones) and to GPCR (cytokines). Also, Ras family GTPases promotes PI3K activation. Once activated, through the release of p85 regulatory subunit, p110 exposes the kinase domain for membrane interaction. PI3K catalyzes the phosphorylation of PIP2 into PIP3. PIP3 recruits AKT and PDK1 to the cell membrane where AKT is phosphorylated by mTORC2 at Ser473, changes its conformation and allows its subsequent phosphorylation at Thr308 by PDK1. Once fully activated, AKT phosphorylates several target proteins (GSK3 β , FoxO1, BAD, FSKHR, MDM2, NF κ B etc.) promoting proliferation, survival and metabolism reversion. The phosphorylation of the tuberous sclerosis complex 1/2 (TSC1/2) cause its inactivation preventing the TSC1/2-mediated block of Rheb GTPase activity. Consequently, Rheb-GDP (inactive) converts to Rheb-GTP (active), which then contributes to the mTORC1 activation. mTORC1, phosphorylating downstream targets (S6K, 4E-BP, etc) stimulates nutrient uptake and metabolism, promoting cellular growth and cell cycle progression. Signal termination is caused by loss of PI3K-PIP2 interaction, via the de phosphorylation of PIP3 to PIP2 by PTEN phosphatase and by the PP2A- and PHLPP- mediated de-phosphorylation of AKT leading to its detachment from the cell membrane. Figure has been adapted from “*Rationale-based therapeutic combinations with PI3K inhibitors in cancer treatment*”, Castel P et al¹²⁶.

1.3.2 Mutations driving PI3K/AKT/mTOR pathway activation

Alterations in the PAM pathway disrupts the canonical regulation of AKT activity, leading to its hyper-activation. This triggers the phosphorylation of multiple substrates, which can either inhibit or enhance their functions promoting cell survival, proliferation, metabolism reprogramming, invasion, metastasis and drug resistance¹¹⁶. Remarkably, about 50% of all tumors exhibit abnormalities in PAM pathway due to aberrant expression or mutations associated to its deregulation and therefore to cancer

onset and progression¹²⁷. PIK3CA, encoding the p110 α catalytic subunit of PI3K, is one of the most frequently mutated genes particularly in breast, colorectal, ovarian, gastric, lung and endometrial cancers¹²⁸. In BC, approximately 30–40% of patients exhibit PIK3CA mutations, which are mainly missense mutations leading to the hyper-activation of the p110 α isoform of PI3K¹²⁹. Frameshift and nonsense mutations have low frequency and contribute to a small percentage of overall PIK3CA mutations in cancer (< 1%)¹³⁰. PIK3CA gene amplification is rare especially in absence of point mutations¹³¹. HR+ BC shows the highest rate of PIK3CA mutations (34.5%), followed by HER2+ (22.7%) and basal-like tumors (8.3%). In particular, among the HR+ subtypes, Lum A tumors show an higher PIK3CA mutation rate compared to Lum B (45% vs 29%)¹³². PIK3CA somatic mutations in BC exhibit high heterogeneity but ~80 % of them clusters in three major hotspots: two in the helical domain and one in the catalytic domain of p110 α . Among the PIK3CA variants, the E542K, E545K and H1047R are the most frequent substitutions with an incidence of 11%, 17% and 35% respectively having different impact on the protein¹²⁹ (Fig. 5). The E542K and E545K hotspots fall into the helical domain and mimic RTKs activation reducing the inhibition of p110 α by the p85 regulatory subunit. On the contrary, H1047R falls near the catalytic domain's end and, increasing the p110 α interaction with the lipid membrane, facilitates access to the PIP2 substrate. Less common PIK3CA mutations, N345K and C420R interfere with the interaction between the C2 and iSH2 domains of p85 activating catalytic subunit also in absence of signals, thus promoting tumor progression. Mutations in other class of PI3K genes are very rare in tumors. However, C-terminal deletions affecting the iSH2 domains of PIK3R1 and PIK3R2 regulatory subunits are the most frequent¹²⁴. Interestingly, ~4% of all BC and ~12% of PIK3CA-mutant BC patients harbor double PIK3CA mutations. Preclinical studies indicate that these double PIK3CA mutations lead to an increased PI3K activity and downstream signaling compared to single hotspot mutants in non-transformed cells and HR+ BC cells¹²⁹.

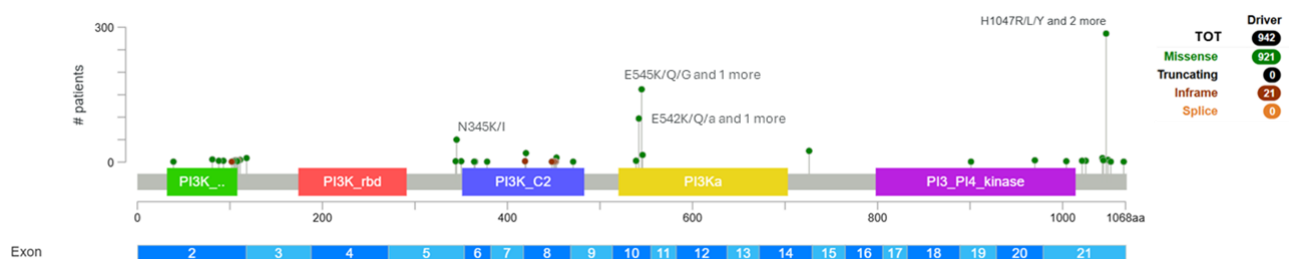


Fig. 5: PIK3CA mutational spectrum in BC. Lollipop plot representing the distribution of PIK3CA mutations positioned by their amino acid coordinates across the protein domain. Data were retrieved from MSK, Cancer Cell 2018 BC (n= 1918) and MSK, Nature Cancer 2020 (n= 141) genomic datasets through cBioportal platform¹³³.

Overexpression or mutations in RTKs genes can also hyperactivate downstream signaling of PI3K. ERBB2 amplification/HER2 overexpression and Fibroblast Growth Factor Receptor 1 (FGFR1) amplification are observed in 10% and 12% of HR+ BC, respectively.

Moreover, in HR+ BCs, AKT1 and PTEN mutations are rare (2-3% and 2-4%, respectively) and usually are mutually exclusive with PIK3CA mutations in early-stage tumors¹³⁴. The most common AKT1 mutation is the E17K missense mutation, which increases AKT1 activity by inducing its constitutive localization to the plasma membrane. Moreover, also post-translational modifications of AKT, including lysine modifications, tyrosine phosphorylation, O-GlcNAcylation, acetylation and sumoylation are crucial for maintaining AKT hyper-activation in cancers, even with normal PI3K and PTEN activity¹¹⁶.

Lastly, PTEN loss, due to deletions, epigenetic changes and post-translational modifications, is found in 5–10% of BCs with loss of heterozygosity at the PTEN locus found in 40–50% of case¹³⁵. TNBC is more frequently associated with PTEN loss (about 30–50%) than PIK3CA mutations (< 10%)¹³⁴.

1.3.3 Cancer therapies targeting the PI3K/AKT/mTOR pathway

PIK3CA activating mutations are highly enriched in LBC⁴¹. The ER and PI3K pathways are intricately connected, interacting at multiple levels. Several kinases, including MAPKs and downstream effectors of PI3K as AKT and S6K, directly phosphorylate ER leading to ligand-independent ER transcription¹⁰⁵. Additionally, PI3K and MAPK pathway lead to the phosphorylation of c-Jun and AIB1 respectively, both ER co-regulatory proteins, thus enhancing ER-dependent transcription. Recently, it was demonstrated that Tamoxifen- or Fulvestrant-resistant ER+ BC cells and xenografts, along with long-term estrogen-deprived ER+ BC cells, displayed hyper-activation of the PAM pathway. Instead, PI3K/AKT/mTOR inhibition triggered cell death blocking hormone-independent cellular growth¹³⁶. Interestingly, low levels of estradiol protect ER+/PIK3CA mutant BC cells from PI3K inhibitors effect¹³⁷. Further preclinical researches indicated that ER+ BC patients with altered PAM pathway activity poorly respond to ET¹³⁸. The phase III clinical trial SOLAR 1 has shown that combining Alpelisib, a PI3K α inhibitor, with Fulvestrant, anti-estrogen drug, significantly improved PFS in HR+/PIK3CA mutant metastatic BC patients from 5,7 to 11 months¹³⁹. Based on this result, FDA approved Alpelisib for post-menopausal women PIK3CA-mutated with HR+/HER2-, advanced or metastatic BC¹³⁹. Notably, the PI3K pathway's upregulation has been identified as a key mechanism also behind adaptive resistance to CDK4/6 inhibitors, leading to increased CDK2 activation¹³⁴. The essential role of PI3K in human cancer has led to the development of PI3K pathway inhibitors (PI3Ki) that have shown promise in preclinical models and have entered phase 1 clinical

trials. They can be divided into different classes based on their target(s) in the pathway: pan-PI3K inhibitors (pan-PI3Ki), isoform-specific PI3K inhibitors (IS PI3Ki), dual PI3K/mTOR inhibitors (dual PI3K/mTORi), AKT inhibitors (AKTi), mTOR inhibitors (mTORi) and PDK1 inhibitors (PDK1i)¹¹⁶.

Pan-PI3Ki are designed to inhibit the catalytic activity of all four class I PI3K isoforms: PI3K α , PI3K β , PI3K γ and PI3K δ thus offering extensive therapeutic efficacy. These inhibitors are effective to treat tumors with elevated PIP3 levels, regardless of PI3K or PTEN mutational status. However, they pose increased risks of both on-target and off-target toxicities¹⁴⁰. Despite several pan-PI3Ki undergoing clinical trials, only Copanlisib, targeting PI3K p110 α and p110 δ isoforms, has demonstrated significant clinical efficacy¹⁴¹.

IS PI3Ki generally exhibit a broader therapeutic index and reduced off-target toxicological effects due to the limited expression of various PI3K isoforms in non-cancerous cells. To date, the FDA has approved three IS PI3Ki: Alpelisib, Duvelisib and Idelalisib¹⁴². Alpelisib, inhibitor of the PI3K α isoform, has demonstrated significant clinical benefits when combined with ET in patients with HR+/HER2- PIK3CA-mutated advanced or metastatic BC¹³⁹.

Dual PI3K/mTORi show efficacy against all PI3K isoforms and mTOR complexes leading to the suppression of three critical intersections of the PAM signaling pathway. Numerous clinical trials are currently investigating dual PI3K/mTORi, such as Apatolisib, Dactolisib and Paxalisib, in monotherapy and in combination with other therapeutic approaches in advanced solid tumors¹¹⁶.

AKTi as Capivasertib, Ipatasertib and M2698 specifically inhibit AKT proteins, thereby impeding the hyper-activation of downstream factors in the PAM signaling pathway. To date, clinical trials are investigating AKTi as monotherapy or in combination with ET in advanced solid tumors¹¹⁶.

mTORi were the first PAM-targeting drugs used in clinic. Targeting both mTORC1 and mTORC2 is crucial to inhibit tumor progression although the efficacy of some mTORi may be compromised by compensatory AKT activation. There are three types of mTORi: first-generation allosteric (non-competitive) mTORC1 inhibitors; second-generation ATP-competitive mTORC1 and mTORC2 inhibitors and third-generation bi-steric mTORC1 inhibitors¹¹⁶. Everolimus, an allosteric mTORi has exhibited moderate clinical benefits in several types of cancers and has been FDA-approved in neuroendocrine tumor (NET) patients, TSC-associated SEGA patients, renal TSC-associated angiomyolipoma adult patients and advanced-stage renal cell carcinoma (RCC) patients⁵⁵.

1.4 Role of TP53 in BC

1.4.1 TP53 structure and activity

TP53 gene encodes for the transcription factor p53 which, upon cellular stress signals, regulates a great number of genes promoting cell cycle arrest, senescence, apoptosis, differentiation, DNA repair and genomic stability to restore cellular homeostasis¹⁴³. Activation of p53 may result various cellular responses according to the type, duration and amplitude of the stress stimulus as well as the context in which it occurs. While minor DNA damage may institute cell-cycle arrest and activate DNA-repair mechanisms, stronger TP53-activating signals induce senescence or apoptosis, effectively preventing the proliferation of damaged cells. Moreover, as a crucial tumor suppressor gene, TP53 can counteract several cancer hallmarks, like new blood vessel formation, invasion, metastasis, stemness and metabolic changes¹⁴⁴.

p53 exhibits the typical transcription factor structure with an amino-terminal trans activation domain (TAD), a core DNA-binding domain (DBD), a tetramerization domain and a carboxy-terminal regulatory domain¹⁴⁵. Several post-translational modifications, including phosphorylation, acetylation, ubiquitination and prolyl-isomerization can affect its transactivation activity, the DNA binding, protein turnover and interaction with other¹⁴⁶.

In the absence of stress signals, p53 levels are maintained low in most cell types by its negative regulators MDM2 and MDMX, which promote p53 degradation through ubiquitination¹⁴⁷. Cellular stress, including oncogenes activation, DNA damage, hypoxia, nutrient deprivation rapidly lead to intracellular p53 protein accumulation and activation. Uncontrolled cell proliferation, induced by oncogenes activation, activates p14ARF, which sequesters MDM2; while DNA damage and chemotherapeutic agents activate protein kinases, as ATM and ATR which, through DNA-dependent protein kinase (DNA-PK) and casein kinase II (CKII), respectively, phosphorylate the amino terminus of p53 to prevent MDM2 binding, and the carboxyl terminus of p53 to increase sequence-specific DNA binding^{148,149}. Stabilized and activated p53 forms tetramers in the nucleus, binds to consensus DNA sequences and regulates gene transcription to restore cellular homeostasis¹⁵⁰ (Fig. 6).

Furthermore, several studies have shown that p53 also controls “non-canonical” pathways, including the apoptotic response, by directly interacting with BCL2 protein family, homeostasis, ferroptosis, stem cell differentiation, autophagy, senescence and tumor microenvironment¹⁵¹.

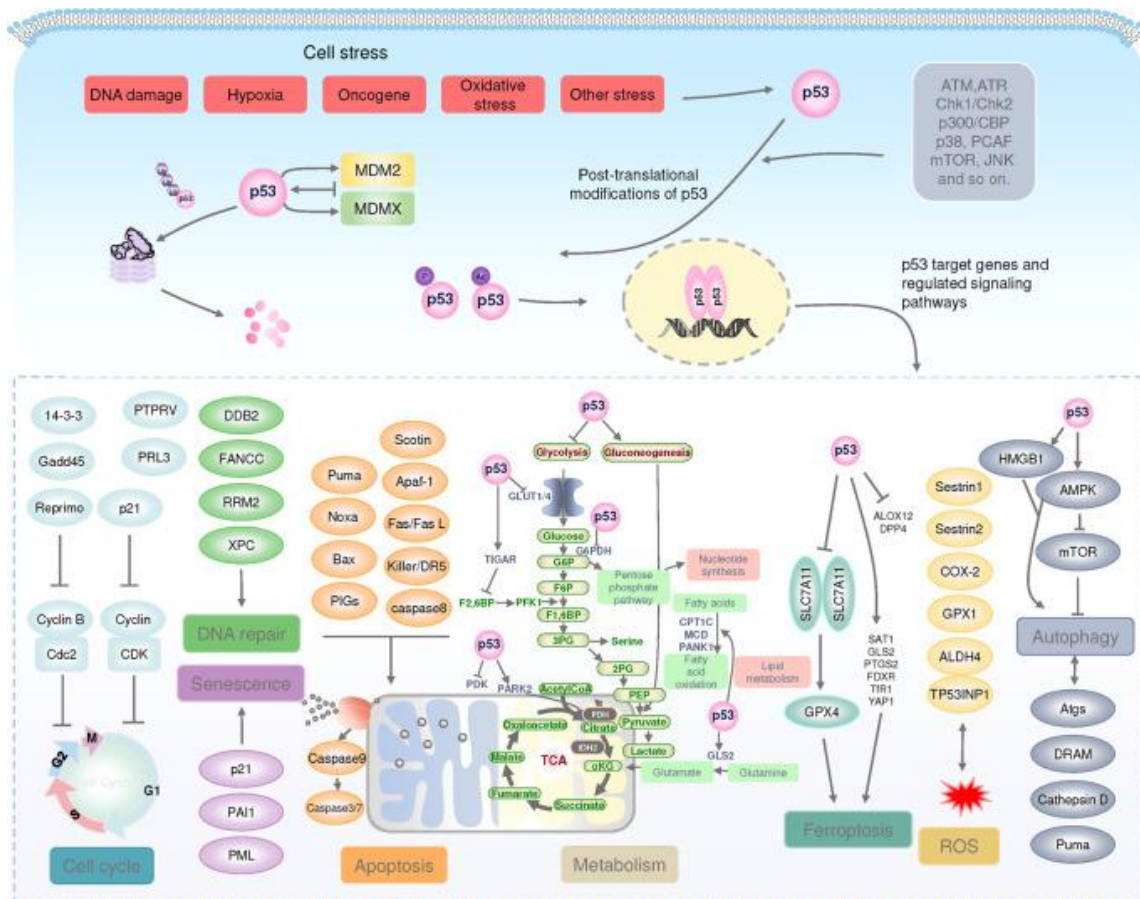


Fig. 6: p53 regulation and transcriptional activity. In normal conditions, p53 protein levels are tightly regulated by MDM2/X, which together ubiquitinate p53 leading to its proteasomal degradation. Under stress conditions, p53 is activated and stabilized by post-translational modifications. Stabilized p53 forms tetramers in the nucleus, binds to consensus DNA sequences, regulates gene transcription and controls many different biological processes restoring cellular homeostasis or inducing cell death or cell cycle arrest. Figure has been adapted from “*Targeting p53 pathways: mechanisms, structures, and advances in therapy*” Wang H et al.¹⁴⁹.

1.4.2 Landscape of TP53 mutations

The tumor suppressor TP53 is the most frequently mutated gene in cancer. The frequencies of reported TP53 mutations vary considerably across cancer types, ranging from ~5% in primary hematopoietic malignancies to 38–50% in ovarian, esophageal, colorectal, head and neck, larynx and lung cancers¹⁵². Notably, TP53 mutations are particularly frequent (60-70%) in advanced tumor stages or aggressive cancer subtypes, such as TNBC or HER2+ BCs¹⁵³. While somatic TP53 mutations are linked to sporadic cancers, TP53 germline mutations contribute to up to 17% of all familial cancer and cause Li-Fraumeni Syndrome (LFS), a rare cancer predisposition syndrome¹⁵⁴.

In 74% of cases, TP53 genetic alterations are monoallelic missense mutations that result in the production of a stable full-length protein (Fig. 7)¹⁵⁵. Approximately 86% of these missense mutations are clustered between codons 125 and 300 within the DNA-binding domain (DBD).

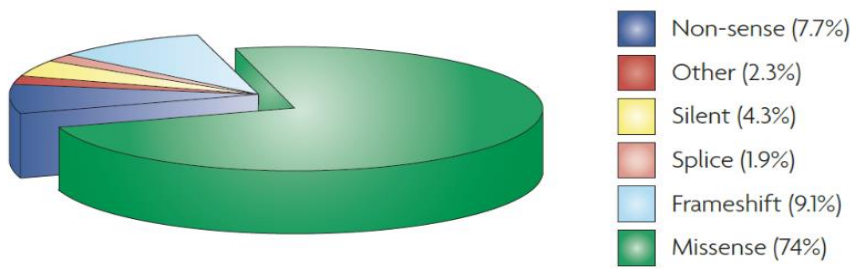


Fig. 7: Distribution of TP53 somatic mutations. Pie chart representing the different tumor-derived mutation types reported in the IARC TP53 Mutation Database. Figure has been adapted from “*When mutants gain new powers: news from the mutant p53 field*”. Brosh, R., Rotter, V.¹⁵⁶

According to their effect on p53 protein's stability, TP53 missense mutations are usually classified as “DNA contact” and “structural” mutants¹⁴⁶. The first type comprises changes in residues that directly interact with DNA (i.e. R248Q, R273H); the second one includes mutations that either cause localized conformational changes (i.e. R249S, G245S), or broader structural distortions (i.e. R175H, R282W)¹⁵⁶.

TP53 nonsense and frameshift mutations typically abrogate the sequence-specific DNA-binding activity towards wild-type (wt) p53 responsive elements causing a Loss of Function (LoF) effect¹⁵⁷. Besides losing their wild-type activities, many p53 mutants also function as dominant-negative (DN) proteins that inactivate wt p53 expressed from the remaining wild-type allele¹⁵⁸. Moreover, some mutant p53 forms also acquire new oncogenic properties that are independent of wt p53, known as ‘gain-of-function’ (GoF) properties¹⁴⁴ (Fig. 8).

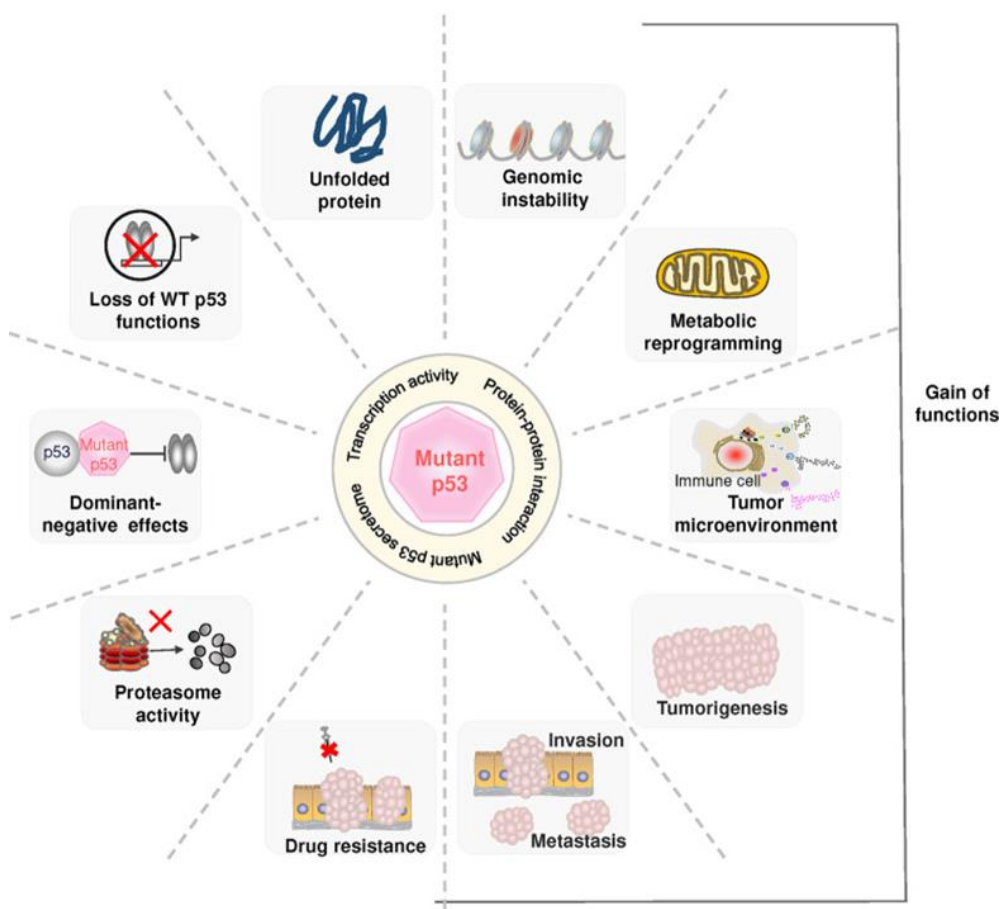


Fig. 8: Functional impact of TP53 mutations. The phenotypic effects of TP53 mutations can be classified into three non-mutually exclusive groups. 1) Loss of Function (LoF) mutations that abrogate or attenuate the binding of p53 to its consensus DNA sequence and, consequently, avoid the transcriptional activation of p53 target genes. 2) Mutations leading to Dominant Negative (DN) mutants which are able to inhibit the function of the wt protein encoded by the second allele. 3) Gain of Function (GoF) mutations that confer mutant p53 with new oncogenic functions independent of wt p53. Mutants p53 interfere with several cellular responses such as genomic instability, metabolic reprogramming and tumor microenvironment

promoting cancer cell proliferation, invasion, metastasis and drug resistance. Figure has been adapted from “Targeting p53 pathways: mechanisms, structures, and advances in therapy”. Wang H et al.¹⁴⁹.

Almost one third of all missense mutations arise in six hotspot residues (R175, G245, R249, R282, R248 and R273) which all result in GoF p53 proteins sharing common characteristics but with unique oncogenic functions¹⁵³ (Fig. 9). Among TP53 mutants, TP53^{R175H} is the most prevalent in cancer patients, as shown in both the TCGA and International Agency for Research on Cancer (IARC) TP53 somatic mutations databases¹⁵⁹. This mutant not only alters global gene expression interacting with new protein partners but also co-aggregates with its wild-type counterpart and other p53 family members (p63, p73) thereby inhibiting their tumor suppressive functions. Recent findings highlight the GoF properties relevance in tumorigenesis. Oncogenic p53 mutants can bind transcriptional factors such as p63, p73, nuclear factor- κ B, NF-Y, E2F1, VDR, SP1, SREBP and ETS, enhancing or repressing targets gene expression through preferential binding to other structural DNA motifs and non-consensus sequences. Mutant p53 (mut-p53) may also regulates chromatin structure and gene transcription upregulating methyltransferases (KMT2A, KMT2D, etc.) and acetyltransferases (KAT6A, etc.)¹⁶⁰, as well as recruiting chromatin-remodeling complexes like SWI-SNF¹⁶¹. GoF p53 mutants actively promote proliferation, survival, metabolic reprogramming, invasion, metastasis and drug resistance¹⁴⁴. Moreover, they play a crucial role in helping cancer cells adapt to proteotoxic and oxidative stress, as well as nutrient deprivation¹⁴⁴.

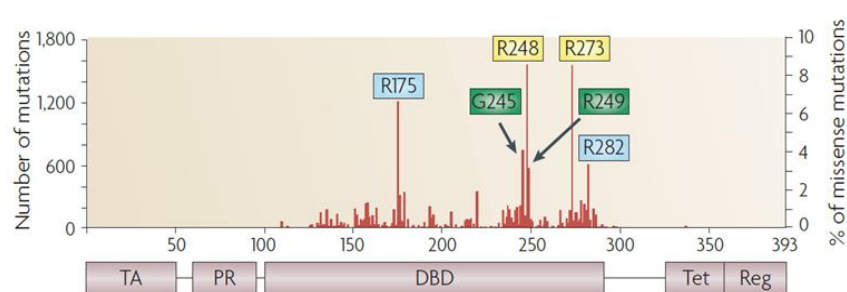


Fig. 9: Distribution of TP53 somatic mutations. The graph represents the distribution of the missense mutations along the 393 amino-acid sequence of TP53. The six most common hotspot mutations are highlighted in yellow for DNA-contact mutations, green for local conformational mutations and blue for global conformational

mutations. They are clustered within the central most conserved region of TP53, the DNA-binding domain, (DBD). In addition to the DBD, the TP53 sequence contains also the Transactivation domain (TD), Proline-Rich domain (PR), Tetramerization domain (Tet) and C-terminal Regulatory domain (Reg). Figure has been adapted from “*When mutants gain new powers: news from the mutant p53 field*” Brosh, R., Rotter, V.¹⁵⁶.

In the Catalog of Somatic Mutations in Cancer (COSMIC) database, TP53 mutations are present in ~23% of BC cases regardless molecular subtype, making TP53 the second most frequently mutated gene after PIK3CA, which has a mutation rate of 26%¹⁶². According to the IARC, around 70% of TP53 mutations in BC are missense variants¹⁵³. Around 20% of these mutations occurs in HR+ BC, while HR- BC, especially the TNBC, shows a higher frequency of TP53 missense mutations (80%)¹⁶³ suggesting that in the absence of hormone-driven signaling of the mammary epithelium, mut-p53 becomes crucial for cancer onset and progression.

The role of TP53 mutations in HR+ BC is still unclear. Scientific evidences indicate that blocking estrogen-dependent signaling leads to a reduction in both wt and mutant p53 protein levels in ER+ BC cells¹⁶⁴. Notably, ER- α and mut-p53 might collaborate to transcribe target genes binding to unconventional promoter regions¹⁶⁵. Despite this potential synergy, ER- α is also known to suppress both the wt and mutated p53-dependent transcription in BC cell lines by directly interacting with the p53 forms and attracting transcriptional repressors. These evidences suggest that in a p53 mutated context, ER- α might shape the transcriptional environment of BC cells harboring these mutants^{166,167}. During BC onset and progression, mutant p53 plays a crucial role in regulating genetic instability, survival, drug resistance, proliferation, invasion, metabolism reprogramming, etc. (Fig. 8).

By interacting with Mre11, mut-p53 recruits MRN/ATM complex to DNA double-strand breaks (DSB), preventing ATM activation¹⁶⁸, thus inhibiting both the activation of the DNA Damage Response (DDR) and the Homologous Recombination (HR)-Mediated DNA Repair. Consequently, BC cells become more resistant to apoptosis and senescence but more sensitive to DNA DSBs, heightening their genomic instability¹⁶⁹.

Certain p53 mutant forms activate the PI3K/AKT signaling pathway boosting cell growth and survival. Normally, the oncosuppressor DAB2IP binds to PI3K and AKT, restricting their activation. However, mut-p53 binds to DAB2IP neutralizing its tumor-suppressing effects and facilitating insulin-driven PI3K–AKT pathway activation¹⁷⁰. Additionally, mut-p53 increases the expression of the insulin-like growth factor I receptor gene (IGF1R) which, once activated, stimulates both the PAM and Ras/mitogen-activated protein kinase (MAPK) pathways¹⁷¹. Mut-p53 also interacts with NF-Y and its cofactor YAP, but unlike its wild type counterpart, it promotes the transcription of NF-Y target genes such as cyclins (Cyclin A, Cyclin B, etc.) and cyclin-dependent kinases (cDKs), consequently leading to cell cycle progression and DNA synthesis¹⁷².

It was demonstrated a positive correlation, in BC context, between mut-p53 and the expression of anti-apoptotic proteins such as survivin¹⁷³ and Bcl-2¹⁷⁴ and that p53 mutations are able to suppress autophagy inhibiting the AMPK kinase or activating the PI3K/mTOR pathway, which further suppress autophagy¹⁷⁵. Mut-p53 can also influence gene expression related to drug resistance by interacting with some transcriptional factors as DNA TopBP1, NFY, p300, ETS1 and ETS2¹⁷⁶. Furthermore, TP53^{R175H} can bind the promoter of the proteasome activator gene REG γ similarly to its wt counterpart. However, unlike the wt, it inhibits the binding of SMAD3 co-repressor, thereby upregulating REG γ expression. This upregulation leads to the degradation of several tumor suppressors, including p53, p21 and p16, thus promoting BC cell proliferation, survival and drug resistance¹⁷⁷. Moreover, TP53^{R175H} promotes epithelial–mesenchymal transition (EMT) and invasion enhancing TWIST1 expression through the downregulation of BMI-1 or inhibiting its paralog p63¹⁷⁸.

1.4.3 Therapeutic strategies against mutants p53

Since p53 is mutated in most malignant tumors, it represents a very promising target for developing new therapeutic approaches. Strategies of treatment might include the reactivation of wild-type p53 function, the mut-p53 degradation, the disruption of oncogenic complexes with specific small molecules or immunotherapies based on mutant p53 neoantigen recognition^{159,176}.

Reactivation of wild-type p53. High-throughput cellular screenings have identified several small molecules capable of restoring the wt function of p53 by binding the cysteine residues of p53-mutated forms. Among them, *PRIMA-1*, a quinuclidinone, effectively inhibits the growth of tumor cells carrying TP53^{R175H} and TP53^{R273H} mutations, but not the TP53 wild type ones¹⁷⁹.

APR-246 (PRIMA-1MET), currently undergoing phase 2 clinical trials, is a methylated derivative of PRIMA-1 even more effective¹⁷⁹. It can both reactivate wt p53 proteins and boost intracellular ROS levels to which Mut-p53 cells are more vulnerable due to their abnormal metabolic pathways¹⁸⁰.

ZMC1, a thiosemicarbazone, acts as a Zn²⁺ chelator and enhances Zn²⁺ binding to TP53^{R175H}. Zn²⁺ is known to be essential for wt p53 stability, while the R175H mutation disrupts the local structure near the Zn²⁺ binding site. By improving Zn²⁺ binding, ZMC1 reactivates mut-p53 and induces oxidative stress generating hydroxyl radicals¹⁸¹.

COTI-2, another thiosemicarbazone, reactivates p53 mutants too and inhibits the PAM pathway reducing phosphorylated-AKT and -caspase-9 levels in tumor cells lines¹⁸².

Disruption of complexes involved in mut-p53 GoF. Designing peptides or nucleotide aptamers to disrupt aberrant complexes formed by mut-p53 may have a putative application in targeted therapy of BC disease. Recently, GFP-KA2 it was used as a chimeric "decoy" protein to disrupt the mut-p53/DAB2IP complex thereby arresting inflammation-driven invasion *in vitro*, as well as xenograft growth and spread in BC models¹⁸³. Additionally, the GFP-KA2 decoy prevented insulin-induced proliferation and invasion in BC cells¹⁷⁰. Similarly, methods aimed at inhibiting mut-p53's interaction with essential partners like NFY, Nrf2, p63 and p73 could be equally valuable to limit cancer progression¹⁷⁶.

Promoting of mut-p53 degradation. The intracellular levels of mut-p53 are regulated and stabilized by the HSP chaperone machinery (Hsp90, Hsp70 and Hsp40/DNAJA1), RhoA, GTPase, HDAC6 and the mevalonate pathway (MVP). Disrupting these interactions can effectively reduce mut-p53 levels through its degradation¹⁵⁹. The inhibition of MVP with Statins (HMG-CoA reductase inhibitors) or Zoledronic Acid (farnesyl diphosphate synthase inhibitor) reduces intracellular geranylgeranyl pyrophosphate (GGPP) levels, which in turn inhibits RhoA activation. Similar outcomes can be

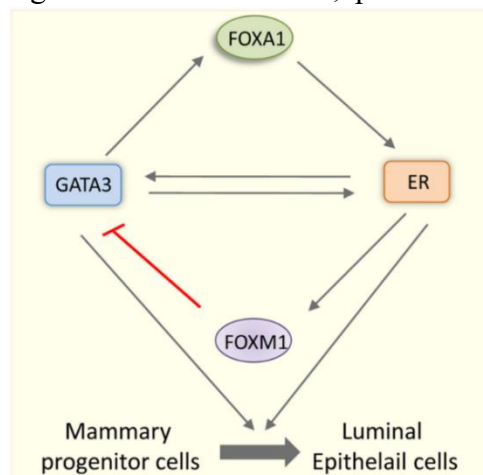
achieved by blocking RhoA geranylgeranylation using GGTI protein geranylgeranyl-transferase type I inhibitors, such as GGTI-298¹⁸⁴. Additionally, the FDA-approved HDAC inhibitor Suberoylanilide Hydroxamic Acid (SAHA) specifically downregulates mut-p53¹⁸⁵. Moreover, combining SAHA with the Hsp90 inhibitors, Ganetespib, disrupts the HDAC6/HSP90/mutp53 complexes, facilitating mut-p53 ubiquitination through MDM2 and CHIP¹⁸⁶.

Immunotherapy based on mut-p53 neoantigen recognition. Recently, Hsiue et al. have developed a bispecific antibody H2-scDb that targets the TP53^{R175H} peptide-HLA-A0201 complex on cancer cells. Simultaneously engaging the TCR-CD3 complex, H2-scDb successfully activated T cells in order to kill cancer cells both in vitro and in vivo¹⁸⁷. These findings emphasize the potential of mut-p53 peptide neoantigens as promising targets for cancer immunotherapies.

1.5 Role of GATA3 in BC

1.5.1 GATA3 functions and mutations

GATA3 is a zinc finger transcription factor involved in regulating several pathways including the differentiation of mammary gland where it is expressed exclusively in the luminal epithelial compartment¹⁸⁸. Here it determines the fate of progenitor cells and maintains the integrity and functions of luminal epithelial cells. GATA3 deletion during mammary gland development leads to significant abnormalities, particularly the failure of Terminal End Bud (TEB) formation during puberty¹⁸⁹, while forced expression of GATA3 in mammary stem cell promotes their differentiation into luminal cells¹⁹⁰.

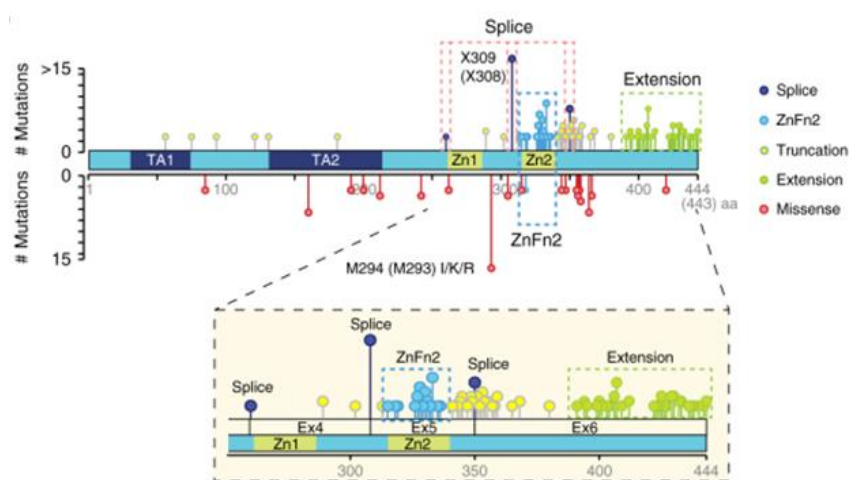


During puberty, pregnancy, lactation and menopause the mammary gland undergoes complex developmental transitions, responding to different acute physiological signals. These transitions are regulated by reproductive hormones as estrogen and progesterone, through transcription factors such as ER- α , FOXA1 as well as GATA3¹⁸⁸ (Fig. 10).

Fig. 10: Interdependence of ER- α , GATA3 and FOXA1 determines mammary cells fate. GATA3 regulates FOXA1, which in turn mediates ER expression. Further, ER induces GATA3 expression in luminal cells. Moreover, FOXM1, an ER- α inducible transcription factor, downregulate GATA3 expression through inducing methylation of its promoter, highlighting that GATA3/FOXA1/FOXM1/ER axis dictates the mammary cell fate. Figure has been adapted from “*Estrogen receptor co-regulators and pioneer factors: the orchestrators of mammary gland cell fate and development*”. Manavathi B et al.¹⁸⁸.

GATA3 protein has two transactivation domains (TA1 and TA2) essential for chromatin remodeling and two zinc finger motifs (ZnFn1 and ZnFn2) for DNA binding. The distal zinc-finger motif (ZnFn2) binds specifically to the GATA DNA motif (A/T)GATA(A/G), whereas the proximal zinc-finger motif (ZnFn1) exhibits broader specificity. Each ZnFn domain is followed by a highly conserved region crucial for post-translational modifications and for homo-hetero dimerization with other GATA family partners¹⁹¹. According to TCGA data, GATA3 is among the top three mutated driver genes in BC (10%), alongside TP53 and PIK3CA⁵¹. GATA3 acts as a tumor suppressor by inhibiting mesenchymal fates, thereby maintaining luminal cell differentiation¹⁹².

GATA3 mutations, mainly frequent in luminal BC rather than basal-like BC, are typically heterozygous, primarily clustered in the C-terminal region, within the exons 5 and 6, near the ZnFn domains. Most of these mutations are frameshifts or indels that can be categorized into five groups: ZnFn2 mutations, splice site mutations, truncation mutations, extension mutations and missense mutations¹⁹³ (Fig. 11). In the METABRIC cohort (n=1980), 230 patients exhibited GATA3 mutations and 75% of them were luminal BC patients (47% Lum A and 28% Lum B)⁸². Interestingly, ZnFn2 mutations were predominantly found in Lum B tumors (52%), whereas splice site and truncation mutations were more frequently observed in Lum A subtypes (62% and 68%, respectively). Moreover,



patients with high GATA3 expression levels carrying ZnFn2 mutations had significantly worse 10-year survival rates than those with other GATA3 variants, suggesting that ZnFn2 mutations may be more harmful than the complete loss of this domain¹⁹³.

Fig. 11: Distribution of GATA3 somatic mutations. The graph represents the distribution of GATA3 mutations along the 444 amino-acid sequence. Different type of variants (Splice, ZnFn2, Truncation, Extension and Missense) are indicated by different colors. Most of these mutations are clustered in the C-terminal region, within the exons 5 and 6, near the ZnFn domains. The mutation data was obtained from the METABRIC cohort via the cBio Cancer Genomics Portal. The positions of zinc-finger motifs were obtained from the Uniprot database. Figure has been adapted from “GATA3 zinc finger 2 mutations reprogram the breast cancer transcriptional network”, Takaku M. et al.¹⁹³.

Mutations occurring in the ZnFn2 domain alter GATA3 DNA binding activity and protein stability, due to conformational changes. Recently, it was demonstrated that R330fs mutation is able to reprogram the breast cancer transcriptional network leading to a partial epithelial to mesenchymal transition and a growth advantage also in xenograft models¹⁹³.

Despite recent large-scale genomic analyses showed that GATA3 mutations are particularly enriched in BC patients, the clinical and molecular outcomes of these variants are poorly understood¹⁹⁴.

2. AIM OF THE STUDY

Breast cancer (BC) is the leading cause of cancer-related deaths and incidence rate among women under 40 years of age is rising, worldwide. Even after adjusting for tumor stage, grade, and subtype, young patients show a poorer response to standard therapies, a higher risk of relapse, and an overall lower survival compared to older patients.

Although the HER2+ and the TN subtypes are typically enriched in young patients, the disparity in clinical outcome is especially notable in the Luminal subtype (LBC), showing a higher prevalence of the more aggressive luminal B subtype over luminal A. However, the biology of LBC arising in young patients is still not well understood and there is a lack of models that accurately reflect the molecular and microenvironmental characteristics of LBC in young patients. Therefore, there is a clinical need for age-specific molecular insights to develop new and tailored treatment options.

In this PhD project, we have tried to deal with this unmet clinical need, aiming at uncovering genetic, molecular and biological bases of this disease.

Therefore, this research work has the following objectives:

1. To investigate the molecular fingerprints of LBC arising in young women, in order to identify unique mutational signatures that may contribute to the high aggressiveness of the disease. By Next Generation Sequencing (NGS) approaches, we aimed at identifying differentially mutated genes and signatures in a “real world” cohort of pre-menopausal (<45, Young, Y) and post-menopausal (>55, non-Young, nY) LBC patients, to identify age-related novel biomarkers of response and therapeutic drugs.
2. Given the enrichment of PIK3CA and TP53 co-mutation in Y patients that we found, we also aimed at characterizing their biological impact on mammary tumorigenesis. To this aim, we have transformed primary luminal breast epithelial (BPE) cells with PIK3CA and TP53 mutants, alone or in combination, to shed light on the molecular mechanisms underlying the increased tumor aggressiveness and identify predictive biomarkers of drug response and new therapeutic targets to transfer to the clinics in patients carrying these mutations.

3. MATERIALS AND METHODS

3.1 Patients's samples

Solid mammary tumor specimens were collected after surgery from patients who signed written informed consent, in accordance with recognized ethical guidelines and following approval by the Institutional Review Board of CRO Aviano, Italy. All studies were performed in compliance with the 1975 Declaration of Helsinki, as revised in 1983. Fresh BC specimens that arrive from the Pathology Unit are immediately processed in our lab. After eliminating adipose or necrotic tissue, every specimen is cut in small pieces (~ 2x2 - 3x3 mm) that are processed separately for different purposes: DNA and RNA extraction, fresh implantation in mice, organoid generation and storage. Histological evaluation of tumor representative sections was performed by the Pathology Units of CRO Aviano. Tumor stage was assessed according to the American Joint Committee on Cancer (8th edition)¹⁹⁵. Expression of hormone receptors (ER clone SP1, cat. # 790-4324, pre-diluted; PgR clone 1E2, cat. # 790-4296, pre-diluted; Ventana, Roche, Basel, Switzerland), HER2 (HER2 clone 4B5, cat. # 790-2991, pre-diluted; Ventana) and proliferation index (Ki-67, clone 30-9, cat. # 790-4286, pre-diluted; Ventana) were evaluated by immunohistochemistry (Ultraview Detection Kit cat. #760-500; Ventana); HER2 status was measured and reported according to ASCO-CAP 2018 guidelines¹⁹⁶. Then, breast tumors were classified in accordance with WHO 2023¹⁹⁷.

3.2 Genomic analyses

3.2.1 DNA purification and quantification

The Maxwell® 16 Tissue DNA Purification Kit (Promega, Madison, WI, USA) was used for automated purification of DNA from fresh solid tumor samples. Before extraction, samples have been stored at -80°C and, during processing, always maintained in dry ice. From the original specimen, we cut a little fragment to use for purification. DNA has been purified in standard elution volume (100-200 µl), already set up following the manufacturer's instructions. Maxwell® purification kit includes Maxwell® 16 Cartridges (MCA), Purification Plungers, Elution Tubes and Elution Buffer. Plunger grinds solid tissue samples directly in the lysis buffer in prefilled reagent cartridges. This integrated grinding replaces time- and labor-intensive use of lytic enzymes, such as proteinase K, or manual tissue grinding prior to purification. Finally, we removed elution tubes from platform-heated slots and placed them onto Magnetic Elution Tube Rack allowing the retention of residual magnetic particles in the magnetized side of the tube.

DNA quantification was performed using the QuantiFluor® ONE dsDNA System (Promega Madison, WI, USA) that contains a fluorescent DNA-binding dye that can sensitively quantify small

amounts of double-stranded DNA (dsDNA) in a purified sample. Quantification of unknown samples always requires a comparison to a dsDNA standard concentration. To prepare a standard, operator can either use the QuantiFluor® ONE Lambda DNA (Promega Madison, WI, USA) or a DNA of similar molecular weight to the sample of interest. The operator just must add the standard or the unknown sample to the dye solution and read on the Quantus Fluorometer (Promega Madison, WI, USA).

3.2.2 Targeted next generation sequencing (NGS)

An input of 25 ng of DNA from solid BC biopsies was used for subsequent mutational analysis by Next-Generation Sequencing (NGS). Mutational status of PIK3CA, AKT1, TP53 and GATA3 was investigated through the NGS approach with an amplicon-based strategy. Specific primers covering the complete open reading frame (ORF) of human GATA3 and TP53 genes or specific regions containing hotspot mutations of PIK3CA (E545; H1047) and AKT1 (E17) were designed and modified adding specific 5' adapter sequences (Merck Life Science, Darmstadt, Germany) which are complementary to the oligonucleotides bound to the surface of sequencing flow cells.

The 5' adapter sequences are the following:

Forward (FW): TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG

Reverse (RV): GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

The primer sequences used to detect hotspot mutations are reported in the following table:

GENE	AMPLICON	SENSE	SEQ
PIK3CA	PIK3CA_EX10	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATGACAAAGAACAGCTCAAAGCA-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCATTTTAGCACTTACCTGTGAC-3'
	PIK3CA_EX21	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTGAGCAAGAGGCTTTGGAGTAT-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATGCATGCTGTTTAATTGTGTGGA-3'
AKT1	AKT1_EX3	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTAGAGTGTGCGTGGCTCTCA-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGAAGGTGCCATCATTCTTGAGG-3'
GATA3	GATA3_EX-2A	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGGGCGCGAGCAGACGCCGAG-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTCCTCCGGCAGCGGTTACTG-3'
	GATA3_EX-2B	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTCAGCCACTCCTACATGGACGCGG-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGCACCCCGGGCGCACTCAC-3'
	GATA3_EX-3A	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTGCCCTTTCCCGTTGCC-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTGGCCCCCGACAAGGAGGAG-3'
	GATA3_EX-3B	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TCCGTCTACCCCGGCGCT-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCGGTCATGCTGCCACGG-3'
	GATA3_EX-3C	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCATGAAGCTGGAGTCGTCCCAC-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTCCAGGGAAGAGCTGGCTCC-3'
	GATA3_EX-4	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCCTAAGTGGCTTATCTGTGC-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCATCCCTTCCGAGAACTTAC-3'
	GATA3_EX-5	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCCTGTCTTCATAGTGATGACA-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTCTTGCTGATCCAGTCCAC-3'
	GATA3_EX-6A	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGTGCGAGAGCCTGTGCAT-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTGAGCCACATCTCGCCCTT-3'
	GATA3_EX-6B	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTCCCAAGAACAGCTCGTTTAA-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAGTCCCTTTCAGATTGCATTTT-3'

TP53	TP53_EX-2	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTTCCAATGGATCCACTCAC-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTTGGAAGTGTCTCATGCTGGAT-3'
	TP53_EX-3	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCATGGGACTGACTTTCTGC-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGGTGAAAAGAGCAGTCAGAG-3'
	TP53_EX-4A	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGCATTCTGGGAGCTTCATCTG-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGCCTTTTACCCATCTACAG-3'
	TP53_EX-4B	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCTGCCCTGGTAGGTTTCTG-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCGATATTGAACAATGGTTCAGTGAAGA-3'
	TP53_EX-4C	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGATACGGCCAGGCATTGA-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCCTGTCTATCTTCTGTCCCTT-3'
	TP53_EX-5A	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTCCGTCATGTGCTGTGACT-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGACTTTCAACTCTGTCTCCTTCT-3'
	TP53_EX-5B	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCCAGACCTAAGAGCAATCAGT-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCCCATGGCCATCTACA-3'
	TP53_EX-6A	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGTCTGTCTATCCAACTCCACA-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTGCTCAGATAGCGATGGTGA-3'
	TP53_EX-6B	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCCACTGACAACCACCCTTAAC-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTCAGCATCTTATCCGAGTGGAA-3'
	TP53_EX-7A	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGCTCCTGACCTGGAGTCTT-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTCATCTTGGGCTGTGT-3'
	TP53_EX-7B	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGGGGTGAGAGGCAAGCAGA-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGGCCATCCTCACCATCAT-3'
	TP53_EX-8A	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTCTCCAGGACAGGCACAAACA-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGAGTAGATGGAGCCTGGTTTTT-3'
	TP53_EX-8B	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTTGCTTACCTCGCTTAGTGCT-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTATCCTGAGTAGTGGTAATCTACTGG-3'
	TP53_EX-9A	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCTAGGAAAGAGGCAAGGAAA-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGAAGAGAATCTCCGCAAGAAAGG-3'
	TP53_EX-9B	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTCCACTTGATAAGAGGTCCCAAG-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCAGTTATGCCTCAGATTCACTTTATC-3'
	TP53_EX-10A	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTTGAGTTCCAAGGCCTCATT-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTTGAACCATCTTTAACTCAGGTACTGT-3'
	TP53_EX-10B	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAGAATGGAATCCTATGGCTTTCCA-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCGTGAGCGCTTCGAGATGTT-3'
	TP53_EX-11A	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCCCTTCTGTCTTGAACATGAGTTT-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAAAGCATTGGTCAGGGAAGG-3'
	TP53_EX-11B	FW	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACCTATTGCAAGCAAGGGTTCA-3'
		RV	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGTCCAAAAGGGTCAGTCTACCT-3'

Amplicon libraries were generated using a modified Illumina protocol. Briefly, multiplex PCR products were generated using Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 5X Phusion Green GC Buffer (Thermo Fisher Scientific, Waltham, MA, USA) and Promega™ Ultra Pure Deoxynucleotide Triphosphates (dNTPs) sets (Promega, Madison, WI, USA).

The PCR reaction is performed using the following thermal cycle protocol:

PIK3CA; AKT1; GATA3 MIX		
Cycles	Temperature (°C)	Time
X1	98 °C	3'
X40	98 °C	20"
	60 °C	20"
	72 °C	20"
X1	72 °C	7'

TP53 MIX		
Cycles	Temperature (°C)	Time
X1	98 °C	45"
X40	98 °C	10"
	60 °C	15"
	72 °C	20"
X1	72 °C	7'

Next, multiplex PCR products are diluted 1:100 and subsequently tagged with specific Index primers (Integrated DNA Technologies, IDT, Coralville, IA, USA), according to manufacturer's instructions.

PCR protocol used for this step is:

PIK3CA; AKT1; GATA3 MIX		
Cycles	Temperature (°C)	Time
X1	98 °C	3'
X10	98 °C	20"
	55 °C	20"
	72 °C	20"
X1	72 °C	7'

TP53 MIX		
Cycles	Temperature (°C)	Time
X1	98 °C	30"
X14	98 °C	10"
	55 °C	30"
	72 °C	30"
X1	72 °C	7'

To purify PIK3CA, AKT1 and GATA3 PCR products, we used Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) that contains Wizard® SV Minicolumns, Collection Tubes, Membrane Wash Solution, Membrane Binding Solution and Nuclease- Free Water for the elution.

To purify TP53 PCR products we used PureLink™ Quick PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) that contains Minicolumns, Collection Tubes, Binding Buffers (B2 and B3), Wash Buffer and 10Mm Tris-HCl Ph8.5 Elution Buffer.

Purified libraries were quantified using NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) and normalized to 2nM. Libraries were then sequenced with paired-end 2 x 250 reads using the Miseq sequencing platform (Illumina, San Diego, CA, USA) with a final concentration of 20pM using MiSeq Reagent Nano Kit v2 (Illumina, San Diego, CA, USA). The minimum depth of coverage (per amplicon) was of approximately 1000/1500X.

Single Nucleotide Variants (SNV) of possible clinical significance were evaluated by analyses of Catalogue of Somatic Mutations in Cancer (COSMIC, <https://cancer.sanger.ac.uk/cosmic>) and International Cancer Genome Consortium (ICGC, <https://dcc.icgc.org/>) data portals.

NGS data were mapped to the human reference genome (GRCh37/hg19, https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.13), analyzed with VariantStudio™ (Illumina, San Diego, CA, USA) and Integrative Genomics Viewer (IGV) software and then annotated. Only somatic variants with an allele frequency (VAF) $\geq 1\%$ called in at least two distinct NGS runs were maintained. Synonymous variants and polymorphisms described in the Single Nucleotide Polymorphism Database (dbSNP138) were removed.

In parallel, we also loaded our data onto a cBioPortal platform local installation, which consents the exploitation of the cBioPortal software, thus providing a user-friendly analysis of molecular data, fully integrated with clinical/pathological features of the tumors analyzed¹³³.

3.2.3 Whole Exome Sequencing (WES)

DNA-Seq libraries were generated using KAPA HyperExome Kit (Roche, Basel, Switzerland, EU) according to the Kapa HyperPlus manufacturer's instructions. This kit is designed to perform enzymatic fragmentation, end repair, A-tailing, and adapter ligation in a single-tube workflow, significantly reducing hands-on time and minimizing sample loss.

Briefly, 120 ng of high-quality genomic DNA, from solid BC biopsies, was used for subsequent mutational analysis. DNAs were enzymatically fragmented using the kit's proprietary enzymatic fragmentation mix. The concentration and the average size of the DNA fragments was assessed using High Sensitivity D1000 Screen Tape System (Agilent Technologies, Inc., Santa Clara, CA, USA). Each DNA sample had an average size of 350-400 bp as recommended by Roche protocol.

Next, DNA fragments were end-repaired and A-tailed in order to allow their ligation to custom sequencing adapters. DNAs were then purified using AMPure XP beads (Beckman Coulter, Brea, California, US) to remove unligated adapters, small fragments and other reaction components.

After the PCR amplification, the libraries have been hybridized with the NimbleGen SeqCap EZ Exome Probes (Roche, Basel, Switzerland, EU) according to the manufacturer's instructions.

Pools of 96 purified libraries were quantified and normalized to 2nM. Libraries were then pooled in equal volumes and sequenced with paired-end 2 x 150 reads using the NovaSeq 6000 sequencing platform (Illumina, San Diego, CA, USA) with a final concentration of 1.7pM.

FastQ files had been processed using the nf-core pipeline Sarek 3.4.0 (<https://nf-co.re/sarek/3.4.0>)¹⁹⁸ on the ORFEO cluster from Area Science Park (Trieste, Italy), thanks to the collaboration with the group of Prof. Giulio Caravagna (University of Trieste). FastQ files were aligned against genome reference GATK.GRCh38 using the aligner Burrows-Wheeler Aligner - Maximal Exact Matches (BWA-MEM)¹⁹⁹. Mark duplicates and recalibration steps were then executed using Sarek default parameters. After the recalibration, data were saved in BAM format and used to perform variant calling. Considering our experimental design, the 96 samples of our study had been divided into two different cohorts: Tumor-normal matched cohort (TNM) (38 samples, both tumoral and normal) and Tumor only cohort (TO) (58 samples). To perform variant calling on the TO cohort, created a Panel-of-Normals (PON) was created following Genome Analysis Toolkit (GATK)²⁰⁰ GATK.4 guidelines (<https://gatk.broadinstitute.org/hc/en-us/articles/360037058172-CreateSomaticPanelOfNormals-BETA>). The custom PON was then used to perform somatic variant calling with Mutect2 for the TO cohort and to obtain additional support in the TNM variant calling step.

Copy number calling was performed using Allele-Specific Copy number Analysis of Tumors (ASCAT)²⁰¹ only for the TNM cohort by following Sarek recommendations to run it on WES

samples. ASCAT returned both information on the copy number state of each identified segment and an estimate of purity and ploidy of the sample. Variant annotation was performed by using the Ensembl-Variant Effect Predictor (VEP)²⁰² with cache version 110 from the corresponding reference genome. Mutations and copy number data (when available) were processed with in-house tools and scripts. BRCA driver list was downloaded by the Integrative OncoGenomics (IntOGen) database²⁰³, in order to identify potential driver genes involved in the onset and evolution of the disease.

3.3 Cell biology experiments

3.3.1 Cell culture

Primary immortalized luminal breast epithelial cells (BPE), isolated by Ince T.A. et al²⁰⁴, were purchased from LTCC (Miami, USA). These cells were cultured on plates with a plastic surface optimized for adhesion and growth of primary cultures (Primo®, EuroClone, Milan, Italy, EU). The culture medium used were WIT-P Basal Medium (LTCC, Miami, Florida, USA), supplemented with WIT supplement (LTCC, Miami, Florida, USA), 1% penicillin-streptomycin (P/S Gibco, Thermo Fisher Scientific, Waltham, MA, USA). and 100ng/mL cholera toxin (Merck Life Science, Darmstadt, Germany, EU) or BMI-P (Breast Modified Ince medium for primary cells) (LTCC, Miami, Florida, USA) supplemented with BMI-P Bullet 1 and 2, 1% PS and cholera toxin (100ng/mL). Cells were cultured at 37°C in a humidified chamber containing 5% CO₂.

3.3.2 Lentiviral transduction and nucleofection of BPE cells

BPE cells overexpressing PIK3CA mutants were generated using lentiviral particles expressing PIK3CA^{H1047R} and PIK3CA^{E545K} (MISSION® CSTVRS02041915MN Merck Life Science, Darmstadt, Germany).

Cells were seeded in 6-well plate (4.5x10⁵ cells/well) and the day after they were transduced according to the manufacturer protocol adding polybrene (8 µg/ml) to enhance transduction efficiency. Supernatants were removed after 24 hours and replaced with fresh medium. After 72 hours from transduction cells were selected by adding to the culture medium puromycin (1.0 µg/ml) (Merck Life Science, Darmstadt, Germany) for PI3K^{E545K} cells, Hygromycin (25 µg/ml) (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) for PI3K^{H1047R} cells and empty vectors control cells. Pools were expanded to evaluate the expression of PI3K mutants by western blot. To overexpress the TP53^{R175H} mutant in BPE cells we used Amaxa™ HMEC Nucleofector™ kit (Lonza, Basel, Switzerland, EU) containing HMEC Nucleofector solution, Supplement 3 and electroporation cuvette. 4µg of pCMV/Neo-TP53R175H (Addgene#16439) and control vector (pCMV/Neo;

Addgene) were mixed with 0.1 ml of cell suspension (2×10^7 cells/ml), transferred to a 2.0 mm electroporation cuvette and nucleofected following the manufacturer's instructions. After electroporation, cells were immediately transferred to 2.0 ml of complete medium in six-well plates and then were selected with 0,3 µg/ml of G418 (Roche, Basel, Switzerland, EU).

Once selected, mutants BPE pools cells were plated in 96-well plate (0.7 cells/well) to perform a single cell cloning. One clone of cells overexpressing mutants PI3K and mutant TP53 was used in *in vitro* assays.

Moreover, TP53^{R175H} BPE clones were additionally transduced with PI3K^{E545} and PI3K^{H1047} lentiviral particles to generate the BPE PI3K^{H1047R}-TP53^{R175H} and PI3K^{E545K}-TP53^{R175H} co-mutant pools. Once selected, the expression of both mutated proteins was evaluated by immunofluorescence. One pool of cells overexpressing mutants PI3K together with TP53 was used for *in vitro* assays.

3.3.3 Cell viability and sensitivity to drugs

For growth curve analyses, BPE cells were seeded into 96-well culture plates (2×10^3 cells/well) and after 24h cell proliferation was measured with CellTiter 96 AQueous cell proliferation assay kit (Promega, Madison, WI, USA) every day for six consecutive days.

This is a colorimetric method to determine the cytotoxicity of substances with which cells are treated, chemosensitivity or the number of proliferating cells. The kit includes saline solution of a tetrazole compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, called MTS, with a coupling electronic reagent, phenazine methosulfate (PMS). The MTS compound is metabolized by living cells in formazan, soluble in the cell culture medium. The absorbance of this substrate is 492 nm and can be measured directly from the 96 multiwell in which the cells were plated.

For kill curve analyses BPE cells were plated in 96-well plate (5×10^3 cell/well) in triplicate in RPMI medium (80%), WIT-P medium (20%) and 1% PS. After 24 h cells were treated with increasing doses of drugs for 72h. Cell viability was determined at the end of treatment using the CellTiter 96 AQueous Cell Proliferation Assay Kit (Promega, Madison, WI, USA).

Tamoxifen (H7904) and Doxorubicin were purchased from Merck (Darmstadt, Germania, EU); Abemaciclib (HY-16297A) was purchased from Clinisciences (Nanterre, Île-de-France, EU).

For the epigenetic drug screening, BPE cells were plated in 96-well plate (5×10^3 cell/well) in WIT-P medium. After 24h, cells were treated with 10 µM of epigenetics screening library (96-well)™ (Cayman Chemical, Ann Arbor Michigan, USA) containing 151 small modulator of histone deacetylases (HDACs), histone acetyltransferases (HATs), histone methyltransferases (HMTs), DNA

methyltransferases (DNMTs) and other epigenetic regulators. Cell viability was measured after 72h using the CellTiter 96 AQueous Cell Proliferation Assay Kit (Promega, Madison, WI, USA).

3.3.4 Sphere-forming assay

To establish primary spheres, cells were collected, disaggregated in trypsin using 25-gauge needle fitted to a syringe. Cells (1×10^4 cells) were plated on 6-well plate coated with Poly-HEMA (P3932 - Poly(2-hydroxyethyl methacrylate) in a medium containing 80% of RPMI, 20% of WIT-P, 1% of PS, B27 supplement (50×, no vitamin A; Life Technologies) and recombinant epidermal growth factor (hEGF, 20ng/ml) (Merck Life Science, Darmstadt, Germany).

Primary spheres were incubated in a humidified atmosphere at 37°C and 5% CO₂ for 7 days without disturbing the plates or replenishing the media. Volocity® software (PerkinElmer) was used to count the spheres (greater than 50 µm diameter) and to measure their area.

Sphere-forming efficiency (SFE%) was calculated using the following formula:

$$SFE\% = N^{\circ} \text{ of spheres per well} / N^{\circ} \text{ of cells seeded per well} * 100.$$

To establish secondary spheres, primary spheres were collected, disaggregated in trypsin using 25-gauge needle fitted to a syringe and plated at the same seeding density of the primary generation.

Sphere self-renewal was calculated as the ratio between the total number of secondary spheres divided for the total number of primary spheres.

3.3.5 Clonogenic Assay

Cells were seeded into 6-well plate (1×10^3 cells/well) in complete medium. The day after, cells were treated in RPMI medium (80%), WIT-P medium (20%) with Tamoxifen and/or Abemaciclib and Doxorubicin for 24h. After 7-10 days, colonies were fixed and stained with 0.5 mg/ml of crystal violet in 20% methanol in shaking 30min at room temperature. Colonies with more than approximately 50 cells were counted manually.

3.4 Molecular biology experiments

3.4.1 Protein extraction and western blot analysis

Total protein extract was obtained scraping cells on ice using an appropriate volume of cold RIPA lysis buffer (NaCl 150 mM, Tris HCl pH 8 50 mM, Igepal 1%, Sodium-deoxycholate 0.5%, SDS 0.1% in deionized water), which was supplemented with a protease inhibitor cocktail Complete™

(Roche, Basel, Switzerland, EU), 1 mM Na₃VO₄ (Merck Lifesciences, Darmstadt, Germania, EU), 100 mM NaF (Merck Lifesciences, Darmstadt, Germania, EU) and 1 mM DTT (Merck Lifesciences, Darmstadt, Germania, EU). Cells were then incubated for 30 minutes on ice and centrifuged at 13200 RPM for 30 minutes at 4°C to obtain proteins lysates that were transferred into fresh tubes. Concentration of isolated proteins was determined using the Bradford protein assay (BioRad, Hercules, California, US) by performing a standard curve using a BSA solution of 1 µg/µl diluted in PBS 1X. Proteins were separated in 4-20% SDS-PAGE Criterion Precast Gel (BioRad, Hercules, California, US) and transferred to nitrocellulose membranes (GE Healthcare). Membranes were incubated with blocking buffer (EveryBlot Blocking Buffer) (BioRad, Hercules, California, US) for 1 hour at room temperature. Subsequently, membranes were incubated at 4°C overnight with appropriate primary antibodies. Membranes were washed in TBS-0.1% Tween20 and incubated 1 hour at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare) for ECL detection (Clarity Western ECL Substrate) (BioRad, Hercules, California, US). Band quantification was performed using the Image Lab software (BioRad, Hercules, California, US) or ImageJ software. The Re-Blot Plus Strong Solution (Merck Life Science, Darmstadt, Germany) was used to strip the membranes, when reblotting was needed.

Primary antibodies used are indicated in the following table:

Primary antibodies	Dilution	Animal	Vendor
PIK3CA	1:1000	Rabbit	Cell Signaling
P53-DO1	1:1000	Mouse	Santa Cruz
pS780-RB	1:1000	Rabbit	Millipore- Sigma
CDK4	1:500	Mouse	Santa Cruz
pS139-H2AX (γH2AX)	1:500	Mouse	Millipore-Sigma
RAD51	1:200	Rabbit	Santa Cruz
VINCULIN	1:1000	Mouse	Santa Cruz
GAPDH	1:1000	Mouse	Calbiochem

3.4.2 Immunofluorescence

For immunofluorescence analyses, cells were seeded in wells containing round coverslips (Thermo Fisher Scientific, Waltham, MA, USA) and then, at specified time points, fixed 10 min in 4% PFA, permeabilized for 5 min in PBS 0.2% Triton, and blocked 1 hour in PBS-10% normal goat serum (NGS). Incubation with primary antibody was performed overnight at 4°C.

Coverlips were washed in cold PBS 1X and incubated 1 hour at room temperature with IR-conjugated AlexaFluor680 (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) or IRDye 800 (Rockland, Limerick Pennsylvania, US) secondary antibodies. Stained cells were observed using a confocal laser-scanning microscope (TSP2 or TSP8 Leica).

Primary antibodies used are indicated in the following table:

Primary antibodies	Dilution	Animal	Vendor
PIK3CA	1:200	Rabbit	Cell Signaling
P53-DO1	1:200	Mouse	Santa Cruz

4. RESULTS

4.1 Unveiling novel mutational signatures in LBC from young patients

4.1.1 PIK3CA, AKT1, TP53 and GATA3 mutational profile in LBC patients

Thanks to the collaboration with the Pathology Unit of our Institute, we have collected fresh specimens of BC, from patients undergoing surgery and consenting to donate their tissues for research purposes. Up to now, we have collected more than 500 samples (primary tumors, recurrences, +/- lymphnodal metastases, +/- healthy peri-tumoral tissue) from 470 patients of different age and BC subtypes.

For this project, we were particularly interested in the luminal subtype (LBC) that remains the most common histotype also in young women ($\sim 65\%$)¹⁰¹, despite the enrichment of more aggressive subtypes (*i.e.* triple negative and HER2+)⁹⁸. In order to uncover the genetic, molecular and biological bases that make LBC in young patients a unique disease entity, we performed a comprehensive mutational profiling analysis using Next-Generation Sequencing (NGS) approaches.

To avoid biases due to tumor type, evolutionary dynamics, treatment history and uncertain menopausal status, we considered different features and adopted several criteria to select the samples for our analyses. First, we selected only patients with primary Hormone Receptor-positive (HR+) tumors (Luminal A, Luminal B, HER2+/- subtypes), sharing the same histological ductal origin. Furthermore, we excluded patients between 45 and 55 years of age, as we were specifically interested in individuals with a well-defined menopausal status. We also excluded patients who had received neoadjuvant therapy prior to sample collection, thus ensuring that the mutational landscape analyzed was reflective of the untreated primary tumor biology (Fig. 1A). Finally, more than 90% of the specimens were from grade 2 and 3 cancer, and disease stages 1-3. Importantly, all these features were perfectly balanced in our subgroups, in order to identify alterations that were not simply associated with other histological and clinical criteria.

Then, the cohort was stratified into two age/menopausal status categories: young pre-menopausal patients (Y) (age <45 years) (n = 119; 56%), and non-Y post-menopausal patients (nY) (age >55 years) (n = 92; 44%) and into three categories, based on their luminal subtype: Luminal A (Lum A, Ki67 <20%) (n=86; 41%), Luminal B (Lum B, Ki67 >20%) (n=102; 48%), and Luminal HER2+ (HR+, HER2+) (n=24; 11%) (Fig. 1B-C).

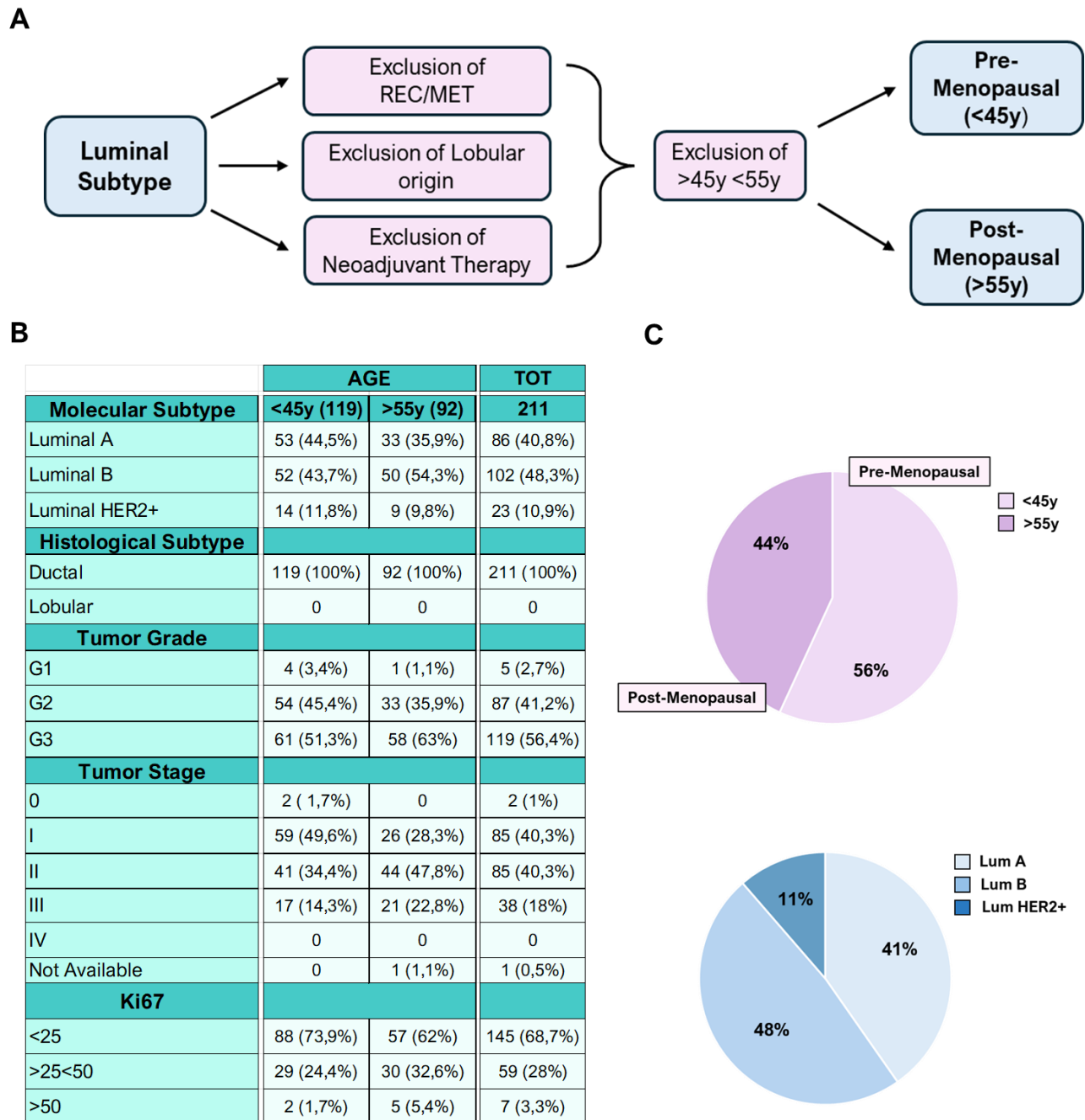
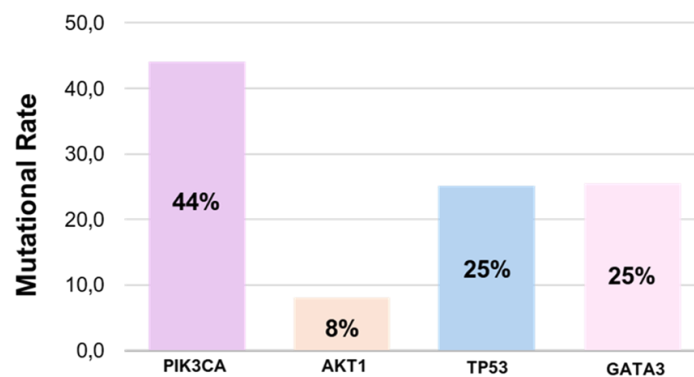


Fig. 1: Luminal Breast Cancer (LBC) cohort overview. **A)** Schematic workflow of the steps for patients' selection. **B)** Table summarizing the histopathological characteristics (molecular and histologic and subtype, Tumor grade/stage, Ki67 levels) of LBC cohort (N= 211). Patients are divided into two age categories: <45y (n=119) pre-menopausal patients and >55y (n=92) post-menopausal patients. **C)** Pie charts display the percentage of the patients based on age/menopausal status (Up) and luminal subtype (Down).

We first analyzed the mutational status of the most relevant genes involved in BC progression. To this aim, we extracted DNA from all samples (N=211) and performed a targeted gene sequencing analysis, using an amplicon-based strategy. A home-made multiplex custom panel was designed and set up to cover the complete Open Reading Frame (ORF) of TP53 and GATA3 genes, two exons (exons 10 and 21) of PIK3CA and one exon (exon 3) of AKT1, bearing the most common mutational hotspot of these genes.

Looking at our whole cohort, PIK3CA, AKT1, TP53 and GATA3 mutational rate was found to be 44%, 8%, 25% and 25%, respectively (Fig. 2A). Across the luminal subtypes, PIK3CA mutations were more enriched in Lum A compared to Lum B and Lum HER2+ tumors (53% vs 36% and 43%, respectively). GATA3 also showed a higher mutational frequency in Lum A compared to Lum B and Lum HER2+ tumors (30% vs 23% and 18%, respectively). On the contrary, TP53 gene was significantly more mutated in Lum B and Lum HER2+ compared to Lum A tumors (33% and 41%, respectively, vs 10%) while AKT1 mutations were much lower and equally distributed within different luminal subtypes (8% vs 8% vs 9%, Lum A vs Lum B vs Lum HER2+) (Fig 2B).

A



B

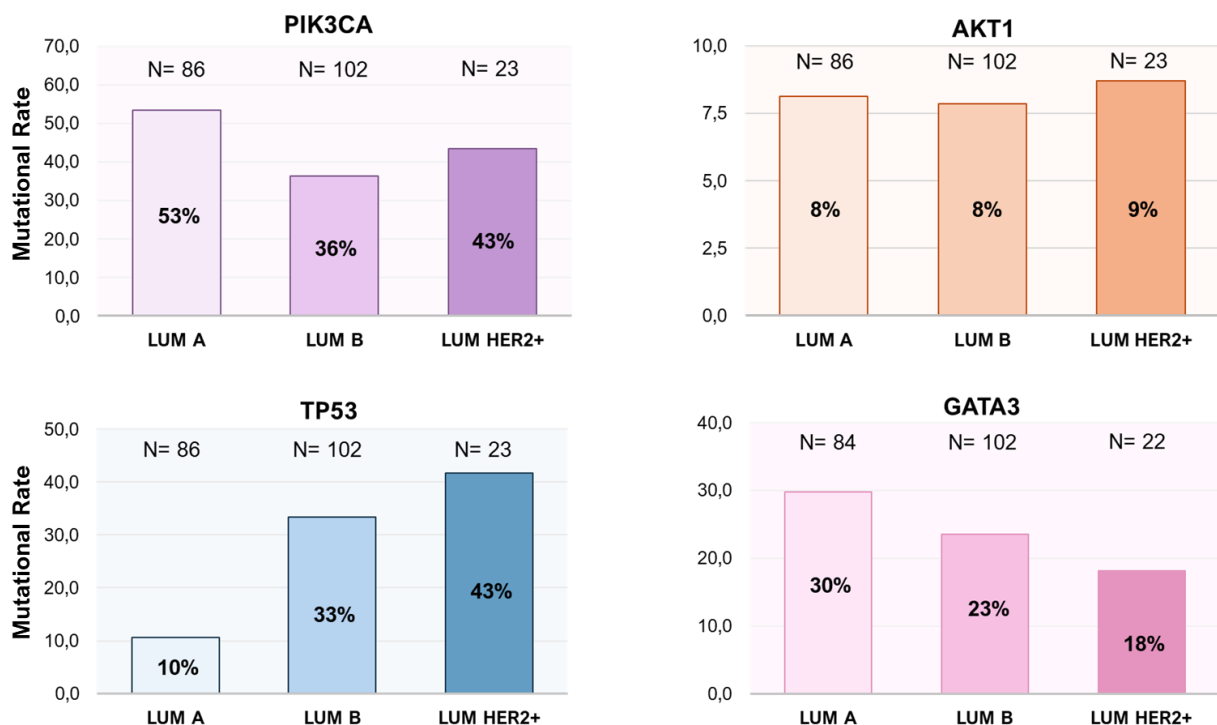


Fig. 2: Mutational profile of PIK3CA, AKT1, TP53 and GATA3 genes in LBC patients. A) Bar plot displaying the mutational rate of PIK3CA, AKT1, TP53 and GATA3 in our patients' cohort. B) Bar plots showing the mutational frequency of the same genes of interest subdividing patients for luminal category (Lum A, Lum B, Lum HER2+). Mutation data are normalized on the total number of patients (N) for each luminal category.

4.1.2 Comparison of PIK3CA/AKT1, TP53 and GATA3 somatic landscape in Y versus nY LBC patients

To evaluate the impact of age/menopausal status in tumor aggressiveness between Y and nY women, we then analyzed the incidence of PIK3CA, AKT1, TP53 and GATA3 mutations in these two groups of LBC patients.

The oncoprint diagram in Fig. 3A shows the mutational status of these genes in the cohort, stratified by age/menopausal class. Mutations in PIK3CA/AKT1 and GATA3 were significantly enriched in Y patients (57% and 34%, respectively, vs 36% and 14%, Y vs nY; $p=0.002$, $p=0.001$). In contrast, TP53 mutations were observed to increase in nY patients compared to their younger counterparts (20% vs 31%, Y vs nY) (Fig. 3C).

Interestingly, 81% of Y LBC patients carried at least one mutation in one of the genes analyzed, compared to 62% of nY ones, overall suggesting that Y LBC patients have a greater propensity to acquire mutations in these genes. This finding is further supported by the evaluation of the frequency of co-mutation in any two of the four genes, which was also higher in pre-menopausal patients (30% vs 16%, Y vs nY) (Fig. 3B).

Despite the well-established significance of the PIK3CA, TP53, and GATA3 pathways in oncogenesis, the role and interactions of co-mutations remain unclear. Several studies indicated that patients harboring co-mutations in PIK3CA and TP53 pathways exhibit more aggressive phenotype and worse survival outcomes^{93,94}. However, in the context of LBC in Y women, the role and mechanisms of action of the co-mutations remain unknown. For this reason, we have considered co-mutants as an additional category in subsequent analyses, but the mutational status of PIK3CA and AKT1 was combined, as they belong to the same pathway and are usually mutually exclusive (Fig. 3A).

Interestingly, we found that Y patients harbor higher frequency of GATA3 and PIK3CA/AKT1 co-mutations (16% vs 7%, Y vs nY; $p=0.051$). On the other hand, co-occurring mutations in PIK3CA/AKT1 and TP53 were equally distributed between the two LBC subgroups (12% vs 13%, Y vs nY), while GATA3 and TP53 alterations appeared to be mutually exclusive, as shown by the low frequency of co-mutated pre- and post-menopausal patients (5% vs 2%, Y vs nY). (Fig. 3C). However, considering the more aggressive Lum B BC patients' category, both GATA-PIK3CA/AKT1 and PIK3CA/AKT1-TP53 co-mutations appeared to be enriched in Y women, although this trend is not statistically significant. Additionally, GATA3 mutational frequency was significantly higher in Lum B Y patients (Y vs nY, $p=0.01$) compared to their older counterpart (Fig. 3D).

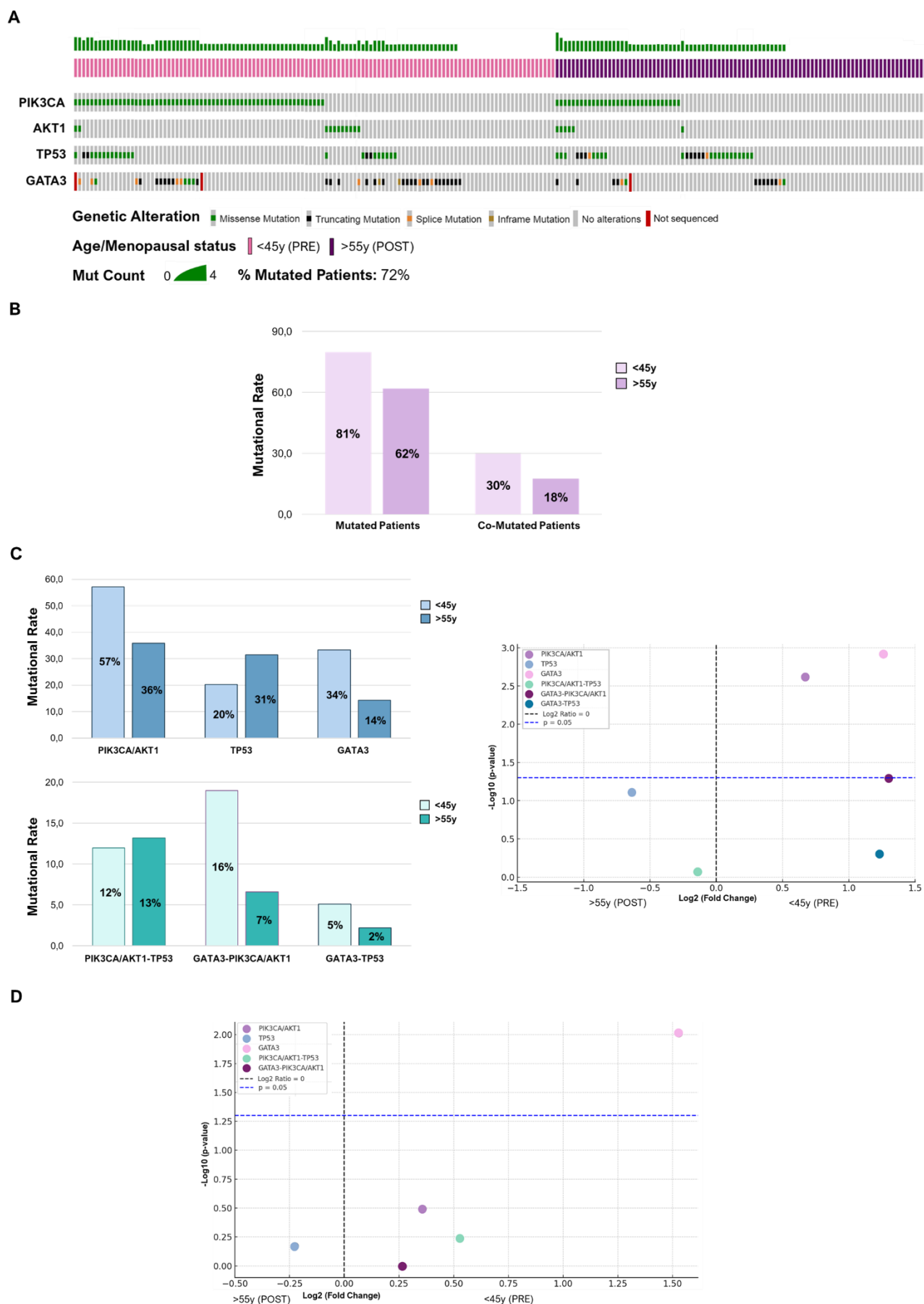


Fig. 3: Evaluation of PIK3CA/AKT1, TP53 and GATA3 mutations and co-mutations in Y pre-menopausal vs nY post-menopausal patients. A) Oncoprint displaying the distribution of our interests' genes mutations subdividing patients

for age/menopausal status. Mutation count for each patient and genetic alteration types are reported on the graph. **B)** Bar plot of total mutations and co-mutations distribution in <45y and >55y patients. **C)** Bar plot showing the distribution of PIK3CA/AKT1, TP53 and GATA3 mutation within the two age/menopausal groups (Up/Left). Bar plot showing the distribution of PIK3CA/AKT1-TP53, GATA3-PIK3CA/AKT1 and GATA3-TP53 co-mutations within the two age/menopausal groups (Down/Left). Volcano plot showing the enrichment of interests' genes mutations between the two age/menopausal categories (Right). The genes are indicated by different colors. Mutation data are normalized on the total number of patients (N) for each age/menopausal category. **D)** Volcano plot showing the enrichment of interests' genes mutations between the two age/menopausal groups of patients stratified for the luminal subtype (Lum A, Lum B, Lum HER2+). Fisher exact test has been used for statistical analysis.

Next, we assessed the impact and localization of the different variants along the protein sequence and domains since they might lead to different oncogenic effects. We focused on TP53 and GATA3 genes, since PIK3CA and AKT1 presented the typical hot spot mutations.

Consistently with literature data, in our cohort, TP53 mutations are predominantly missense mutations located in the DNA-binding domain (DBD)¹⁶³ (72% vs 28%, missense vs other mutation types) (Fig. 4A), whereas GATA3 mutations are largely frameshift mutations (68% vs 32%, frameshift vs other mutation types), primarily localized within their DNA-binding zinc finger domains¹⁹³ (ZnFn1 and ZnFn2). Additionally, in GATA3, the most harmful ZnFn2 frameshift mutations represented the 21% of mutated patients (Fig. 4B).

Next, we analyzed whether the distribution of these TP53 and GATA3 variants was different between the two age/menopause groups. As shown in the TP53 lollipop and bar plots, nY patients displayed a higher number of frameshifts, nonsense, and splice site mutation, usually associated with a loss of function of p53 (16,5% vs 38%, Y vs nY), while Y patients most frequently displayed missense mutations, usually associated with a gain of function of the encoded protein (83% vs 62% Y vs nY) (Fig. 4C and D).

Next, we evaluated the distribution of specific GATA3 variants, across the two age/menopausal group of patients. The lollipop plot shows that the Y patients present a higher number of GATA3 variants compared to the older counterpart and that the most enriched variant in the group of Y patients is the X309_splice site mutation (Fig. 4D). When we stratified patients for the luminal subtypes, we observed that Y Lum B BC patients showed a significantly high frequency of frameshift mutations within the ZnFn2 domain (33,3% vs 0%, Y vs nY; $p = 0.027$) while Y Lum A BC patients carried frameshift mutations located in other regions (42,1% vs 0%, Y vs nY; $p=0.02$).

Overall, these data suggest that our selected cohort of pre-menopausal patients had distinct mutational features compared to the post-menopausal counterparts, at least using this small gene panel. In particular, these patients significantly harbor more aggressive mutations in these driver genes, such as missense mutations in TP53 and mutations in the Znfn2 domain of GATA3, particularly in the Lum B subtype, known to be enriched in Y patients. Additionally, the presence of a higher frequency of

PIK3CA/AKT1 mutations in combination with GATA3 or TP53, may contribute, at least in part, to the increased aggressiveness of their tumors compared to those of nY patients.

We decided to investigate deeper into this possibility, by conducting *in vitro* mechanistic studies in LBC cells (see section 4.2).

However, the approach that we used was heavily biased since we only evaluated the most frequently mutated genes in LBC. In search of possible novel alterations or profiles, we also analyzed the same patients' cohort using whole exome sequencing (WES).

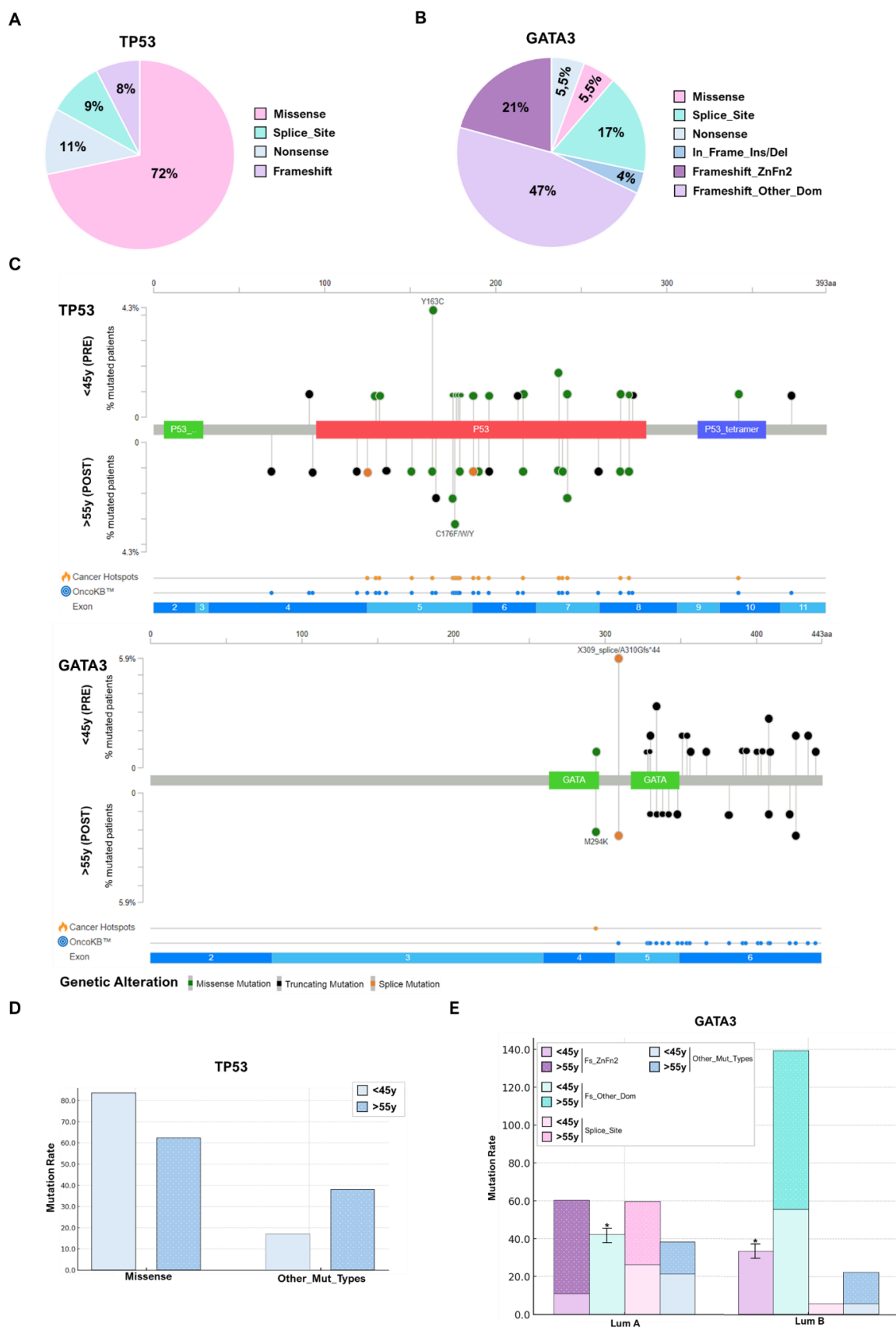


Fig. 4 Distribution of different types of TP53 and GATA3 mutations in Y pre-menopausal vs nY post-menopausal patients across the luminal subtypes. A) Pie chart displaying the distribution of TP53 different mutation types (missense, splice site, nonsense, frameshift). **B)** Pie chart displaying the distribution of GATA3 different mutation types (splice site, missense, non-sense, in frame_ins/del and frameshift). The pie chart shows also the distribution of GATA3

frameshift variants within the protein domains (ZnFn2, other) in our cohort. **C)** Lollipop plots displaying the distribution of specific amino acidic variants of TP53 (Up) and of GATA3 (Down) in <45y and >55y patients. The genetic alteration and Clinvar/ OncoKB pathogenicity classification are reported in the graphs. **D)** Bar plot showing the distribution of TP53 missense and other mutations (non-sense, splice site and frameshift variants) in <45y and >55y patients. **E)** Bar plots showing the distribution of different types of GATA3 mutations across different Lum A and Lum B subtypes in <45y and >55y patients. Mutation types are reported in the graph. The category Other_Mut contains missense, non-sense, in frame_ins/del variants. Mutation type data are normalized on the total number of mutated patients for each age/menopausal category. Fisher exact test has been used for statistical analysis; asterisks indicate significant differences *P ≤ 0.05.

4.1.3 Whole exome sequencing analysis of Y versus nY LBC patients

In order to assess a broader genetic profile and identify novel mutational signatures in Y LBC patients, we performed a WES analysis on a cohort of LBC patients (n=96) stratified by age and menopausal status: Y pre-menopausal patients (age <45 years) (n=54) and nY post-menopausal patients (age >55 years) (n=38). Additionally, a panel of matched normal samples (PON, n=38) was also sequenced, allowing us to reduce the background noise of germline polymorphisms²⁰⁰ (Fig. 5A).

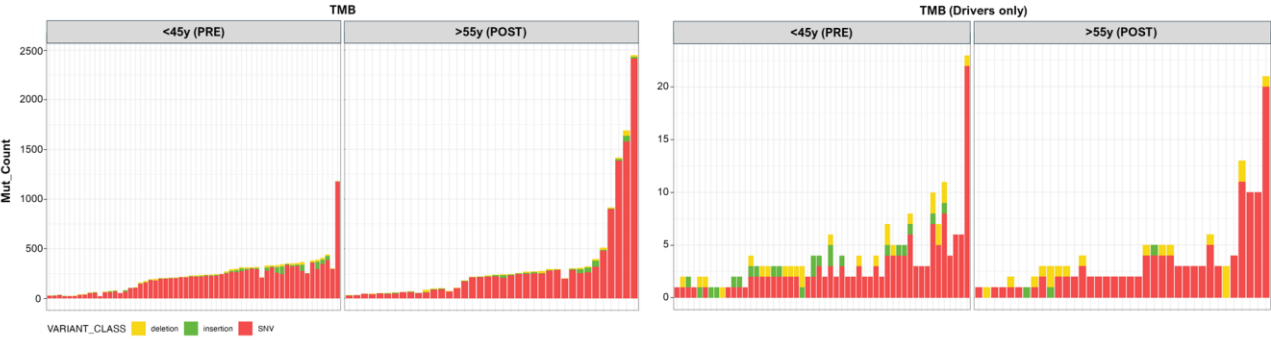
Tumor Mutational Burden (TMB) has been evaluated considering both the total number of genetic mutations and only mutations occurring in driver genes for BC onset. As expected, nY patients showed a higher number of total variants per sample compared to Y patients, in line with the positive correlation between age and mutational events. There was no significant difference in the number of mutations occurring in BC driver genes between the Y and nY group (Fig. 5B).

Among the top thirty mutated BC driver genes in the whole cohort, PIK3CA (36%), TP53 (17%), and GATA3 (14%) showed the highest mutational frequency, followed by MAP3K1 (11%), KMT2C (10%) and CBFB (8%) (Fig. 5C). A different mutational profile between the Y and nY groups of patients was observed. In line with our targeted gene sequencing data, shown above, PIK3CA (40% vs 32%, Y vs nY) and GATA3 (19% vs 5%, Y vs nY) resulted more frequently mutated in Y compared to nY women, followed by ERBB2, FAT3, CBFB and MAP3K1 genes. On the other hand, LRP1B, EPHA3 and CSMD3 mutations were enriched in the nY subgroup.

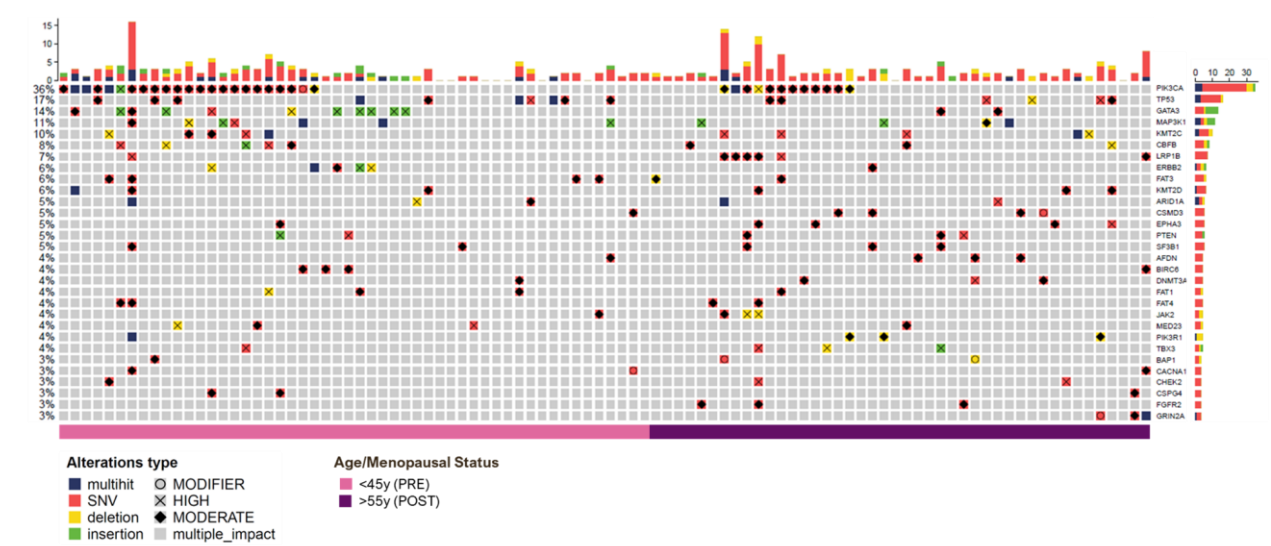
A

Menopausal Status	Pre Menopausal				Post Menopausal	
Age group	<45y				>55y	
Luminal type	A	B	A	B	A	B
Tumor Samples (n = 96)	14	23	8	13	16	22
	58				38	
Healthy Samples (n = 38)	4	9	1	8	7	9
	22				16	

B



C



D

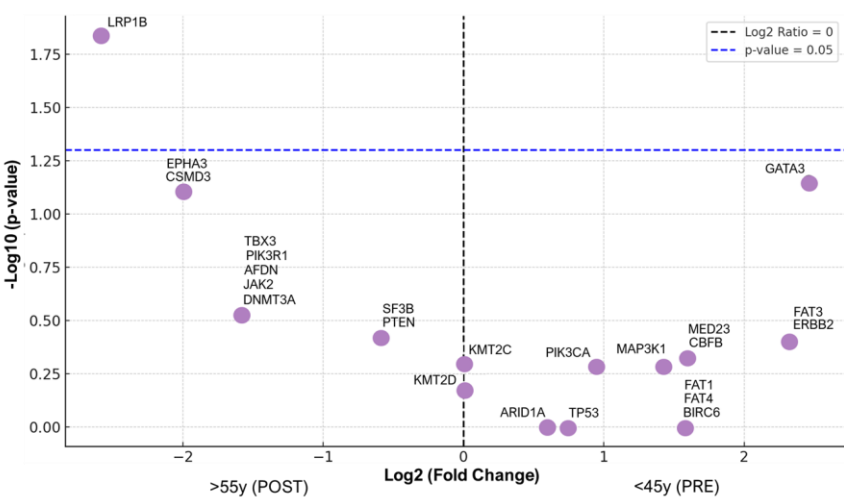


Fig. 5: The genomic landscape of LBC internal cohort. **A)** Table displays the LBC patients' samples selected for WES analysis. Patients have been divided into two age and menopausal status categories: <45y (n=58) and >55y (n=38). Additionally, 38 healthy matched samples have been sequenced. **B)** Bar plots show the tumor mutation burden (TMB) in <45y vs >55y. The graphs show the number of total mutations (Left) and BC driver genes mutations (Right) detected for each patient. **C)** OncoPrint graph shows the top mutated BC driver genes in our cohort. Mutation count for each patient, genetic alterations and effect predictions are reported. IntOGen database was used to obtain the list of driver genes. **D)** Volcano plot shows the enrichment of top 24 mutated BC driver genes between the two age/menopausal groups. Fisher exact test has been used for statistical analysis.

Next, we evaluated the copy number alterations (CNA) distribution in the tumor-matched samples from Y (n=22) and nY (N=16) patients. Both the age/menopausal groups exhibited gains and deletions alteration across multiple chromosomes. In detail, within the whole cohort, the long arm of chromosome 16 (16q) and the short arm of chromosomes 8 (8p) and 17 (17p) were the most deleted regions; while chromosomes 1 (1q), 8 (8q), 17 (17q) and 16 (16p) showed highest frequency of duplications. Taking into consideration the two age/menopausal groups, Y patients exhibited an overall greater number of chromosomal amplifications compared to nY (~23,000 Mb vs ~15,000 Mb, Y vs nY), while the number of deletions remained roughly comparable between the two groups (Fig. 6A).

Then, we analyzed CNA profiles of single chromosomes in which known oncogenes and tumor suppressors involved in BC are located. As shown in Fig. 6B, chromosome 3, containing PIK3CA gene sequence, did not display significant differences in CNA profile between the two groups, while the region with TP53, in the chromosome 17p, exhibited a higher frequency of deletions in Y patients compared to nY (55% vs 44%, Y vs nY), suggesting that Y patients may be more susceptible to TP53 loss and, consequently, to genomic instability. Interestingly, within chromosome 10, the region containing GATA3, exhibited similar rates of duplication and deletion in nY patients (25% and 38%, respectively), while it resulted selectively amplified rather than deleted in Y (0% and 45%, respectively), supporting the hypothesis that a high frequency of GATA3 amplification may enhance the oncogenic effect of its mutations, particularly enriched in Y women.

Overall, these findings, whose analyses are still ongoing, further elucidate the genetic landscape of LBC patients, suggesting that other driver genes, in addition to PIK3CA, GATA3 and TP53, may be involved in promoting tumor aggressiveness in Y patients.

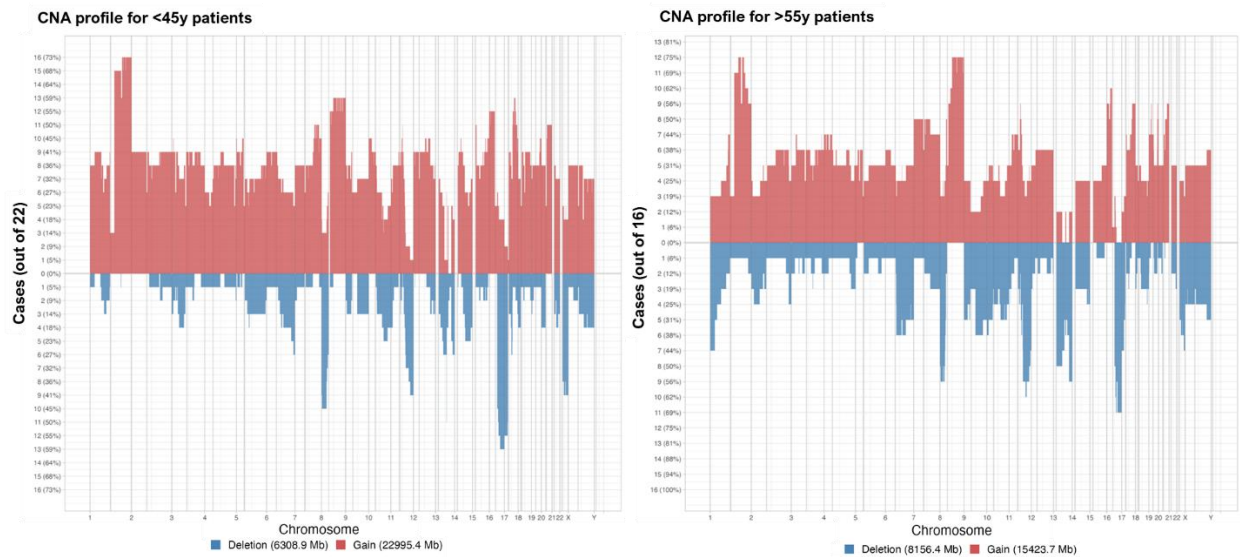
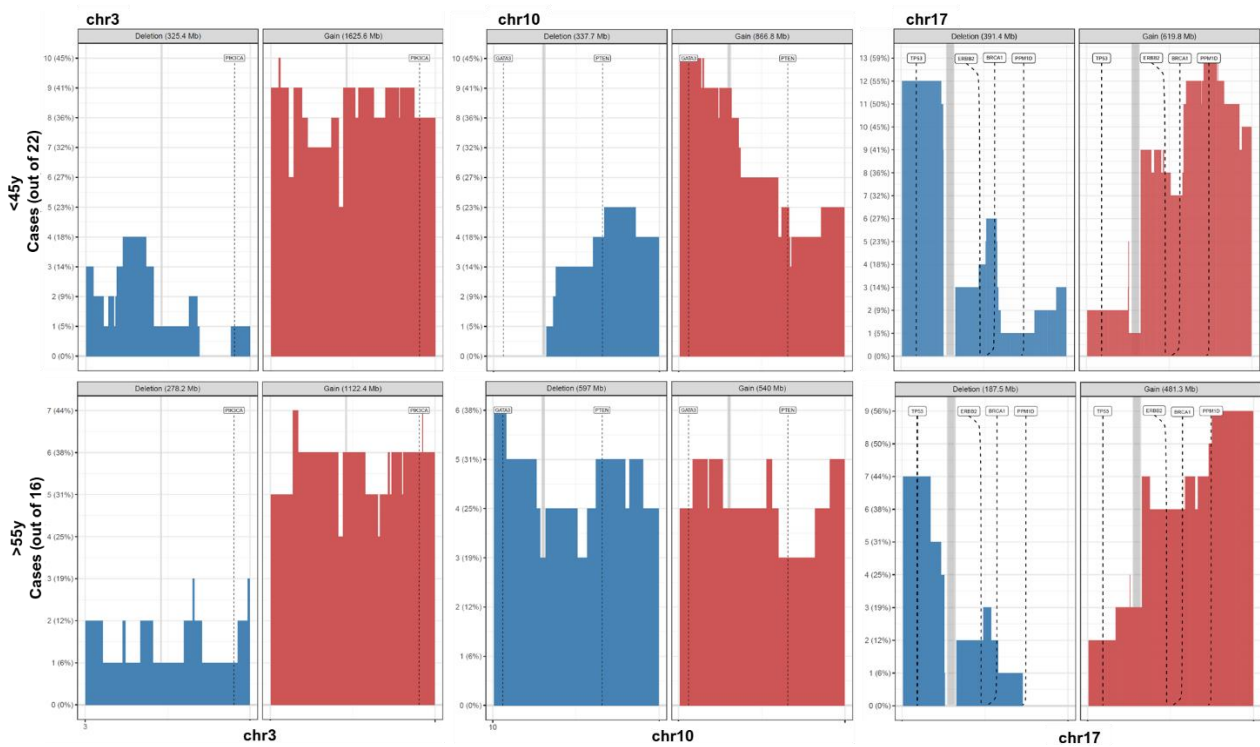
A**B**

Fig.6: Genomic distribution of CNAs in LBC internal cohort. A) Plots representing CNAs (deletions and gains) distribution between Y (<45y, n=22, left) and nY (>55y, n=16, right) patients. Short (p) and long (q) arms of the chromosomes are to the left and right, respectively, of the thicker line, which indicates the centromere. **B)** Plots representing CNAs in chromosomes (chr) 3, 10 and 17 of Y (<45y, Up) and nY (>55y, Down) patients. Genes of interest are indicated in each chromosome (PIK3CA; Chr3) (GATA3, PTEN; Chr10) (TP53, BRCA1, PPMID, ERBB2; Chr17).

4.2 Dissecting the relevance of PIK3CA/AKT1 and TP53 co-mutations in LBC aggressiveness and drug resistance

4.2.1 PIK3CA/TP53 co-mutations alter the growth and the drug response of luminal breast primary epithelial cells

Data obtained from NGS analysis performed on our cohort of patients demonstrated that mutations in PIK3CA/AKT1, as well as co-mutations in PIK3CA/AKT1 and TP53, are more prevalent in Y, patients compared to their nY counterpart. Although literature data suggest that PIK3CA/TP53 co-mutations (PIK3CA/TP53^{co-mut}) confer increased aggressiveness to cancer cells⁹³, the role and mechanisms of action of these mutations remain unclear in the context of LBC in Y women. Therefore, we decided to thoroughly dissect the biological impact of PIK3CA and TP53 mutations on mammary tumorigenesis, by introducing single and combined mutations into a primary immortalized luminal breast epithelial cell line (BPE) to evaluate if and how these alterations could drive transformation and resistance to therapy, in LBC context.

Using lentiviral particles, we first generated BPE clones over-expressing PIK3CA^{E545K}, PIK3CA^{H1047R} or TP53^{R175H} and then, BPE pools overexpressing both PIK3CA and TP53 mutations (TP53^{R175H} + PIK3CA^{E545K} and TP53^{R175H} + PIK3CA^{H1047R}) (Fig. 7A-B). Once selected, we characterized these modified BPE cells testing them in proliferation, survival, 3D-morphogenesis and in response to therapy. Cell viability assay clearly indicated that, as expected, both PIK3CA/TP53^{co-mut} BPE clones proliferate significantly faster compared to the control counterpart (Fig. 7C). To further investigate the tumorigenic potential of these mutations, we performed anchorage-independent growth assays, by culturing the modified BPE clones on polyHEMA-coated dishes, notably a “hostile” microenvironment to grow, due to the absence of a substrate to attach. While BPE control cells were not able to survive in this condition, all BPE clones overexpressing mutated PIK3CA and TP53 displayed a higher ability to grow in suspension, forming more spheres, also larger in size, especially when both pathways are altered (Fig. 7D). Next, we analyzed the stem-like feature of the same clones, evaluated by mammosphere assay. PIK3CA/TP53^{co-mut} BPE cells significantly increase the mammosphere-forming and self-renewal abilities, in terms of both number and size of the spheres (Fig. 7E), thus confirming that these cells acquired more stem-like features. These results confirmed the well-established roles of PIK3CA¹¹⁶ and TP53¹⁴⁴ mutations in promoting cellular growth and survival and demonstrated that the co-occurrence of both mutations can further enhance the oncogenic effect of the single alterations.

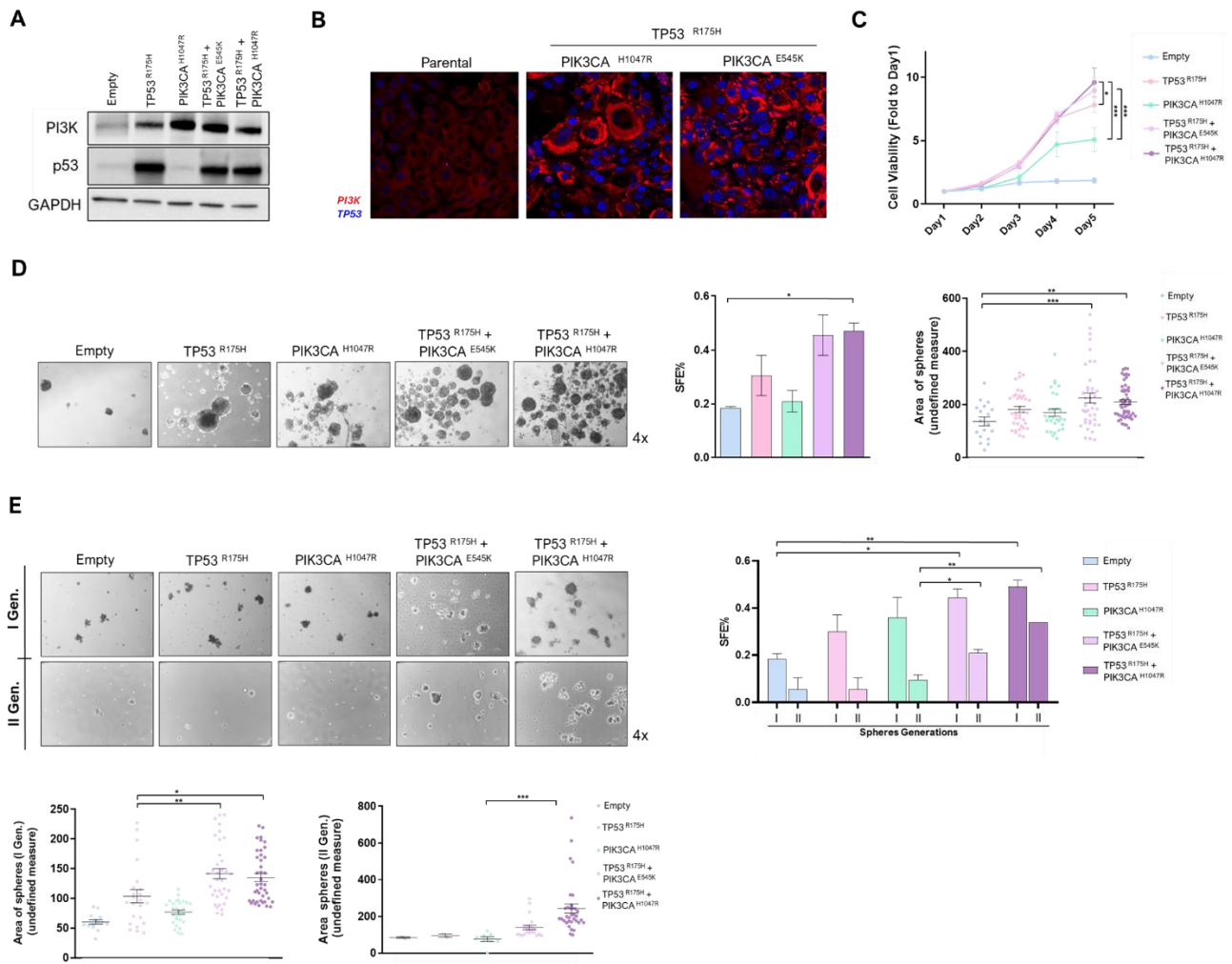


Fig. 7: PIK3CA and TP53 mutations alter the growth and the stem-like features of BPE cells. **A)** Western Blot analysis of PI3K and p53 expression in BPE modified clones. GAPDH was used as loading control. **B)** Immunofluorescence analysis on BPE modified pools stained for PI3K (red) and p53 (blue) expression. **C)** Growth curve of BPE modified cells, expressed as fold increase over the number of cells plated on day 1. Anova test has been used for statistical analysis. Data represent the mean (± SD) of three independent experiments performed in sextuplicate. **D)** Left, representative bright field images (4X) of spheroids from BPE modified cells grown on polyHEMA for one week. Middle, graph reporting the spheres forming efficiency (SFE%) of BPE modified cells. Right, graph reporting the area of the spheroids. Each dot represents one analyzed sphere. Data represent the mean (± SD) of three independent experiments performed in duplicate. **E)** Left, representative bright field images (4X) of mammospheres formed in first and second generations. Right, graph reporting the first and second generation mammospheres forming efficiency (SFE%). Down, the graphs report the area of first (left) and second-generation (right) mammospheres. Each dot represents one analyzed sphere. Data represent the mean (± SD) of three independent experiments performed in duplicate. Spheroids size was measured using Image Lab TM software. Mann–Whitney test and Student's t-test have been used for statistical analysis. Asterisks indicate significant differences, *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

To further evaluate the behavior conferred by the presence of PIK3CA/TP53^{co-mut}, we tested the response of mutated and co-mutated cell lines to Endocrine Therapy (ET), which represents the first line treatment for LBC patients, either alone or in combination with targeted therapy or chemotherapy. In particular, we treated BPE cells with Tamoxifen (TM), an estrogen receptor modulator (SERM), and Palbociclib or Abemaciclib (AB), two different CDK4/6 inhibitors used in clinics, alone or in combination. The presence of PIK3CA/TP53^{co-mut} mutations increased significantly the resistance of

BPE cells to these drugs, both when administered as single agents and in combination (Fig. 8A-B-C). The effect is particularly evident when both pathways are altered, highlighting the potential effect of the PIK3CA and TP53 altered pathways in diminishing the efficacy of the therapeutic approaches used in clinics. Notably, PIK3CA/TP53^{co-mut} BPE cells significantly increased their survival and ability to form colonies under TM treatment, alone and in combination with AB (Fig. 8D). Under these treatment conditions, an increased expression level of phosphorylated Rb protein was observed, suggesting that PIK3CA/TP53^{co-mut} BPE cells are more able to escape cell cycle arrest induced by CDK4/6 inhibition (Fig. 8E).

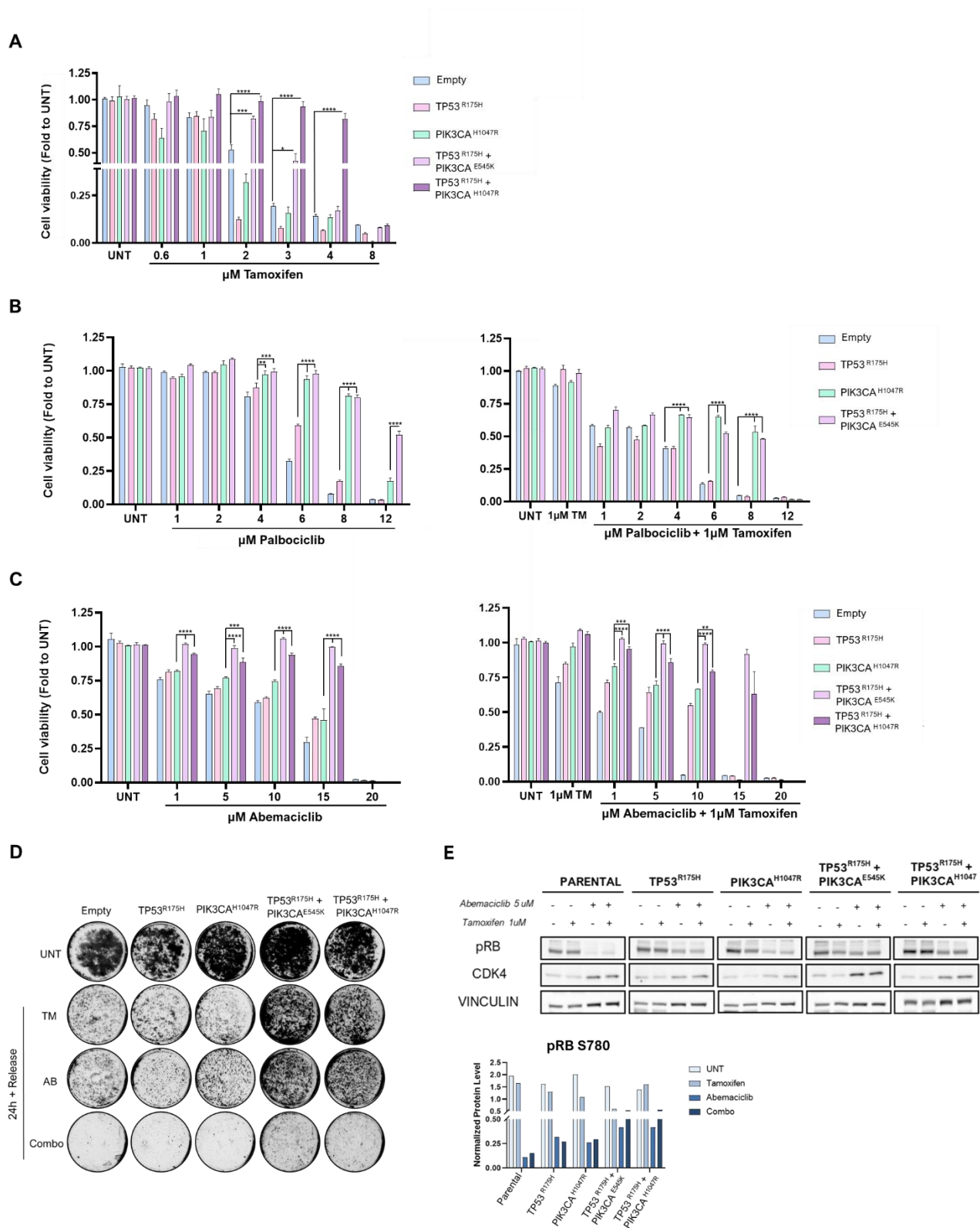


Fig. 8: PIK3CA /TP53 co-mutations increase the resistance of BPE cells to endocrine therapy plus CDK4/6i.

A-B-C) Graphs reports cell viability of BPE modified clones, treated with increasing doses of Tamoxifen (**A**), Palbociclib (**B**) and Abemaciclib (**C**) alone or in combination with standard doses of Tamoxifen for 72h. Data represent the mean ($\pm$ SD) of three independent experiments, each performed in sextuplicate. Cell viability was tested using MTS cell viability assay and Anova test has been used for statistical analysis. **D)** Colony formation assay on BPE modified clones treated with TM and/or AB for 24 hours + 7 days of release. **E)** WB analysis of Rb S780 phosphorylation (pRB S780) and CDK4 level in BPE modified clones treated as indicated for 72h. VINCULIN was used as loading control. Below, the graph

reports the normalized protein levels of pRB S780 obtained through ImageJ software. Asterisks indicate significant differences, * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$, **** $P \leq 0.0001$.

Furthermore, we observed that PIK3CA/TP53^{co-mut} BPE cells were much more resistant than single PIK3CA mutant cells to Alpelisib, a selective PI3Ki used in clinic to treat metastatic BC patients mutated for PIK3CA, both alone and in combination with Tamoxifen (Fig. 9A).

Next, to test whether PIK3CA and TP53 mutations might be also implicated in tumor response to standard treatment of ET in combination with chemotherapy, we treated BPE modified cells with Doxorubicin (DX), a topoisomerase II inhibitor known for inducing double-strand breaks, as a single agent and in combination with Tamoxifen. Interestingly, we observed that presence of PIK3CA and TP53 mutations increased significantly the resistance of BPE cells to DX, both when administered alone and in combination with TM, and this is also more evident when both the pathways are altered (Fig. 9B). Under these treatment conditions, PIK3CA/TP53^{co-mut} BPE cells (both TP53^{R175H} + PIK3CA^{E545K} and BPE TP53^{R175H} + PIK3CA^{H1047R}) showed higher ability to survive and to form a large number of colonies (Fig. 9C). Moreover, PIK3CA/TP53^{co-mut} BPE cells displayed lower γ H2AX (marker of DNA damage) and higher of RAD51 (marker of DNA repair) expression levels, overall suggesting that these cells were protected from ET plus chemotherapy-induced death (Fig. 9D).

Taken together, these results indicated that concomitant mutation of PIK3CA and TP53 pathways alters the growth of BPE cells, leading to a more tumorigenic phenotype and higher resistance to ET, targeted therapies (CDK4/6i and PI3Ki) and chemotherapy (Doxorubicin), thus highlighting the possible impact that these mutations could have on therapy response of LBC patients.

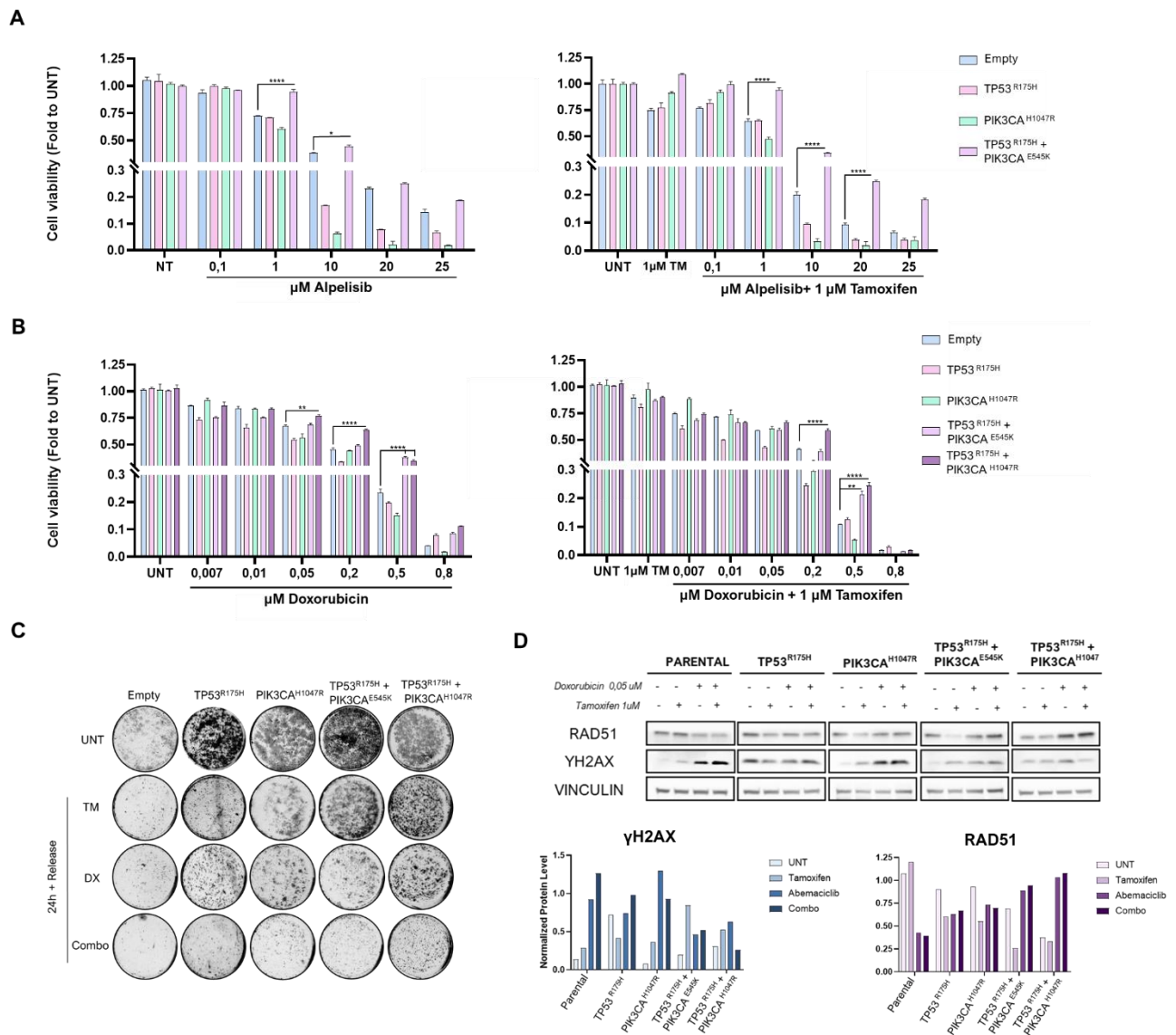


Fig. 9: PIK3CA /TP53 co-mutations increase the resistance of BPE cells to endocrine therapy plus PI3Ki and chemotherapy. **A-B)** Graphs reports cell viability of BPE modified clones, treated with increasing doses of Alpelisib (**A**) and Doxorubicin (**B**) alone or in combination with standard doses of TM for 72h. Data represent the mean ($\pm$ SD) of one experiment, performed in sextuplicate. Cell viability was tested using MTS cell viability assay and Anova test has been used for statistical analysis. **C)** Colony formation assay on BPE modified clones treated with TM and/or Doxorubicin (DX) for 24 hours + 7 days of release. **D)** WB analysis of RAD51 and γ H2AX level in BPE modified clones treated as indicated for 72h. VINCULIN was used as loading control. Below, the graphs report normalized protein level of γ H2AX (left) and RAD51 (right) obtained through ImageJ software. Asterisks indicate significant differences, * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$, **** $P \leq 0.0001$.

4.2.2 Co-mutation in PIK3CA and TP53 genes sensitizes BPE cells to inhibition of EZH2.

Scientific evidences have recently demonstrated that in some tumoral context, such as lung cancer or acute myeloid leukemia, PIK3CA and TP53 mutations might sensitize tumors to therapeutic strategies

based on the use of epigenetic inhibitors^{205,206}. Thus, we wondered whether the concomitant alteration of these two pathways could unveil new therapeutic vulnerabilities also in LBC context.

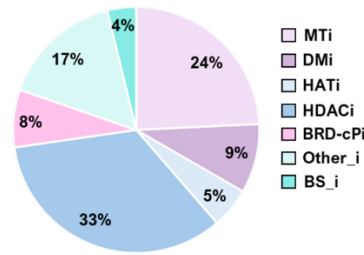
To this aim, we performed a drug screening using an epigenetic drug library, evaluating the response of PIK3CA/TP53^{co-mut} BPE clones to a broad spectrum of epigenetic regulators inhibitors. We used an epigenetic drug library (Cayman Chemicals™) (N=133) that included inhibitors of histone methyltransferases (MTi; n=32; 24%), of de-methylases (DMi; n=12; 9%), of histone acetyl-transferases (HATi; n=7; 5%) and inhibitors of histone de-acetylases (HDACi; n=44; 34%). Additionally, library included inhibitors targeting bromo domain-containing proteins (BRD-cPi; n=10; 8%), compounds targeting other epigenetic factors and DNA damage response proteins, indicated as others (Others-i; n=21; 16%) and broad-spectrum epigenetic inhibitors (BS-i) (n=5; 4%) (Fig. 10A).

First, we observed that while PIK3CA/TP53^{co-mut} does not affect the sensitivity of the BPE cells to the most epigenetic inhibitors, used at 10uM concentration, some drug classes, particularly G9A inhibitors (G9Ai) and some HDAC inhibitors (HDACi), induced a dramatic reduction in the viability of all BPE cells, regardless of their mutational status. However, we interestingly observed that EZH2 inhibitors, a class of drugs targeting methyl-transferases of the EZH2 family, were able to selectively reduce the viability of PIK3CA/TP53^{co-mut} BPE cells (Fig. 10B).

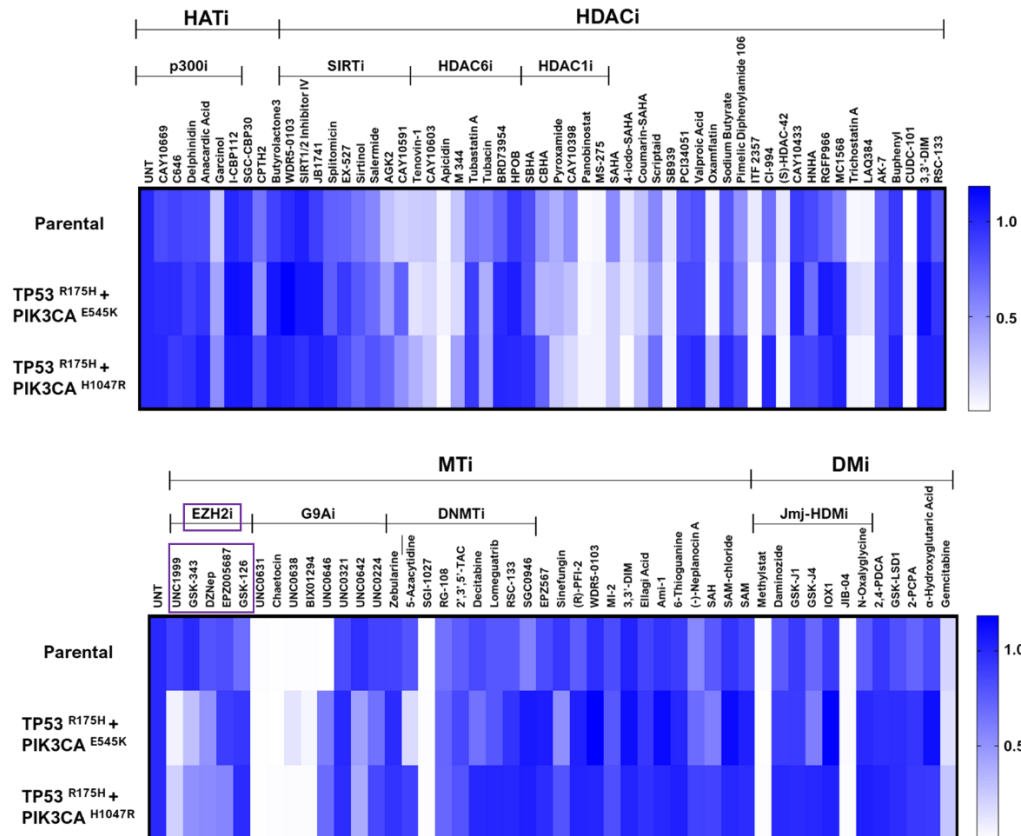
To validate these findings and determine whether the vulnerability to this class of inhibitors was conferred by the presence of mutations on both pathways or if only one of the two was sufficient, we assessed the response of BPE-modified cells to increasing doses of UNC-1999 and GSK-343 (EZH2 inhibitors), including single-mutant clones, eventually confirming that PIK3CA/TP53^{co-mut} sensitized BPE cells to inhibition of EZH2 (Fig. 10C).

Overall, these results suggested that inhibition of the EZH2 methyltransferase acted in a synthetic lethal manner with PIK3CA/TP53 co-mutation in LBC, possibly providing new tailored therapeutic strategies for all LBC patients carrying these mutations.

EPIGENETIC SCREENING LIBRARY	
Methyl-Transferases Inhibitors (MTi)	32
De-Methylases Inhibitors (DMi)	12
Histone Acetyl-Transferases Inhibitors (HATi)	7
Histone De-Acetylases Inhibitors (HDACi)	44
Bromodomain-containing protein inhibitors (BRD-cPi)	11
Other Inhibitors (Other_i)	22
Broad spectrum Inhibitors (BS-i)	5
tot	133



B



C

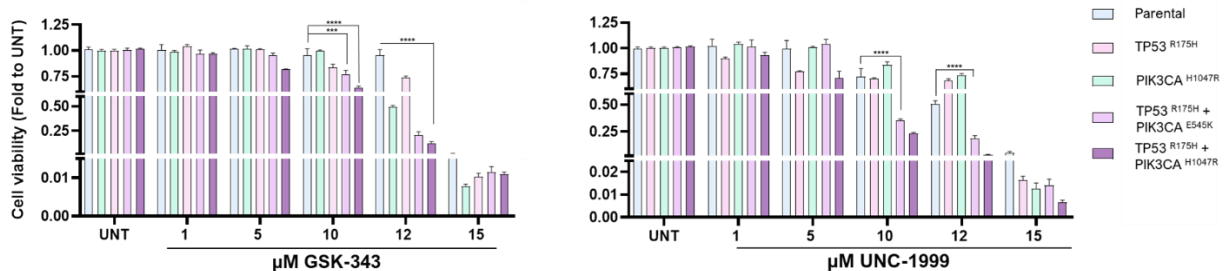


Fig.10: PIK3CA and TP53 co-mutated BPE cells are more sensitive to EZH2i compared to the parental ones.

A) The table (Left) and the pie chart (Right) summarize the epigenetic inhibitors (n=133) of the epigenetics screening library divided by drug classes. **B)** Heatmaps showing cell viability of BPE modified cells treated with 10μM of Histone Acetyl-Transferases Inhibitors (HATi) and Histone De-Acetylases Inhibitors (HDACi) (Up), Methyl-Transferases Inhibitors (MTi) and De-Methylases Inhibitors (DMi) (Down). The names of the individual drugs for each epigenetic class are indicated. **C)** The graphs report cell viability of BPE modified clones treated with increasing doses of GSK-343 (Left) and UNC-1999 (Right) (EZH2i) as indicated. Cell viability was measured after 72h using MTS cell viability assay and Anova test has been used for statistical analysis. Asterisks indicate significant differences: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001

5. DISCUSSION

BC is the most diagnosed cancer globally¹. This is especially challenging from a clinical perspective when the diagnosis occurs in young women. Young age at diagnosis of BC is an independent factor associated with higher risk of relapse and death in several large studies, even when more aggressive therapies are administered^{207,208}. As reported by many prospective studies evaluating the pathological features of BC in young women, these patients showed higher proportion of aggressive subtypes, such as basal-like and HER2-enriched tumors compared to their older counterparts⁹⁸ and their tumors are often poorly differentiated, with higher grade, multifocality, and, more frequently, nodal involvement¹⁰¹.

Up to now, few clinical studies have focused their attention on the young subgroup of BC patients, leaving a gap in understanding whether this disease could display a distinct biology.

Recent analyses on molecular profiling of BC in young women showed that they displayed enrichment of gene sets representing mammary stem cells, luminal progenitors, BRCA1-mutation signatures and higher expression of c-kit and RANK-ligand, independently from tumor subtype, stage and grade. These results also showed that young age represented an independent risk factor, not only for the higher incidence of more aggressive subtypes, but also for a worse prognosis of the disease, after correction for subtype and stage, particularly evident in the Luminal subtype (LBC). In fact, the disparity in clinical outcome is especially notable in LBC showing a higher prevalence of the more aggressive luminal B subtype over luminal A¹⁰¹. This finding strongly suggested that the biology of BC in young women might be distinct from that of older BC patients, especially in LBC however, further biological validations are needed.

To address this point and, possibly, discover new molecular signatures leading to increased aggressiveness in young patients, we performed a comprehensive mutational profiling analysis using different Next Generation Sequencing (NGS) approaches on LBC specimens derived from young pre-menopausal (Y, <45y) and non Y post-menopausal (nY, >55y) patients treated in our institute.

Custom amplicon-based NGS was performed to target four of the most relevant genes involved in BC progression, PIK3CA, AKT1, TP53 and GATA3 genes. The analysis revealed that Y subgroup included a higher number of mutated and co-mutated patients compared to the nY subgroup, suggesting that younger age or pre-menopausal status may influence the driver genes mutational status of BC, leading to a more complex patients' mutational profile. Additionally, the elevated mutation rates in BC driver genes might partially explain the more aggressive tumor phenotype observed young patients.

In detail, the analysis of driver gene mutation patterns in Y and nY BC patients revealed notable differences in the prevalence of specific genetic alterations. For instance, in Y patients, there was a significant enrichment in PIK3CA and GATA3 mutations further confirmed also by WES analysis.

Moreover, while TP53 appeared slightly more mutated in nY patients from the TGS analysis, in the WES it was found to be equally mutated between the two categories. However, within the luminal B subgroup, Y patients exhibited a higher rate of co-occurring mutations in the PIK3CA/AKT1 pathway alongside GATA3 or TP53 mutations but also of the most aggressive mutation types in these driver genes, such as missense mutations in TP53 and mutations in the Znfn2 domain of GATA3. This is consistent with reports showing that luminal B tumors, known for their more aggressive profile and higher proliferation rates, are often overrepresented in Y BC patients¹⁰¹.

The 4-genes TGS approach, evaluating the most frequently mutated genes in LBC, was of course heavily biased, while through WES we were able to identify other genes selectively mutated or amplified/deleted in the two categories, thus delineating a specific mutational profile of Y and nY patients. While most of these analyses are still ongoing, especially those on CNA, we were able to detect ERBB2, FAT3, CBFB, and MAP3K1 as the most mutated genes in Y patients, after PIK3CA and GATA3, while LRP1B, EPHA3, and CSMD3 in the nY subgroup. So far, CNA profiles were different between the two groups. We detected higher frequency of chromosome 17p deletions in Y patients compared to nY, specifically in the region containing TP53 gene, suggesting that Y patients may be more susceptible to TP53 loss and, consequently, to genomic instability, a finding that would have been completely missed if we had not done the CNA analysis. Moreover, the region containing GATA3 within chromosome 10, resulted selectively amplified in Y. Since GATA3 is involved in luminal differentiation maintenance of mammary cells, loss of GATA3 expression is usually associated with aggressive tumor phenotypes¹⁹¹. However, certain frameshift mutations, particularly in the ZnFn2 domain, have been associated with increased tumor aggressiveness and poor patient prognosis¹⁹³. Ongoing analysis will clarify whether some patients carry both GATA3 amplification at chr10 and frameshift mutation and whether this, leading to an amplification of the mutant GATA3 allele, also drives amplified aggressive phenotypes in the cancer cells.

Recently, a genomic profiling analysis of LBC has been published, showing some different distribution of gene alterations between pre-menopausal and post-menopausal patients, compared to our analysis¹⁰⁴. While GATA3 mutations were enriched in the Y group, in line with our findings, Luen et al. also observed a lower frequency of PIK3CA and a higher of TP53 mutations in the same group. The patients analyzed were from the SOFT cohort²⁰⁹ and were *in silico* compared to post-menopausal patients from the BIG 1-98²¹⁰ and TCGA-Breast⁴¹ cohorts (>55y). Many features differentiate this work from ours, possibly explaining the discrepancies observed. First, in our cohort, samples, both Y

and nY, were collected fresh from surgery and analyzed together, with the same NGS, coverage and bioinformatic pipeline. As for our internal experience in analyzing the results from targeted sequencing and WES, this consistency makes a lot of difference in terms of alteration retrieval, especially for those at subclonal frequency. Furthermore, our cohort was also highly selected at histological and pathological level. Only patients with primary LBC, same histological origin (ductal), similar grade and stage were included. None of our samples was collected after neoadjuvant therapy, ensuring that the mutational landscape analyzed was reflective of the untreated primary tumor biology. In other published cohort analysis, patient samples are from FPPE, often include both ductal and lobular LBC, are collected after neoadjuvant treatment and, as in the case of Luen et al., one of the two arms to be compared is *in silico*. Finally, geographic and ethnic variability between multi-institutional mainly American and Asian cohorts might also contribute to the differences observed with our internal cohort.

Overall, our NGS analyses suggested that the molecular profile of Y women with LBC is distinct from that of nY ones. The significantly higher frequency of GATA3 frameshift variants, TP53 missense variants and PIK3CA/AKT1 mutations, both alone and in combination with GATA3 or TP53 alterations, may contribute, at least in part, to the increased aggressiveness of tumors of the Y compared to the nY ones. Since we have also carried out a wide transcriptomic analysis (not shown) in the same samples, the integration of these different omics information will hopefully lead to even more interesting and solid findings.

Interestingly, literature data suggested that PIK3CA/TP53 co-mutations (PIK3CA/TP53^{co-mut}) confer increased aggressiveness and drug resistance to cancer cells⁹³, but the role and mechanism of action of these co-mutations remain unclear in the context of LBC. Our results showed that stable overexpression of PIK3CA^{E545K}, PIK3CA^{H1047R} and TP53^{R175H} mutations alters the growth of primary immortalized luminal breast epithelial (BPE) cells leading to a more tumorigenic phenotype. Next, we tested the impact of these co-mutations in the response to therapies, since drug resistance is one of the most powerful drivers of disease progression⁵⁷. Inhibitors of Cyclin-Dependent Kinase 4/6 (CDK4/6) or PI3K, in combination with endocrine therapy (ET) represent the first line treatment for patients with advanced-stage LBC^{54,139}. We thus tested ET, based on the use of tamoxifen, plus targeted therapies (CDK4/6i and PI3Ki) and chemotherapy (Doxorubicin), in BPE cells carrying alterations in TP53, PIK3CA pathways or both. Our results highlighted the potential role of PIK3CA/TP53^{co-mut} in diminishing the efficacy of different therapeutic approaches used in clinics for the treatment of LBC patients.

It has been demonstrated that PIK3CA and TP53 are involved in cancer cell epigenome reprogramming^{211–213} and their mutations may be responsible of epigenetic dependency of cancer

cells^{205,206}. However, whether this is true in BC is unknown and we thus tested whether the alteration of these two pathways in LBC could unveil new therapeutic vulnerabilities, using drug screening library composed by 133 small epigenetic modulators. We observed that EZH2 methyl-transferase inhibitors were particularly effective to reduce the viability of co-mutated BPE cells, compared to the parental and the single mutants alone. EZH2 is the catalytic component of the polycomb repressive complex 2 (PRC2), which plays a crucial role in gene silencing through the methylation of histone H3 at lysine 27 (H3K27me3)²¹⁴. Moreover, through the direct interaction with different proteins, EZH2 can also modulate different pathways involved in BC onset and progression, such as pRB-E2F, p53, PI3K/AKT, WNT and estrogen receptor pathways^{215,216}. Thus, the inhibition of EZH2 might disrupt its crosstalk with these oncogenic pathways, possibly explaining why PIK3CA/TP53^{co-mut} cells rely on EZH2 activity for their survival. Further ongoing experiments, aimed at identifying the molecular mechanisms behind this dependency, particularly the EZH2-dependent transcriptional reprogramming in a PIK3CA/TP53^{co-mut} context, might reveal who are the players and what are their roles in this crosstalk. Together, our results, suggesting that PIK3CA/AKT1 and TP53 mutational status may correlate with response to EZH2i, may provide a new personalized therapeutic strategy for LBC patients carrying these alterations.

Overall, this PhD project provides new insights into the molecular fingerprint of LBC patients, supporting the hypothesis that BC arising in young pre-menopausal women may represent a unique biological entity. Moreover, we shed a new light in the biological impact of PIK3CA/TP53^{co-mut} on tumorigenesis and drug resistance in BC context, suggesting that both PIK3CA and TP53 altered pathways might represent novel prognostic/ predictive biomarkers for both ET plus targeted therapies (CDK4/6i) and epigenetic therapies (EZH2i).

To assess the preclinical impact of our findings we also aim at characterizing the biological relevance of our findings *in vivo*. To reach this goal, we are planning to use LBC Patient-Derived Organoids (PDO) and then, Patient-Derived Xenograft (PDX), generated and already available in our lab, carrying the alterations that we have identified and treated with ET plus targeted therapies (CDK4/6i) and epigenetic therapies (EZH2i). If these models will confirm our previous results, we might extend the significance of our finding to any patient carrying these alterations, whether Y or nY.

Altogether, these studies will allow us to clarify the molecular mechanisms through which these co-mutations drive the LBC aggressiveness and, possibly, reveal novel druggable targets to reach more effective and personalized therapeutic approaches.

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