UNIVERSITY OF MODENA AND REGGIO EMILIA

PhD Program of Clinical and Experimental Medicine (CEM)

XXXII Doctorate Cycle

Director of the PhD Program: Prof. Giuseppe Biagini

MUTATIONAL PROFILE OF HER2 POSITIVE EARLY BREAST CANCER TREATED WHIT NEOADJUVANT CHEMOTHERAPY

SUPERVISOR:

Dr. Federico Piacentini

CANDIDATE:

Dr. Claudia Omarini

TABLE OF CONTENTS

ABBREVIATIONS

SUMMARY

RIASSUNTO

1. BREAST CANCER LANDSCAPE
   1.1. Epidemiology
      1.1.1 Incidence
      1.1.2 Mortality
   1.2 Risk factors
   1.3 Breast cancer carcinogenesis

2. HER2 POSITIVE BREAST CANCER
   2.1 Anti-HER2 targeted treatments
      2.1.1 Mechanism of resistance to anti-HER2 targeted therapy
   2.2 Neoadjuvant treatment in HER2 positive breast cancer

3. STUDY RATIONALE AND AIMS

4. MATERIALS AND METHODS
   4.1 Population and samples
   4.2 Next generation sequencing technology
   4.3 DNA isolation
   4.4 Gene analyses
      4.4.1 The multi-gene panel
   4.4 Statistical analyses

5. RESULTS
   5.1 Population and sample characteristics
   5.2 Primary tumor mutation (pCR group versus residual disease group)
   5.3 Comparison between primary tumor and residual breast cancer disease4
   5.4 Prognostic value of detected gene mutations

6. DISCUSSION

7. CONCLUSION

REFERENCES

ABBREVIATIONS

ARF Alternate Reading Frame

BC breast cancer

BER base excision repair

BRCA BReast CAncer gene

CDK cyclin-dependent kinase

DSBs double-strand breaks

EGFR epidermal growth factor

ERK Extracellular-signal-Regulated Kinase

FFPE formalin-fixed and paraffin-embedded

FGFR fibroblast growth factor receptor

IGF-1 insulin-like growth factor 1

IHC immunohistochemistry

INDEL insertion or deletion

HER2 Epidermal Growth Factor Receptor

HR hormone receptor

HSP90 heat shock protein 90

JAK Janus Kinase

MAPK mitogen-activated protein kinase

MDM2 Mouse double minute 2 homolog

mTOR mammalian target of rapamycin

NACT neoadjuvant chemotherapy

NER nucleic acid excision repair

NF-kB nuclear factor of kB

NGS next-generation sequencing

NHEJ non-homologous end joining

NOTCH1 Notch homolog 1, translocation-associated (Drosophila)

OS overall survival

PARP Poly(ADP-ribose) polymerase

pCR pathological complete response

PFS progression free survival

PIK3 phosphatidylinositol 3-kinase

PTEN Phosphatase and tensin homolog

RFS Relapse Free Survival

RTK tyrosine kinases

SNV single nucleotide variation

SSBs single-strand breaks

STAT Signal Transducer and Activator of Transcription

TILs tumor infiltrating lymphocytes

VEGF Vascular Endothelial Growth Factor

Summary

Neoadjuvant chemotherapy (NACT) (define as chemotherapy administered before breast cancer surgery) is a treatment option in patients with early-stage HER2 positive breast cancer. In particular, the achievement of the pathological complete response (pCR) (define as no residual invasive disease in the breast and the axillary lymph nodes) is a powerful indicator of long-term outcome. Patients achieving less than pCR have a worse prognosis, however, this group is heterogeneous, including patients with a good response even if not in complete response as well as patients with primary resistant disease. To date, there are no clear predictive biomarkers of response to NACT as well as prognostic biomarkers in patients with residual tumor disease.

On these bases, using a next-generation sequencing (NGS) technology, we evaluated a panel of 21 genes involved in treatment resistance mechanisms in a group of HER2 positive breast cancer patients treated with NACT. We compared the breast cancer diagnostic biopsy of women with residual disease after NACT to a control group of patients who achieved the pCR. The residual breast cancer taken from the surgical specimen was analysed and compared to the matched diagnostic biopsy too.

Overall, the detection rate of mutations was 79% in the No-pCR group versus 90% in the pCR cohort and 98% in the residual breast cancer disease one. The high rate of detected mutations underlines the fact that the analysed genes are highly involved in both, first steps of cancer progressions and treatment resistance. The most frequently mutated genes were TP53 and PIK3CA in all the three subgroups analyses. No correlations between single gene mutations and predictive and prognostic value have found.

A substantial discordance between primary tumors and residual disease, in term of mutational profile was found. More than half of the patients had different genes status in the residual tumor compared to matched diagnostic biopsy. In

particular, 69% of them had an increased in the number of the detected mutated genes. Mutational profile changes from diagnostic biopsy to residual breast cancer were negative prognostic factors in term of relapse free survival. All the recurrence were in the subgroup of patients with treatment induced genes mutations (42% vs 0% p value 0.019). This finding shown that NACT induced a selective pressure on residual breast cancer cells able to confer a more aggressive tumor behaviour reflecting in worse survival outcomes.

This study demonstrated that during NACT cancer often modify gene mutational profile due to treatment selective pressure on tumor cells. Treatment-induced gene mutations significantly increase the risk of relapse. Profiling primary and residual breast cancer may is a major step in order to personalized adjuvant treatment strategy.

Riassunto

La chemioterapia neoadiuvante (preoperatoria) è una strategia di trattamento nelle paziente con diagnosi di tumore mammario HER2 positivo in stadio precoce. In particolare, l'ottenimento della risposta patologica completa (definita come l'assenza di malattia tumorale residua sul pezzo operatorio) è un indicatore di ottima prognosi a lungo termine. Al contrario, i pazienti con residuo di malattia tumorale hanno una prognosi più infausta. Questo gruppo è però eterogeneo, perché include sia pazienti che hanno comunque ottenuto una buona risposta al trattamento, sia pazienti con tumore resistente alla terapia somministrata. Ad oggi, non ci sono marcatori biomolecolari predittivi di risposta alla chemioterapia neoadiuvante così come non ci sono marcatori prognostici nei pazienti con malattia tumorale residua.

Partendo da queste basi, utilizzando una tecnologia di next-generation sequencing (NGS), abbiamo valutato, in un gruppo di pazienti con tumore mammario HER2 positivo sottoposte a chemioterapia pre-operatoria, un panello di 21 geni coinvolti nei meccanismi di farmaco resistenza. Abbiamo quindi confrontato il tessuto tumorale proveniente dalla biopsia diagnostica mammaria in un gruppo di pazienti con malattia residua al termine della chemioterapia neoadiuvante con un gruppo di controllo di pazienti che avevano ottenuto la risposta patologica completa. Inoltre, abbiamo analizzato l'espressione genica del tumore residuo proveniente dal pezzo operatorio confrontandolo con la corrispettiva biopsia diagnostica.

Complessivamente il tasso di mutazioni trovate era del 79% nel gruppo di pazienti con tumore mammario residuo, del 90% nel gruppo di pazienti con risposta patologica completa e del 98% sul residuo tumorale dopo chemioterapia. L'alto tasso di mutazioni trovato evidenzia come i geni analizzati fossero geni altamente coinvolti sia nelle fasi precoci della tumorigenesi, sia nella resistenza ai trattamenti antineoplastici. I geni trovati maggiormente mutati erano TP53 e PIK3CA in tutti e tre i sottogruppi. Nessuna delle singole mutazioni geniche trovate ha dimostrato un significativo valore prognostico e/o predittivo di risposta al

trattamento.

Considerando le mutazioni trovate sul tumore mammario pre- e post-trattamento chemioterapico neoadiuvante, abbiamo trovato una sostanziale discordanza tra i profili mutazionali. Più della metà dei pazienti aveva un profilo mutazionale differente tra la biopsia diagnostica e il corrispettivo residuo tumorale. In particolare, nel 69% dei casi c'era un aumento dei numero dei geni trovati mutati sul tumore residuo rispetto alla biopsia. La presenza di un diverso assetto genico tra la biopsia diagnostica e il tumore residuo si è dimostrato essere un fattore prognostico negativo in termini di rischio di recidiva. Tutte le recidive infatti si sono verificate nel gruppo di pazienti con un assetto mutazionale diverso tra la biopsia e la chirurgia (42% vs 0% p value 0.019). Questo dato evidenzia che il trattamento chemioterapico neoadiuvante è in grado di determinare modifiche nello stato mutazionale del tumore capaci di conferire maggior aggressività al tumore che si riflette in una peggior prognosi per il paziente.

In conclusione, il nostro studio dimostra che durante la chemioterapia neoadiuvante il tumore mammario spesso modifica il suo assetto mutazionale come conseguenza della pressione selettiva indotta dal trattamento sulle cellule tumorali. La presenza di un diverso assetto mutazionale del tumore residuo rispetto alla biopsia diagnostica può essere considerato un fattore prognostico negativo che aumenta il rischio di recidiva. La valutazione dell'assetto mutazionale sia sulla biopsia diagnostica e sul residuo di malattia è uno step fondamentale per poter personalizzare la strategia adiuvante successiva.

1. Breast cancer landscape

1.1 . Epidemiology

1.1.1 Incidence

Breast cancer is a global problem. Worldwide, breast cancer accounts for nearly a quarter of all cancers in women and it is estimated that 2.1 million women will be diagnosed with the disease in 2019 [1]. The chance of a woman being diagnosed with breast cancer during her lifetime has increased from about 1 in 11 in 1975 to 1 in 8 today [2]. Although cancer exists anywhere in the world, the incidence rate of breast cancer varies among different parts of the world, varying from 27 per 100,000 in Middle Africa and East Asia to 92 per 100,000 in Northern America [2].The median age at diagnosis is 62 years [1]. Incidence rates of invasive breast cancer have remained stable over the past several decades among women <50 years of age while substantial changes in rates have been observed over time among women ≥50 years of age. In particular, rates increased sharply over the 1980s due to the introduction and utilization of mammographic screening [1].

1.1.2 Mortality

Breast cancer is the fifth leading cause of cancer death in 2012 worldwide, with a record of 324,000 deaths in 2012. Moreover, with 197,000 deaths accounting for 15.4% of all deaths, breast cancer was the second cause of death in developed countries after lung cancer [1]. The mortality rate varies from six cases per 100,000

people in East Asia to 20 cases per 100,000 people in Western Africa (4). Due to improved therapeutic and diagnostic methods and the promotion of breast cancer management in the high-income countries, a significant reduction in mortality rate is seen in developed countries. The survival rate is increasing; 1-year survival rate in European countries varies from 94.1% in Scotland to 97.1% in Italy. The average 10-year survival rate is 83%. Patients with early stage breast cancer diagnosis (62% of patients with breast cancer) have a 5-year survival rate of 99% [1, 2].

1.2 Risk factors

Epidemiologic studies have established a number of risk factors for breast cancer [3]. These studies provide information about risk factors on a population level, but have not proven to be effective in predicting an individual's risk of breast cancer. Evidence attributes the majority of cancers to not one single factor but various physical, hormonal, environmental and genetic factors [4, 5]. It has been estimated, using data collected from both, the first National Health and Nutrition Examination Survey and the Epidemiologic Follow-up Study, that no more than 41% of breast cancer were attributable to not modifiable risk factors such as age, family history, reproductive history, ages at menarche/menopause, BRCA status and breast density. Moreover, potentially modifiable factors such as obesity, exogenous environmental exposures, alcohol consumption, smoking and being physically inactive, influence the development of the disease too. However, all of

these factors are only weakly to moderately associated with breast cancer risk, with relative risks of <2.0 [6, 7].

1.3 Breast cancer carcinogenesis

Several evidence in literature has already pointed out that cancer is the result of subsequent genetic mutations in somatic cells [2, 8]. These mutations affect and activate a number of cellular pathways, which are responsible for growth, proliferation and differentiation of breast cancer cells:

1. **Epidermal Growth Factor Receptors (HER) family** (Fig. 1). The Epidermal Growth Factor Receptor (HER) family includes four different receptors: erbB1 (HER1 or EGFR), erbB2 (HER2), erbB3 (HER3), erbB4 (HER4). This family of tyrosine kinase receptors regulates several biological processes and is particularly involved into cell proliferation control, differentiation and survival [9]. This receptor family transduces signals by homo- or hetero-dimerization. HER1, HER3, and HER4 need to be ligand-bound in order to undergo conformational changes and rapid dimerization, whereas HER2 has a constitutively activated dimerization unit. Of the ten different HER homo- and hetero-dimer combinations, those containing HER2 endure for a long time and transmit strong signals, and are therefore associated with malignant growth. Moreover, HER2 overexpression promotes formation of HER2 heterodimers. HER2/HER3 dimerization has a particularly strong intracellular signal activity. In general, these receptors

are composed by an extracellular domain for ligand binding, a transmembrane segment and an intracellular domain with tyrosine kinase activity. The ligand binding causes conformational changes in the receptor that allows dimerization (homo or hetero-dimerization) with the other Epidermal Growth Factor Receptors and induces intracellular kinase domains phosphorilation with the activation of “downstream” signaling pathways, including PI3K/AKT/mTOR, MAPK and JAK/STAT pathways, which promote proliferation and survival [10, 11].

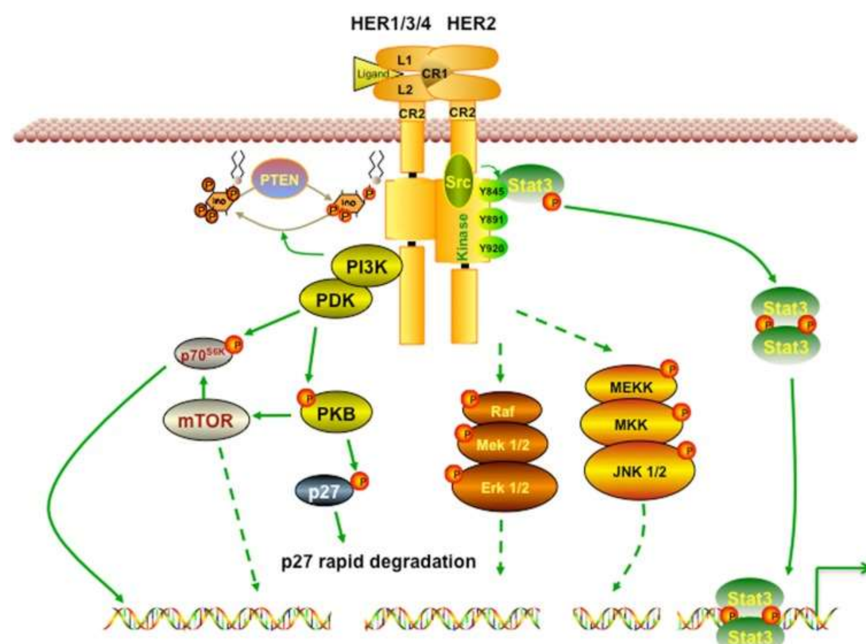


Figure 1 *HER2 signaling pathway. Receptor homodimerization or heterodimerization is the prerequisite step for HER2 activation that triggers downstream cascades to promote cell growth, proliferation, and survival. PI3K/Akt as well as Ras/Raf and MEK pathways are activated by HER2 [12]*

2. **Estrogen signalling pathway** (Fig. 2). Steroid hormones contribute to carcinogenesis in breast cancer acting on cell growth, development, differentiation, and homeostasis. Estrogens activate both nuclear estrogen

receptors (ERα and ERβ, genomic pathway) and membrane estrogen receptors (mER, non-genomic pathway) [13]. In the genomic pathways, ligand-activated nuclear estrogen receptor dimerizes and translocates in the nucleus where it binds to DNA to regulate the activity of different genes. On the other hand, in the non-genomic pathway, the mER activates a variety of signal transduction pathways, including the MAPK pathway and the PI3K/AKT/mTOR pathway [13].

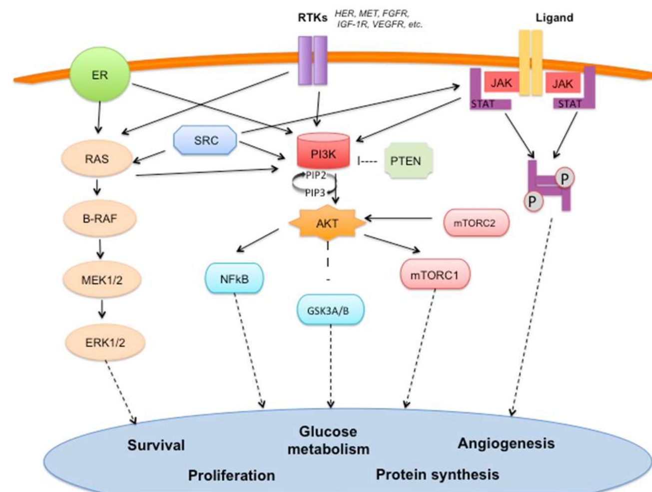


Figure 2 *The cross-talking network of signalling pathways involved in breast cancer development and progression: estrogen receptor (ER) signalling pathway, receptor tyrosine kinase (RTK) pathway, PIK3/AKT/mTOR pathway, MAPK signalling pathway, angiogenic pathway, SRC pathway and JACK/STAT pathway [14].*

3. **PI3K/AKT/mTOR pathway** (Fig. 1). PI3K/AKT/mTOR pathway is one of the main downstream pathways involved in cancer cell proliferation. It is activated by several receptor tyrosine kinases (RTKs), such as EGFR, IGF-1, FGFR, MET, etc. PI3K represents a family of kinases classifiable into 4 main classes. Class I PI3Ks have a catalytic subunit known as p110, with four isoforms: p110 alpha (encoded by *PIK3CA*), p110 beta (encoded by *PIK3CB*), p110 gamma (encoded

by *PIK3CG*) and p110 delta (encoded by *PIK3CD*) [15]. This first Class of PI3Ks is the one mainly involved in oncogenesis and has been the target for anti-cancer drug development. The principal function of Class I catalytic subunits is to phosphorylate an inositol-containing lipid, the phosphatidyl-inositol(4,5)P2, with conversion into phosphatidylinositol(3,4,5)P3 [15]. After phosphorylation, it activates AKT. Activated AKT recognizes a wide range of substrates, with their activating or inhibiting functions, such as mTOR, NF-kB (nuclear factor of kB), MDM2 (a negative regulator of the oncosoppressor p53), GSK3beta (involved in cell cycle and glucose metabolism processes) etc. Therefore, activated AKT mediates and regulates different biological processes, including growth independence, apoptosis and proliferation [16]. PTEN (Phosphatase and Tensin homolog) is the negative regulator of PI3K/AKT/mTOR pathway due to its dephosphorylating action. PTEN is a tumor suppressor with diverse functions, including regulation of cell cycle, apoptosis and metastasis [17]. Mutations or a reduced expression of PTEN gene are associated with a wide variety of human tumors, including breast cancer. Somatic mutations in all points of this pathway have been identified in breast cancer [17]. Particularly, mutations of PIK3CA have been found in almost 30% of all sporadic breast cancer with a wide range of frequencies among breast cancer subtypes, whereas the frequency of PTEN loss is 30-40% and the somatic intragenic PTEN mutation frequency is <5% [17].

4. **MAPK signalling pathway** (Fig. 2). MAPK may lead to uncontrolled cell cycle, resistance to apoptosis and to chemotherapy, targeted therapies and radiotherapy. The interaction between the RTKs (such as EGFR, PDGFR, FGFR,

etc.) and their ligands allows RAS (a family of small GTPases) to activate the protein kinase activity of RAF, a serine/threonine kinase. RAF kinase, as a cascade, phosphorylates and activates MEK (Mitogen-activated protein kinase). MEK phosphorylates and activates a mitogen-activated protein kinase, ERK (Extracellular-signal-Regulated Kinase, also called MAPK), which translocates into the nucleus where triggers several transcription factors that mediate expression of oncogenes involved in proliferation and survival [18].

5. **RB-E2F and p53 pathways** (Fig. 3). A wide range of mechanisms, including the activation of both RB-E2F and TP53 pathways, regulate cell cycle. RB is one of the best-known oncosuppressor gene, responsible for turning on or off the cell cycle [13]. One downstream consequence of RB activation is the inhibition of E2F activity, which is important for the transcription of several genes that are required for cell growth and progression. Particularly, E2F up regulates the cyclin E gene and then, the cyclinE-CDK2 holoenzyme completes the phosphorylation and inactivation of RB [19, 20]. In addition, the Cyclin D1, up regulated by growth factors like EGF and estrogen, binds to CDK4/6 and partially phosphorylates and inactivates RB [21]. In the p53 pathway, signals such as DNA damage induce the tumor suppressor ARF (Alternate Reading Frame) to increase p53 levels by sequestering MDM2, which facilitates the degradation and inactivation of p53. Among the p53 target genes there are WAF1, an inhibitor of cyclin-dependent protein kinases (CDKs) that causes cell-cycle arrest, and BAX which promotes apoptotic cell death [22]. RB also regulates p53 activity through a trimeric p53-MDM2-RB complex [22].

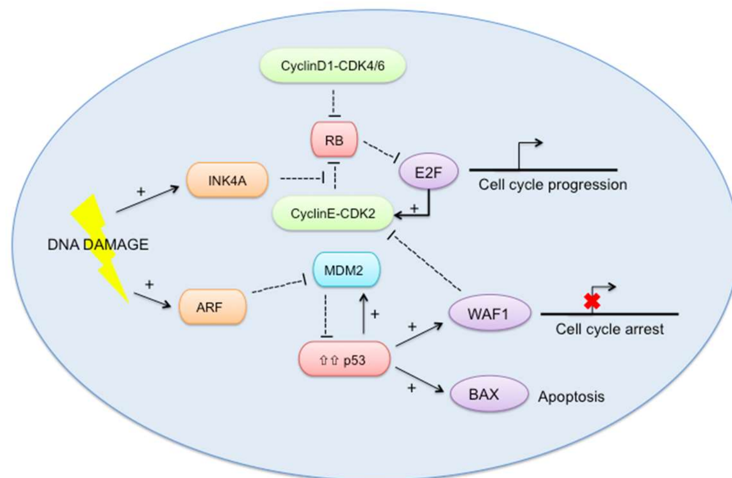


Figure 3 *The RB-E2F and p53 pathways [14].*

6. **Angiogenic pathway**_(Fig. 2). Tumor angiogenesis means the growth of new blood vessels, which are needed by the tumor in order to grow [23]. A huge number of molecules are involved in this process, some of them with a facilitating role (pro-angiogenic factors, such as the Vascular Endothelial Growth Factor VEGF), others with an inhibiting role (anti-angiogenic factors). Activation of pro-angiogenic pathways in cancer cells is critical to cancer development [24]. Particularly, signal transduction induced by VEGF involves binding to tyrosine kinase receptors and results in endothelial cell proliferation, migration, and new vessel formation [24].
7. **HSP90 mechanism of action** (Fig. 4). Under stressful conditions, the heat shock protein 90 (HSP90) molecular chaperone protects from degradation via the ubiquitin-proteasome pathway. HSP90 is upregulated in cancers, and this

contributes to increase proliferation and decreased apoptosis [12].

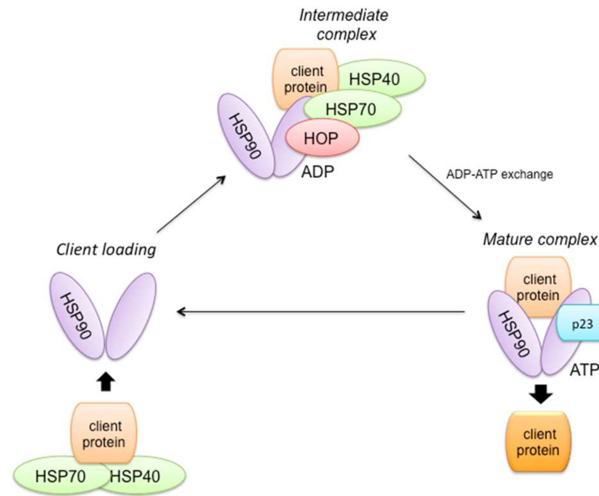


Figure 4 *HSP90 mechanism of action. The binding of a client protein to HSP90 requires the co-operation of another chaperone (HSP70 and its co-factor HSP40). HOP mediates interaction between HSP70 and HSP90. The exchange of ADP to ATP induces dissociation of HSP70 and its co-chaperones from the complex that associate then with p23, forming a mature complex. Under stressful conditions, HSP90 protects oncoproteins (such as HER2, AKT, c-MYC etc.) from degradation [14].*

8. **DNA repair mechanisms** (Fig. 5). Several mechanisms are involved in the repair of DNA damage, which includes single-strand breaks (SSBs) and double-strand breaks (DSBs) [25]. The SSB repair is accomplished by the base excision repair (BER), the nucleic acid excision repair (NER) and the mismatch repair (MMR). Poly(ADP-ribose) polymerase (PARP) is an enzyme involved in the BER. DSBs are corrected by the homologous recombination and non-homologous end joining (NHEJ) systems [25]. When a defect occurs in one of the enzymes involved in HR, such as BRCA1 and BRCA2, the DSBs are repaired from error prone mechanisms, mostly NHEJ. The NHEJ activation results in increased risk of new chromosomal defects and thus, the development of cancer [26].

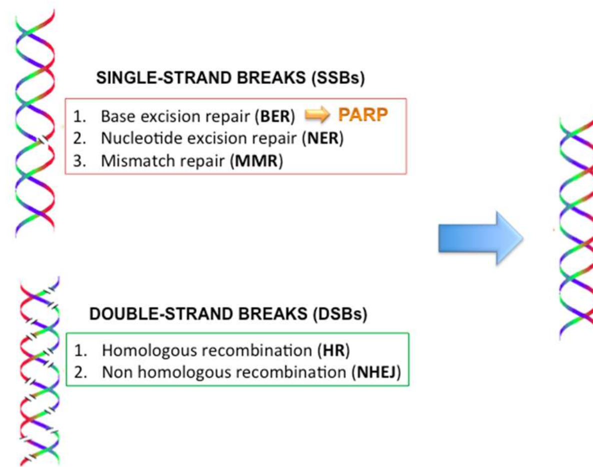


Figure 5 *DNA repair mechanisms and the role of PARP enzymes [14]*

9. **JACK/STAT pathway** (Fig. 2). The interaction between the RTKs or the cytokine receptors and their ligands allows a conformational change in the JAK (Janus Kinase) inactive form, placed on the intracellular tails of the receptor. Active JAKs phosphorylate tyrosin residues of the intracytoplasmic domain of the receptor itself, creating a binding domain for STAT protein (Signal Transducer and Activator of Transcription) that floats around in the cytoplasm [27]. Phosphorylated STAT dimerizes with other STAT proteins and the activated dimer translocates into the nucleus and promotes transcription of genes involved in proliferation, differentiation and apoptosis processes [27]. Dysregulations in JAK-STAT functionality result in immune disorders and cancers [27].

2 HER2 positive breast cancer

The assessment of HR and HER2status by immunohistochemistry (IHC) or ISH for HER2 status, together with traditional clinico-pathological variables (e.g. tumor size, tumor grade and nodal involvement) are conventionally used for breast cancer patient prognosis and management [28]. In every day clinical practice, hormonal receptors (including estrogen and progesterone receptors expression) and HER2 status group breast cancers into three major classes:

1) Hormonal receptors positive breast cancer (estrogen-receptor and/or progesterone-receptor positive, HER2 negative)

2) HER2 positive breast cancer (HER2 positive);

3) Triple negative breast cancer (estrogen-receptor and/or progesterone-receptor negative/HER2 negative).

In particular, HER2 positive breast cancer represents 15–20% of the breast cancers diagnosed [29]. Before the development of HER2-targeting agents, overexpression of HER2 was associated with poor survival outcomes due to the aggressive tumor, proliferation and metastatic activity [29]. Further understanding of the molecular mechanisms underlying HER2-positive oncogenesis has led to the development of a series of HER2-targeting agents, which have revolutionized the standard of care for HER2-positive breast cancers [30-32].

2.1 Anti-HER2 targeted therapy

The clinic-pathological importance of HER2 signaling pathways for treating HER2-positive breast cancers has become well recognized since the introduction of anti-HER2 targeted treatments [30-32]. A better understanding of tumor biology and HER2 signaling has led to the development and approval of HER2-targeted agents [30-32]. Nowadays the anti-HER2 therapy approved for the treatment of HER2 positive breast cancer are trastuzumab, pertuzumab, T-DM1 and lapatinib.

- **Monoclonal antibodies - TRASTUZUMAB and PERTUZUMAB (Fig. 6)**

TRASTUZUMAB

The first Food and Drug Administration (FDA)-approved targeted therapy for breast cancer was trastuzumab, a recombinant humanized monoclonal antibody directed against the extracellular domain IV of HER2 [33]. Clinical studies have shown that the combination of trastuzumab with standard chemotherapy produces better response rates than chemotherapy alone [30, 34]. Thus, the combinations that include trastuzumab have been considered as the standard of care for HER2 positive breast cancer patients. The major mechanisms of trastuzumab actions are [35]:

- **HER2 DEGRADATION** Trastuzumab has been proposed to trigger HER2 internalization and degradation through promoting the activity of tyrosine kinase – ubiquitin ligase c-Cbl [36].
- **ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY** As an antibody, it attracts immune cells to tumor sites that overexpress HER2, by a

mechanism called antibody-dependent cellular cytotoxicity (ADCC) [35]. Xenografts study by Clynes et al. showed that natural killer cells could target HER2-overexpressing cells coated with trastuzumab via a CD16-mediatedADCC mechanism [37]. Arnould et al. later confirmed the immune by using samples from patients with locally advanced breast cancer [38]. After treating the tumor samples with trastuzumab and docetaxel, the group observed an increase in the number of both natural killer cells and cytotoxic proteins in tumor infiltrates [38]. These findings provide strong support for ADCC as a mechanism of action of trastuzumab and help explain why cancer cells with high HER2 expression are more responsive to the drug.

- **MAPK AND PI3K/Akt INTERFERENCE** The most known effect of trastuzumab is the inhibition of the MAPK and PI3K/Akt pathways which leads to an increase in cell cycle arrest and suppression of cell growth and proliferation [39]. It is widely accepted that by interfering with the dimerization of HER2, trastuzumab inhibits HER2 activation and suppresses Akt phosphorylation [39]. Moreover, trastuzumab can block tyrosine kinase Src signaling and thus, increases PTEN level and activity [40]. This also results in the suppression of PI3K/Akt signaling and reduction in cell growth and survival. Finally, trastuzumab was demonstrated to induce cell cycle arrest by restoring p27 and suppressing CDK2 activity in BT474 and SKBR3 breast cancer cells [41].

PERTUZUMAB

Pertuzumab is a recombinant, humanized, monoclonal antibody that binds to the extracellular dimerization domain II of HER2 [42]. Domain II is located on the opposite side of domain IV, where trastuzumab binds. Pertuzumab inhibits heterodimerization of HER2 with HER1, HER3, HER4 and IGF-1R31 [42]. Pertuzumab efficiently inhibits in vitro tumor cell growth by blocking ligand-mediated HER2/HER3 heterodimerization, whereas trastuzumab is more efficient at inhibiting cell growth in the absence of HER3 ligand [39]. These findings suggest that a combination of trastuzumab and pertuzumab can be used for treating HER2-overexpressing tumors. Evidence of phase III clinical trials have shown that the double anti-HER2 targeted treatments significantly improved survival outcome in metastatic breast cancer patients as well pathological complete response in early breast cancer setting [32, 43-45].

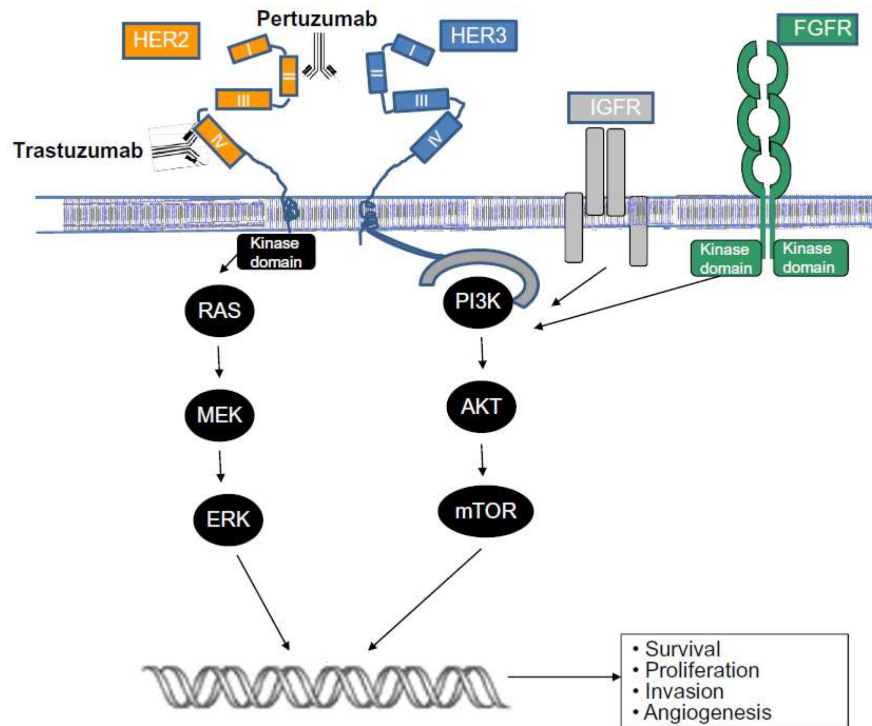


Figure 6 *Schema outlining the activation of the human epidermal growth-factor receptor 2 pathway and antibody blockade by trastuzumab and pertuzumab [14].*

- Antibody-drug conjugate - **T-DM1 (Fig. 7)**

TDM-1 also known as ado-trastuzumab emtansine combines the monoclonal antibody trastuzumab with the cytotoxic emtansine (DM1), a maytansinoid class anti-microtubule agent, linked by a stable thioether [46]. After T-DM1 binds to the HER2 receptor, the complex of HER2 and T-DM1 enters target cells through receptor-mediated endocytosis. This results in antibody degradation within the lysosome, intracellular release of DM1, and subsequent cell cycle arrest and apoptosis induction. A specific feature of T-DM1 includes the selective delivery of the cytotoxic component to the tumor, which minimizes systemic toxicity and generally improves tolerance of T-DM1 [46]. T-DM1 significantly improved both PFS and OS compared with lapatinib plus capecitabine as a second-line treatment

and as a later line in patients with advanced HER2-positive breast cancer previously treated with trastuzumab [47]. Based on those results, T-DM1 is currently the standard second-line therapy for advanced HER2-positive disease [47]. In early stage HER2 positive breast cancer results from the phase III Katherine trial suggested a potential benefit in survival outcome in patient with residual breast cancer disease after neoadjuvant chemotherapy treated with adjuvant TDM-1 instead of trastuzumab [48].

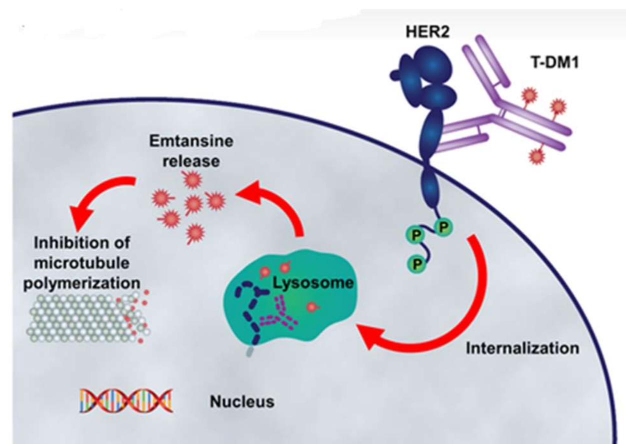


Figure 7 TDM-1 mechanism of action [49]

- Tyrosine kinase inhibitor (TKI) – **LAPATINIB (Fig. 8)**

Lapatinib is an oral tyrosine kinase inhibitor (TKI) that reversibly inhibits HER1/EGFR and HER2 kinases [50]. It acts as a dual reversible TKI for both these receptors, thus blocking the downstream MAPK/Erk1/2 and PI3K/AKT pathways. Lapatinib has been shown to enhance the trastuzumab-dependent cell-mediated cytotoxicity against breast tumor cells, in vitro studies [51]. A preclinical study showed that lapatinib inhibited the growth of HER2-positive breast cancer cells

that were resistant to trastuzumab and increased the apoptotic effect of anti-HER2 antibodies [50]. The approval of lapatinib was based on the improvement in progression-free survival (PFS) found in a phase III trial when combined with capecitabine versus capecitabine alone [52].

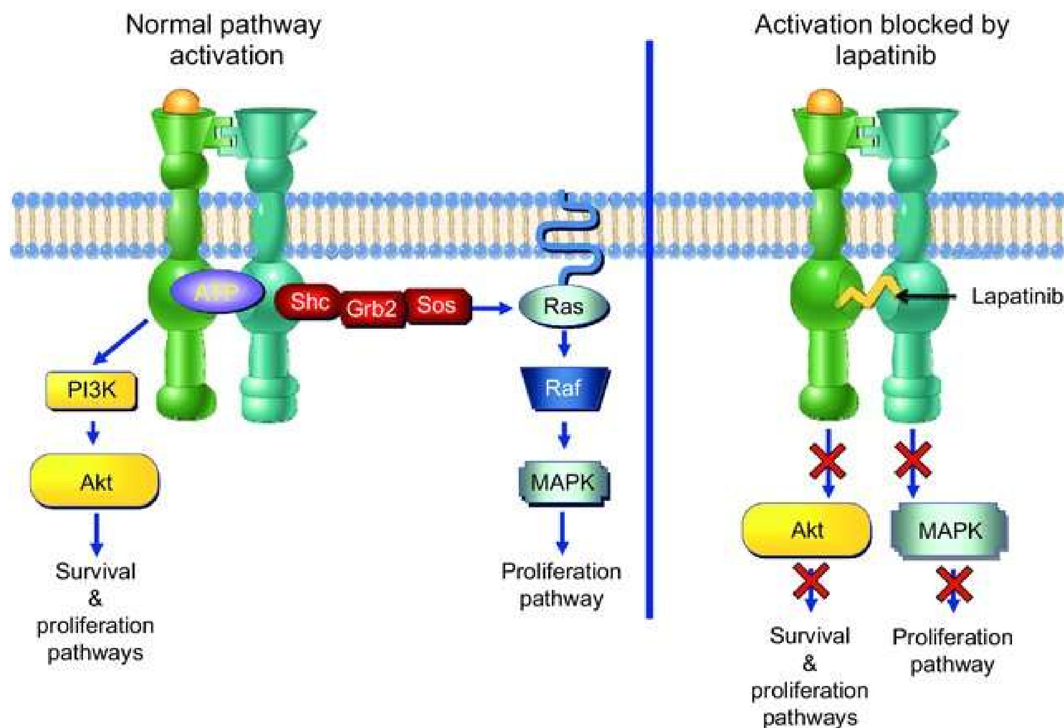


Figure 8 *Mechanism of action of lapatinib. Lapatinib blocks the catalytic cleft of the eRBB1 and eRBB2 receptors, thereby preventing adenosine triphosphate binding and subsequent receptor phosphorylation leading to inhibition of downstream mitogenic signaling cascades [14].*

2.1.1 Mechanisms of resistance to anti-HER2 therapy

Despite the outstanding improvement in survival with the introduction of anti-HER2 therapies alone or as dual HER2-blockade in the standard treatment of adjuvant/neoadjuvant HER2 positive BC about 10-15% of patients ultimately

develop metastatic disease [53]. For that reasons, therapeutic resistance to trastuzumab has become an increasingly important clinical issue. Relapse after adjuvant/neoadjuvant therapy indicates the presence of de novo or acquired resistance to previous treatments. The mechanisms underlying the development of resistance to HER2 targeted agents, mainly trastuzumab, are still under active investigation. Many potential resistance mechanisms to anti-HER2 therapy have been described that ultimately lead to reactivation of the HER2 pathway or its downstream signaling, through pathway redundancy or stimulation of alternative survival pathways [53]. Known mechanism of resistance are (seen Fig. 9) [54]:

1) **Escape from antibody-dependent cell mediated cytotoxicity**. Trastuzumab covers HER2 and, by binding to Fc receptors expressed on natural killer cells, antigen-presenting cells, or immune effector cells, it causes them to become active and lyse the antibody-coated tumor cell. This response is modulated by monoclonal antibodies binding, expression of different polymorphic receptors on immune cells, level of tumor antigen expression by tumor cells and the frequency and reactivity of immune cells in the tumor microenvironment [55].

2) **Crosstalk between estrogen receptor and HER2 pathways**. Tumors that express both ER and HER2 are less sensitive to endocrine therapy than ER-positive and HER2-negative tumors, and ER can act as an escape pathway to HER2 inhibition. These observations suggest the existence of a bidirectional cross-talk between both pathways, so that targeted therapy against a signaling pathway can be followed by tumor growth through the others. ER is mainly a nuclear receptor and functions as a ligand-dependent transcription factor that regulates expression of

different genes, such as IGF1R, cyclin D1, bcl-2, VEGF-R and receptors of HER family. There is also a small pool of ER located in the cytoplasm and non-nuclear subcellular fractions. Activation of these ER increases the levels of cyclic adenosine monophosphate and other second messengers such as IGF-IR, EGFR, and HER2 [56].

3) **Intrinsic alterations in HER2 protein receptor** (such as p95HER2). HER2 carboxy-terminal fragments, also known as p95HER2 fragments, are a subtype of HER2 receptors that are characterized by the lack of ECD, where the binding point of trastuzumab is located. These fragments can arise by the shedding of ECD by a metalloprotease or by alternative initiation or translation of the mRNA-encoding HER2. This expression has been associated with trastuzumab resistance in some retrospective studies [57].

Moreover, a HER2 splice variant with enhanced transforming activity, HER2Δ16, has been described in BC cell lines and tumors. It is characterized by an imbalance in the number of cysteines in the ECD portion and by the constitutive generation of stable HER2 homodimers. Its appearance is a tumor-specific event, and it is associated with trastuzumab resistance [58].

4) **Expression levels of HER2 mRNA and protein**. In the EMILIA trial, a greater benefit in OS was also observed in patients treated with T-DM1 and high HER2 mRNA expression [59].

5) **Aberrant activation of pathways downstream of HER family proteins** (ie PI3K/Akt/mTOR pathway) by reduced levels of tumor suppressor genes (like PTEN

and INPP4-B), or by activating mutations in PIK3CA (phosphatidylinositol-4,5 bisphosphate 3-kinase catalytic subunit). In particular, PIK3CA mutations seems to be associated with significantly shorter PFS and OS in patients treated with capecitabine plus lapatinib, but not in T-DM1 treated patients. Data are available from studies with dual HER2 blockade: various neoadjuvant trials with a combination of trastuzumab and pertuzumab or trastuzumab and lapatinib showed better pCR rates in those patients without PI3KCA mutations. In the CLEOPATRA trial, tumors with PI3KCA mutations had a worse prognosis independent of the treatment arm [60].

6) **Alterations in apoptosis and cell cycle control**. In particular the activation of cyclin D1-CDK4 pathway. HER2 signal activation determinates cell death inhibition. It is reasonable that alterations in the apoptotic machinery can induce resistance to trastuzumab. For example, overexpression of t-Darpp, a truncated form of the dual kinase/phosphatase inhibitor Darpp-32, has been linked to acquired resistance to trastu-zumab [61]. P27Kip1 is a CDK inhibitor that blocks cyclin E/CDK2 complexes, which induce cell cycle arrest. Amplification/overexpression of cyclin E has been associated with lower response rate and PFS in a small study with 34 patients treated with trastuzumab [62].

7) **Expression of other HER family proteins** in the cellular membrane and their interaction that activates compensatory mechanisms within the HER family (such as HER3). Trastuzumab may not be able to completely inhibit the signaling pathway because of redundant ligands and receptors that enable alternative

dimerization patterns. In this category, epidermal growth factor receptor (EGFR/HER1) and HER3 are the receptors with a more significant role in trastuzumab resistance. Coexpression of EGFR in HER2-overexpressed BC has been associated with worse survival [63].

8) **Activation of membrane receptors outside of the HER family** such as insulin-like growth factor 1 receptor (IGF-1R) and MET [59]

9) **Host and tumor microenvironment components**, such as tumor infiltrating lymphocytes (TILs) and FCγR polymorphisms. For example, an increased quantity of stromal TILs was significantly associated with improved OS in patients with advanced HER2-positive breast cancer treated with docetaxel, trastuzumab, and pertuzumab or placebo in the CLEOPATRA trial [32]

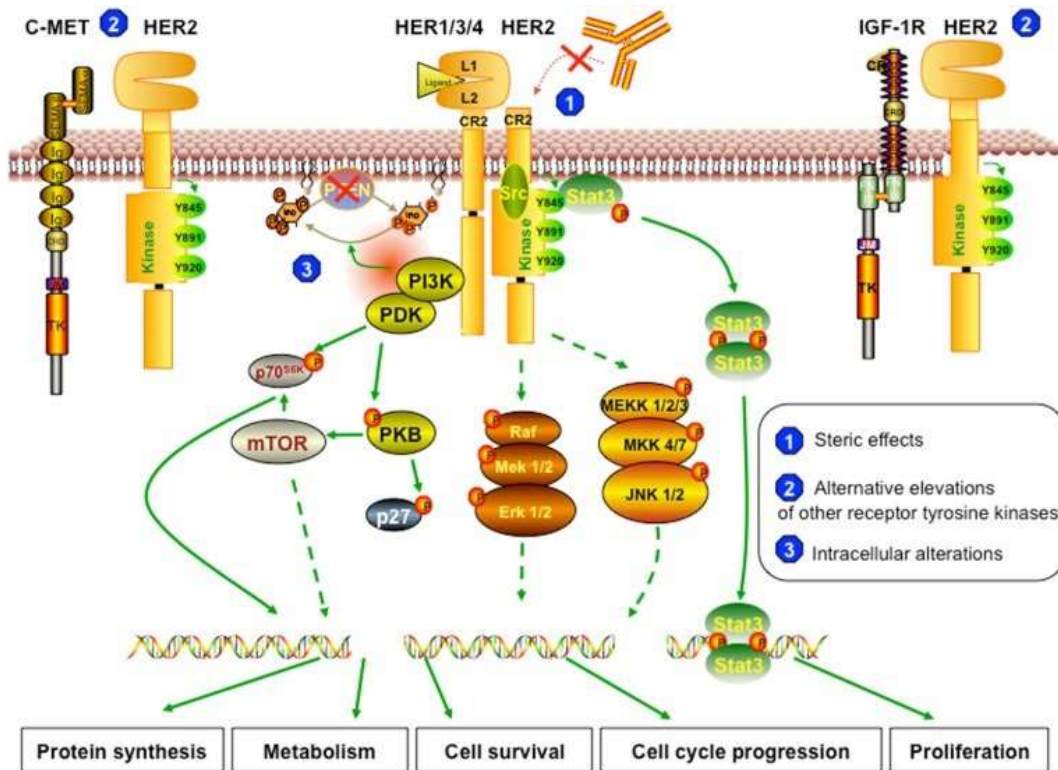


Figure 9 *Resistant mechanism to trastuzumab. (1) Steric effects or masking of the trastuzumab-binding sites. The truncated form of HER2 (p95HER2) that lacks trastuzumab-binding domain can no longer be inhibited by trastuzumab. The remaining structure can still dimerize with other receptors and therefore can still trigger downstream cascades. (2) Alternative elevations of other receptor tyrosine kinases. The overexpression of other growth factor receptors, such as c-Met, IGFR1 etc [14].*

2.2. Neoadjuvant treatment in HER2 positive breast cancer

In early-stage breast cancer (define as breast cancer that has not spread beyond the breast or the axillary lymph nodes including stage I-III breast cancers) the treatment strategy depend on tumor subtype and stage at the diagnosis. In particular, in HER2 positive breast cancer treatment strategy includes: surgery +/- radiation therapy, adjuvant or neoadjuvant chemotherapy with targeted anti-HER2 antibody and endocrine therapy in case of hormone receptor positive tumors [28].

Considering systemic treatment, neoadjuvant chemotherapy (NACT) (define as chemotherapy administered before breast cancer surgery) instead of adjuvant chemotherapy is widely used HER2 positive breast cancer [64]. NACT was introduced in the 1970s, aiming to downstage locally advanced (inoperable) disease and make it operable. Other reasons that supported the use of NACT are: 1. NACT might be somewhat more likely to eradicate micro-metastatic disease than might chemotherapy delayed until after surgery; 2. NACT might mitigate the hypothesized stimulatory effect of surgery on occult disease and reduce tumor cell shedding during surgery; 3. NACT might also provide useful in-vivo information about the chemo sensitivity of the tumor to different chemotherapy regimens, helping to guide subsequent drug selection. In particular, it is well known that the achievement of pathological complete response (pCR) (define as no residual invasive disease in the breast and the axillary lymph nodes) significantly predicts long-term survival outcomes (Fig. 10) [64]. The results of EBCTCG meta-analysis, published in The Lancet Oncology in 2018, including 4756 women randomly allocated in ten trials to either NACT or adjuvant chemotherapy, showed that patients treated with NACT had a higher rates of breast-conserving surgery than those treated with adjuvant one without compromising survival outcomes [65].

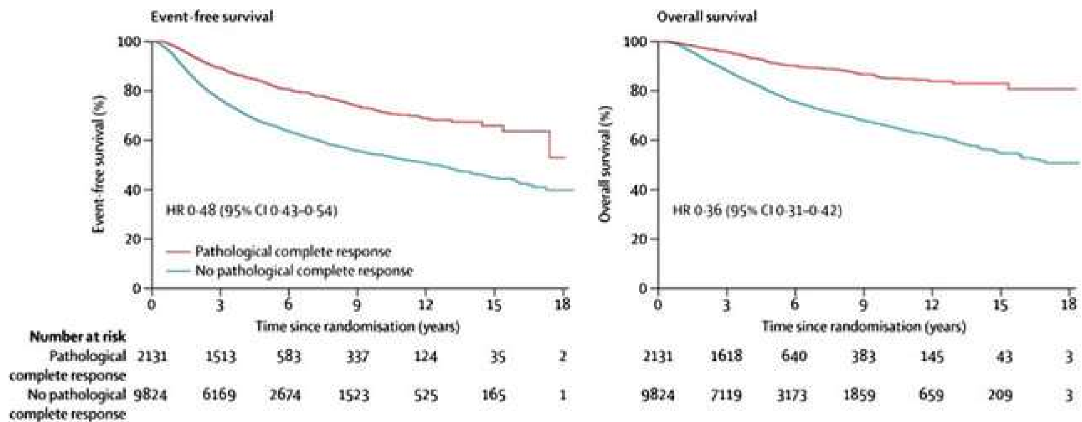


***Figure 10** Associations between pathological complete response and event-free survival and overall survival. ypT0/is ypN0 definition of pathological complete response (ie, absence of invasive cancer in the breast and axillary nodes, irrespective of ductal carcinoma in situ). HR=hazard ratio [14].*

Considering HER2 positive breast cancer, the introduction of trastuzumab +/- pertuzumab significantly increased the rate of pCR [44, 45]. Several trials have reported consistently high pCR proportions of up to 66% among HER2-positive breast cancers treated preoperatively with combination chemotherapy and (dual) targeted anti-HER2 agents [44, 45]. On the other hand, the presence of residual disease after NACT indicates the existence of partial treatment resistance in the tumor. The current guidelines recommend maintaining anti-HER2 treatment with trastuzumab after surgery in all the HER2 positive breast cancer patients to complete one year of treatment [28]. However, the benefit of this consolidated strategy is uncertain for patients with residual disease after NACT. To evaluate the activity of TDM1 in the post-neoadjuvant scenario, the KATHERINE phase III trial randomized 1486 patients with HER2-positive breast cancer and residual disease after NACT to receive 14 cycles of TDM1 or to maintain trastuzumab for 14 cycles [48]. In the interim analysis with a median follow up of 41.4 months, the 3-year invasive disease free survival rates were 88.3% in the TDM1 *versus* 77% in the

trastuzumab group (HR 0.50; 95% CI 0.39–0.64, $p < 0.001$ log-rank test). The rate of distant recurrences was 10.5% with TDM1 *versus* 15.9% with trastuzumab, and all subgroups benefited from TDM1. Around 18% of the patients included in the study received both trastuzumab and pertuzumab in neoadjuvant setting. Given the impressive results observed in the KATHERINE trial, post-neoadjuvant TDM1 represents the new standard of care treatment for HER2-positive patients with residual disease after NACT [48]. Takes to these evidence, nowadays the most important role of NACT is the possibility to adapt and further personalize the adjuvant strategy. In fact, patients with a residual disease will be candidate for an alternative adjuvant treatment, whereas patients achieving the pCR will stay on the same treatment used before surgery.

3 Study rational and aims

Neoadjuvant chemotherapy (NACT) is a treatment option in patients with early-stage HER2 positive breast cancer. In that setting the addition of trastuzumab and pertuzumab to standard chemotherapy resulted in a significantly higher activity when compared to chemotherapy alone. In HER2+ breast cancer subtypes, the achievement of the pathological complete response (pCR) is a powerful indicator of long-term outcome, in particular. Patients achieving less than pCR have a worse prognosis; however, this group is heterogeneous, including patients with a good response but not complete as well as patients with primary resistant disease. To date, there are no clear prognostic biomarkers in patients with residual tumor disease. Actually, the main challenge remains the identification of a mutational profile able to predict treatment sensitivity and survival outcomes prior any intervention.

The aim of the study was to investigate the mechanisms of treatment resistance in a sample of HER2 positive breast cancer patients treated with NACT. We compared the mutational profile of HER2 positive breast cancer with residual disease after NACT to a control group of patients who achieved the pCR. Particularly, we aimed to evaluate which mutations were the most involved in primary treatment resistance analyzing the detected mutated genes on the diagnostic breast cancer biopsy. Moreover, we aimed to identify the treatment-induced mutations analyzing the surgical specimens of patients with residual

tumor after primary systemic treatments. Finally, an exploratory analysis in terms of treatment outcome and genes status was performed.

4 Material and methods

Patients with diagnosis of HER2 positive early breast cancer treated with neoadjuvant chemotherapy (NACT) with residual disease after systemic treatment were identified. A control group of patients with HER2 positive disease who achieved pathological complete response (pCR) after NACT was selected too. In order to be enrolled, patients must have tissue samples taken from the diagnostic breast cancer biopsy and from matched surgical specimens. All the breast cancer tissue samples were formalin-fixed and paraffin-embedded (FFPE) and archived in the Pathology Department of Azienda Ospedaliero-Universitaria Policlinico of Modena. Using a next-generation sequencing (NGS) technology, we evaluated a panel of 21 genes known to be involved in the mechanisms of treatment resistance. Firstly, we compared the mutational profile of breast cancer with residual disease after NACT to the mutational profile of pCR ones. Secondarily, in order to detect the treatment-induced mutations, we compared the gene profile founded in the diagnostic biopsy to the matched residual tumor in patients without pCR.

The study was approved by the local ethics committee (protocol number 0024589/19). Written informed consent was obtained from each alive patient.

4.1 Population and samples

One hundred and ninety-six patients with HER2 positive early breast cancer treated with NACT at the Azienda Ospedaliero-Universitaria Policlinico of Modena from 2008 to 2018 were identified. One hundred and seventy-eight of them received an anti-HER2 agent (trastuzumab +/- pertuzumab) as part of their neoadjuvant systemic treatment. Among these women, 31 underwent to breast cancer surgery outside Modena University Hospital. Considering patient with known breast surgery outcome, 111 did not achieved the pCR. Among these, 83 women had available formalin-fixed and paraffin-embedded (FFPE) breast cancer tissues taken from both diagnostic biopsy and surgical specimens archived in our Pathology Department. Of note, all the samples from the breast biopsy were taken before the initiation of any systemic treatment. In order to select the real treatment-resistant breast cancer, we decided to perform the gene analysis only in breast cancer sample with at least 1 cm of residual invasive tumors. The final study population included 32 patients with residual breast cancer disease after NACT (**No pCR group**) and a control group of 32 patients who achieved the pCR (**pCR group**). The control pCR group was balanced for patients and tumor characteristics (Fig. 11)

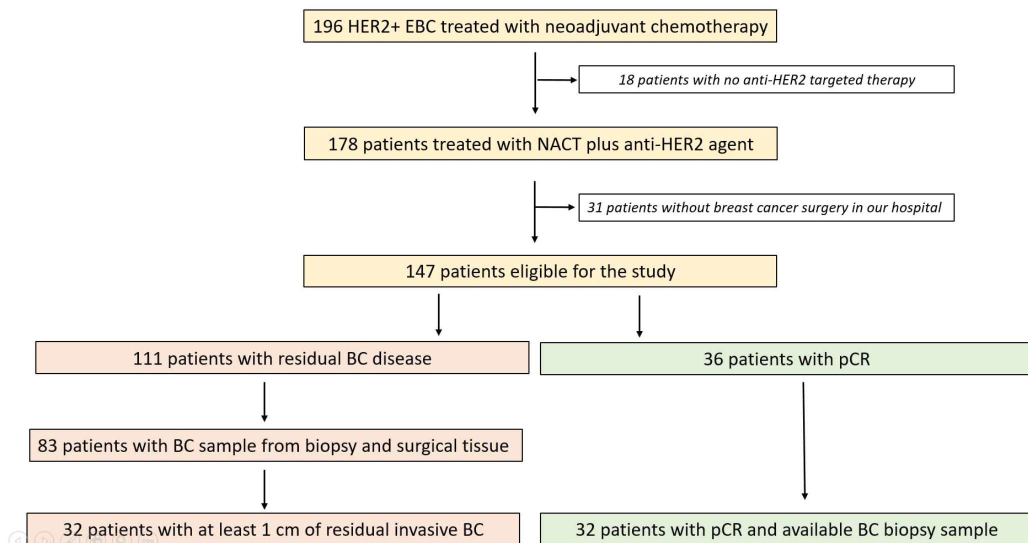


Figure 11 *Flowchart of the study population.*

NACT: neoadjuvant chemotherapy; BC: breast cancer; pCR: pathological complete response

4.2 Next generation sequencing technology

The Next-Generation Sequencing (NGS) technology describes a number of different modern technologies that consist in massively parallel sequencing reactions. This high-throughput approach allow to obtain big amount of sequencing data, starting from restricted targeted gene panel to largest applications such as Whole Exome or Whole Genome Sequencing (WES/WGS), thanks to the capability of analysing millions or even billions of sequencing reactions at the same time. According to capability to sequence each single position many times (coverage), NGS can reach very high sensitivity in variant detection.

The different available platforms recognise some principal common steps:

1. *Library preparation*: libraries are created using random fragmentation of DNA using enzymatic reaction or by sonication. DNA segments are modified through a ligation reaction aimed to link a sample-specific index.

2. *Amplification*: the library is amplified by Emulsion PCR or Bridge PCR

3. *Sequencing*

4. *Bioinformatic analysis*: raw data obtained by sequencing need a 'deconvolution step' to acquire biological significance. Bioinformatic analysis is usually supported by software containing specific pipelines that offer automated primary and secondary analysis and support variants annotation to identify those that are biologically relevant. (Fig 12).

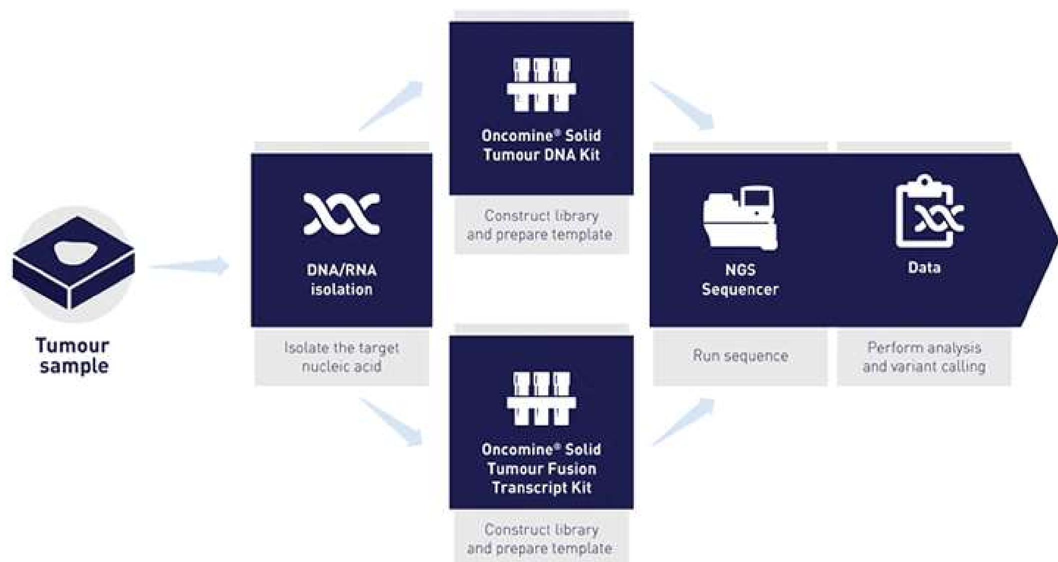


Figure 12 *Example of parallel workflows for DNA analysis using NGS resulting in single nucleotide variant and indel data for the DNA workflow and fusion detection and expression data for the RNA workflow.*

4.3 DNA isolation

DNA extraction was performed in the Molecular Biology Laboratory of Modena Pathology Department. DNA extraction was performed with QIAamp DNA Mini Kit (Qiagen) from 10 µm-thick sections of FFPE tissues. Tumor-representative areas containing at least 20-50% tumor cells were selected by pathologist and isolated by manual microdissection, as suggested from guidelines. DNA Extraction Protocol performed follows:

1. Scrape selected areas from at least 2 10 µm-thick sections and put it in 1,5ml Eppendorf tube.

2. Add 180 µl Buffer ATL and 20 µl Proteinase K, mix by vortexing and incubate at 56°C until completely lysed (1–3 h). Vortex occasionally during incubation.

3. Add 200 µl Buffer AL. Mix thoroughly by vortexing for 15 s.

4. Incubate at 70°C for 10 min. Briefly centrifuge the tube to remove drops from the lid.

5. Add 200 µl ethanol (96–100%). Vortex for 15 s. Briefly centrifuge the tube to remove drops from the lid.

6. Pipet the mixture onto the QIAamp Mini spin column (in a 2 ml collection tube). Centrifuge at 6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tube.

7. Place the QIAamp Mini spin column in a new 2 ml collection tube and add 500 µl Buffer AW1. Centrifuge at 6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tube.

8. Place the QIAamp Mini spin column in a new 2 ml collection tube and add 500 µl Buffer AW2. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min. Discard the flow-through and collection tube.

9. Place the QIAamp Mini spin column in a new 2 ml collection tube and centrifuge at full speed for 1 min. This eliminates the chance of possible Buffer AW2 carryover.

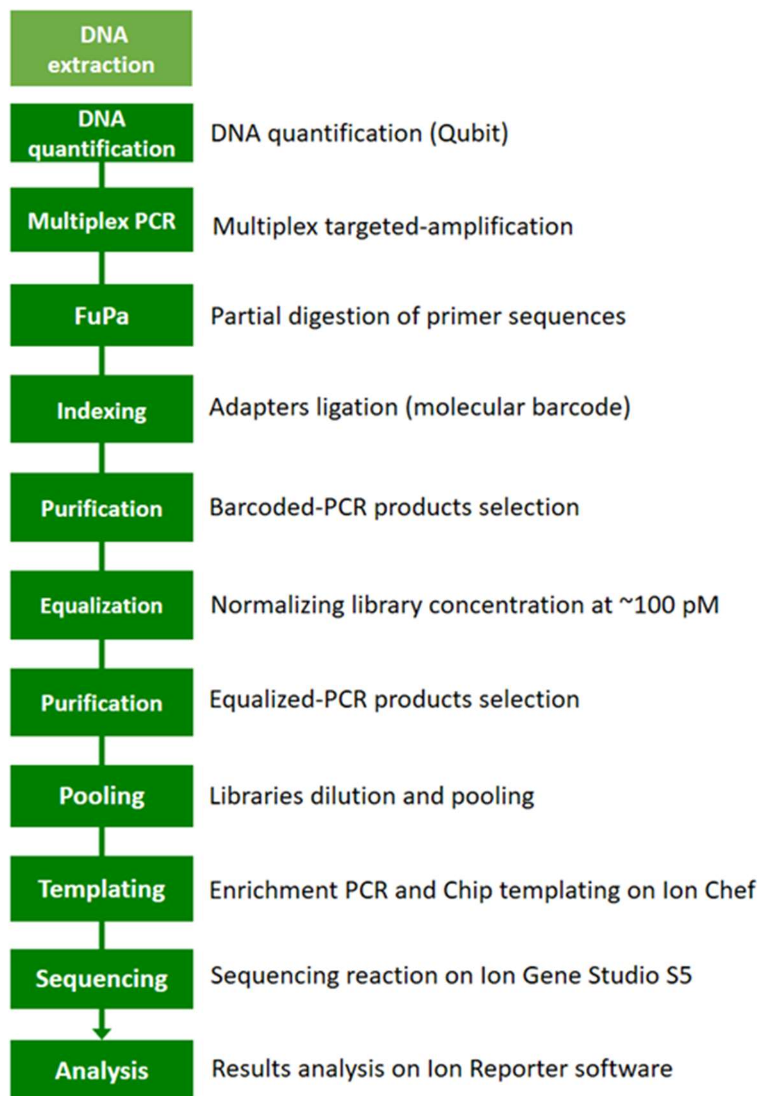
10. Place the QIAamp Mini spin column in a new 1.5 ml microcentrifuge tube, add 200 µl Buffer AE and incubate at room temperature for 1 min. Centrifuge at 6000 x g (8000 rpm) for 1 min to elute the DNA.

Extracted DNA was quantified with Qubit fluorometer (Life Technologies) and 10 ng from each sample were amplificated in single-tube multiplex PCR.

4.4 Gene analyses

Mutational analysis was performed with targeted amplification-based NGS panel ‘Oncomine Solid Tumour DNA’ (ThermoFisher Scientific). The Oncomine Solid Tumour DNA Kit allowed the identification of somatic variants (SNV, MNV, INDEL) in selected regions of a define list of cancer-related genes. The Ion Torrent workflow consists in manual library preparation over-night templating on Ion Chef

system (ThermoFisher Scientific) and sequencing on Ion GeneStudio S5 Sequencer (ThermoFisher Scientific). In particular, DNA libraries preparation required a preliminary step of PCR to amplify target DNA. PCR primers were partially digested and conjugated with specific adapters that acted like molecular barcodes for samples tagging. Using specific capture solution and magnetic beads, the equalization of library was performed and loading by Ion Chef System that provides automated high-throughput template preparation and chip loading. After that, first chip was placed in a specific clamp on Ion GeneStudio S5 Sequencer. Analysis of raw sequencing data was conducted with Ion Reporter software. Only variants with a minimum coverage of 500X were considered to ensure variant specify. The reference of specific databases, such as 1000Genome or ExAC, and the evaluation of allelic frequency allowed us to rule out germinal variants. Integrative Genomic Viewer was used for variant visualization.



4.3.1 The multi-gene panel

To perform the gene analysis, we used the Oncomine Solid Tumour next-generation sequencing (NGS) kits for DNA enabling analysis of solid tumour FFPE samples. The multi-gene panel is able to analyze genetic status of 21 cancer-related genes. These genes are known to be involved in the mechanisms of treatment resistance and cancer progression. The analysed genes were: EGFR,

ALK, ERBB2, ERBB4, FGFR1, FGFR2, FGFR3, MET, DDR2, KRAS, PIK3CA, BRAF, AKT1, PTEN, NRAS, MAP2K1, STK11, NOTCH1, CTNNB1, SMAD4, FBXW7, TP53.

According with molecular pathways and gene function, these genes can be classified as follow:

- **EGFR, ERBB2 and ERBB4**: this family of tyrosine kinase receptors is involved in cell proliferation control, differentiation and survival. The mutation rate of ERBB2 in breast cancer is about 2–3% [20, 66].
- **FGFR1, FGFR2, FGFR3, MET**: in HER2 positive breast cancer, are one of the possible mechanisms of resistance to anti-HER2 agents, is an alternative signalling from other receptor tyrosine kinases [19].
- **AKT1, PIK3CA, PTEN**: the PI3K/AKT/mTOR pathway is one of the main downstream pathways involved in cancer cell proliferation. Mutations of PIK3CA have been found in almost 30% of all sporadic breast cancer with a wide frequency range in breast cancer subtypes [19, 21]. The majority of the mutations, "Hotspot" mutation, occurred in three sites: E542 and E545 in the helical domain, and H1047 in the kinase domain. These mutations cause gains in protein enzymatic function and induce oncogenic transformation [67]. The somatic intragenic PTEN mutation frequency is <5% [40].
- **BRAF, KRAS, NRAS, MAP2K1, DDR2**: the MAPK signaling pathway may lead to uncontrolled cell cycle, resistance to apoptosis, to chemotherapy and to targeted therapies [68].

- **ALK, SMAD4**: ALK activates multiple pathways, including phospholipase C γ, Janus kinase (JAK)-signal transducer and activator of transcription (STAT), PI3K-AKT, mTOR, sonic hedgehog (SMO and GLI), and MAPK signaling cascades, which affect cell growth, transformation and anti-apoptotic signaling [69].
- **NOTCH1**: NOCHT pathway is involved in cell proliferation, invasion and chemo-resistance process [68].
- **CTNNB1**: CTNNB1 mutations determinate the constitutive activation of the Wnt/β-catenin signaling pathway known to be involved in the first step of tumorigenesis [70].
- **FBXW7**: FBXW7 is a critical tumor suppressor and one of the most commonly deregulated ubiquitin-proteasome system proteins in human cancer. FBXW7 controls proteasome-mediated degradation of oncoproteins such as cyclin E, c-Myc, Mcl-1, mTOR, Jun, Notch and AURKA [71].
- **TP53**: p53 is a nuclear transcription factor with a pro-apoptotic function. Since over 50% of human cancers carry loss of function mutations in p53 gene. Activated p53 promotes cell cycle arrest to allow DNA repair and/or apoptosis to prevent the cancer development [14].

4.5 Statistical analyses

Statistical analysis was performed using STATA 13 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP). Baseline clinical and tumor characteristics were compared between the two tumor-response subgroups (No pCR vs pCR) by chi-square test for categorical variables (i.e. breast cancer subtypes, grade, histotype, neoadjuvant treatment, stage, recurrence and death) and by Wilcoxon Mann-Whitney test for continuous variables (i.e. age at diagnosis). Survival outcomes of interest were OS defined as the time from the diagnosis of breast cancer to the death/last follow up and Relapse Free Survival (RFS) defined as the time from the date of the diagnosis of breast cancer to the date of the first documented recurrence/death. OS and RFS were addressed by the Kaplan–Meier method and log-rank test. A p-value < 0.05 was considered statistically significant; hazard ratio was estimated with 95% of confidence limits.

5 Results

5.1 Patient and sample characteristics

Tumor and patient characteristics are described in Table 1. The two group were well balanced according to baseline clinical and tumor characteristics. As expected, the percentage of hormone receptors positive breast cancer were higher in No pCR group than pCR cohort (*p value 0.002*). All the enrolled patients received trastuzumab combined with systemic chemotherapy, in 6 cases with the addition of pertuzumab too. Considering survival outcomes, in the pCR group, 2 patients had a recurrence and are still alive while in the No pCR cohort, there were 8 relapse and 4 deaths. The median follow up was 45 months in both patients groups.

***Table 1** Baseline clinical and tumor characteristics according with tumor response to neoadjuvant treatment (No pCR vs pCR). p value was calculated by chi-square test for categorical variables and by Wilcoxon Mann-Whitney test for continuous variables*

	No pCR		**pCR**		
	32		32		**p value**
Characteristic	**N**	**%**	**N**	**%**	
Age (range)	50 (28-70)		50 (26-80)		
Clinical stage					
II	24	(75)	23	(72)	1
III	8	(25)	9	(28)	
BC subtypes					
HER2+ HR+	20	(63)	7	(22)	0.002
HER2+ HR-	12	(24)	25	(78)	

Histotype					
ductal	29	(93)	32	(100)	0.238
lobular	3	(7)	0		
Grade					
2	4	(10)	2	(6)	0.671
3	28	(90)	30	(94)	
Neoadj pertuzumab					
Yes	3	(7)	3	(7)	1
No	29	(93)	29	(93)	
Neoadj cht					
Taxane	5	(6)	4	(10)	1
Anthracycline + Taxane	27	(84)	28	(90)	
Recurrence					
Yes	8	(25)	2	(6)	0.081
No	24	(75)	30	(94)	
Death					
Yes	4	(10)	0		0.113
No	28	(90)	32	(100)	

Among the 64 enrolled patients, 28 with residual breast cancer disease and 30 with pCR had successfully gene analysis performed on the diagnostic breast cancer biopsy. Considering the No pCR group, in 25 cases the gene analysis was successfully performed in both biopsy and residual breast cancer tissue. Overall, the detected mutations were 113: 37 in the pCR group, 35 in the No pCR group and 41 in the residual breast cancer samples (Table 2). The majority of the mutations (77%) were single nucleotide variation (SNV) while in the other cases were insertion or deletion (INDEL).

Table 2 *Number and type of the detected mutations in the breast cancer biopsy and residual disease*

	Breast cancer biopsy		**Residual BC tissue**
	pCR (N= 30)	**No–pCR (N=28)**	**(N=25)**
Mutations	*N. detected mutated genes*		
Number	**37**	**35**	**41**
Type			
TP53	20 (54%)	12 (34%)	13 (32%)
PIK3CA	8 (21%)	8 (23%)	8 (19%)
MET	1 (3%)	2 (6%)	3 (6%)
KRAS	0	2 (6%)	1 (3%)
SMAD4	0	2 (6%)	1 (3%)
NOTCH1	0	1 (3%)	2 (5%)
FGFR3	1 (3%)	2 (6%)	4 (9%)
ERBB2/4	2 (5%)	1 (3%)	2 (5%)
PTEN	0	1 (3%)	3 (6%)
DDR2	2 (5%)	1 (3%)	1 (3%)
ALK	1 (3%)	0	1 (3%)
BRAF	1 (3%)	0	0
STK11	1 (3%)	1 (3%)	0
NRAS	0	1 (3%)	0
EGFR	0	1 (3%)	1 (3%)
AKT1	0	0	1 (3%)

5.2 Primary tumor mutation (*No-pCR group versus pCR group*)

Overall, we found 72 mutations in the diagnostic breast cancer biopsy. Thirty-seven of them were in the pCR group and 35 in residual disease cohort. Considering the mutational burden, in No pCR cohort 6 patients had no detected mutation (21%), 9 one detected mutation (32%), 12 two detected mutations (43%) and 1 with three detected mutations (4%). In the pCR group, we found 3 patients with no mutation (10%), 18 with one mutation (60%), 8 with two mutations (27%) and 1 with three mutations (3%) (Table 2, Fig. 13).

***Table 3** Patients mutational burden according to No pCR and pCR groups.*

	No-pCR *N. of patients (%)*	pCR *N. of patients (%)*
No mutation	6 (21%)	3 (10%)
1 mutation	9 (32%)	18 (60%)
2 mutations	12 (43%)	8 (27%)
≥ 3 mutations	1 (4%)	1 (3%)

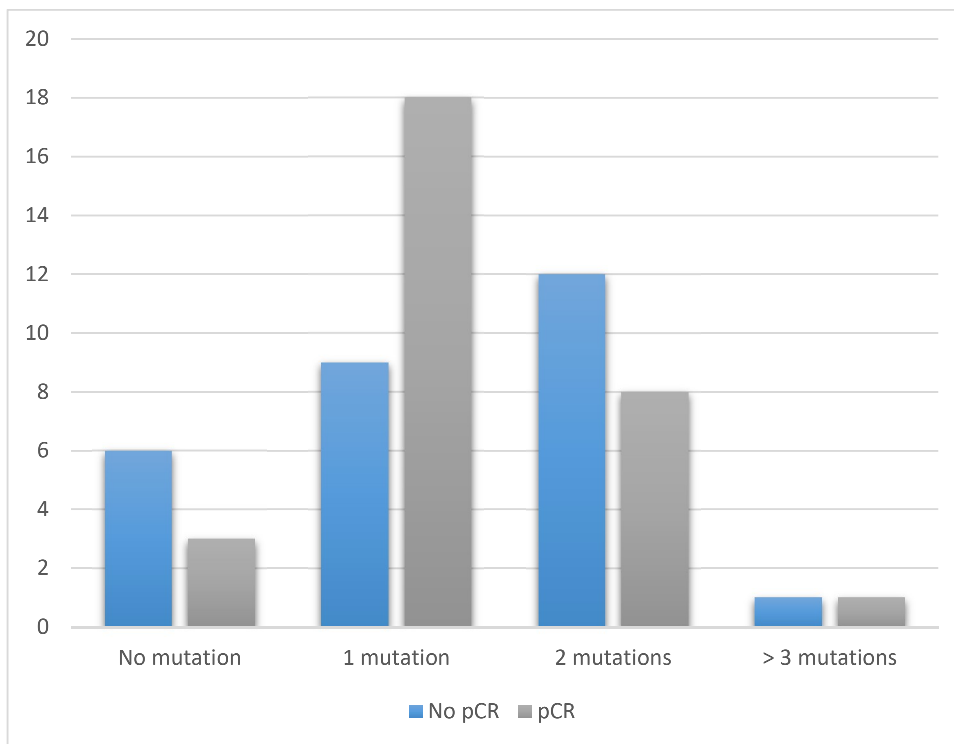


***Figure 13** Patients mutational burden according to No pCR and pCR groups*

Regarding the meaning of the detected mutations, in both groups, 15 mutations were known to be pathogenic/likely pathogenic ones (PI3KCA, TP53, SMAD4, DDR2, on PTEN). Moreover, in the pCR cohort, we found a MET mutation with

uncertain significance. The following tables list all the detected mutated genes and their significance.

LIST OF DETECTED MUTATIONS IN No-pCR GROUP

MUT	**TP53**	**N.**	NOTE
TYPE	SNV	7	
	INDEL	5	
CLINICAL VAR		3	PATHOGENIC
VARIANT EFFECT	MISSENSE	5	
	NONSENSE	2	
	FRAMESCHIFT/DEL	5	

MUT	**PIK3CA**	**N.**	NOTE
TYPE	SNV	8	H1047R n.5, E548K n.2, T1025A n.1
	INDEL		
CLINICAL VAR		8	PATHOGENIC
VARIANT EFFECT	MISSENSE	8	
	NONSENSE		
	FRAMESCHIFT/DEL		

MUT	**MET**	**N.**	NOTE
TYPE	SNV	2	
	INDEL	0	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	2	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

MUT	**NOTCH1**	**N.**	NOTE
TYPE	SNV	0	
	INDEL	1	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE		
	NONSENSE	0	
	FRAMESCHIFT/DEL	1	

MUT	**FGFR3**	**N.**	NOTE
TYPE	SNV	2	
	INDEL	0	
CLINICAL VAR			
	MISSENSE	2	

VARIANT EFFECT	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

MUT	**ERBB4**	**N.**	NOTE
TYPE	SNV	1	
	INDEL	0	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	1	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

MUT	**KRAS**	**N.**	NOTE
TYPE	SNV	2	
	INDEL	0	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	2	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

MUT	**NRAS**	**N.**	NOTE
TYPE	SNV	1	
	INDEL	0	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	1	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

MUT	**SMAD4**	**N.**	NOTE
TYPE	SNV	1	
	INDEL	1	
CLINICAL VAR		1	PATHOGENIC W398Ter
VARIANT EFFECT	MISSENSE	0	
	NONSENSE	1	
	FRAMESCHIFT/DEL	1	

MUT	**DDR2**	**N.**	NOTE
TYPE	SNV	1	
	INDEL	0	
CLINICAL VAR		1	PATHOGENIC W398Ter
VARIANT EFFECT	MISSENSE	1	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

MUT	**STK11**	**N.**	NOTE
TYPE	SNV	1	

	INDEL	0	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	1	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

MUT	**EGFR**	**N.**	NOTE
TYPE	SNV	1	
	INDEL	0	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	1	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

MUT	**PTEN**	**N.**	NOTE
TYPE	SNV	0	
	INDEL	1	
CLINICAL VAR			PATOGHENIC
VARIANT EFFECT	MISSENSE	0	
	NONSENSE	0	
	FRAMESCHIFT/DEL	1	

LIST OF DETECTED MUTATIONS IN pCR GROUP

Mutation	**TP53**	**N.**	**NOTE**
TYPE	SNV	15	
	INDEL	5	
CLINICAL VAR		6	PATHOGENIC
VARIANT EFFECT	MISSENSE	12	
	NONSENSE	3	
	FRAMESCHIFT/DEL	5	

Mutation	**PIK3CA**	**N.**	NOTE
TYPE	SNV	8	H1047R n.4, E548K n.3, G1049R n.1
	INDEL		
CLINICAL VAR		8	PATHOGENIC
VARIANT EFFECT	MISSENSE	8	
	NONSENSE		
	FRAMESCHIFT/DEL		

Mutation	**DDR2**	**N.**	NOTE
TYPE	SNV	2	

	INDEL	0	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	2	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

Mutation	**STK11**	**N.**	NOTE
TYPE	SNV	0	
	INDEL	1	associated with PT53, DDR2
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	0	
	NONSENSE	0	
	FRAMESCHIFT/DEL	1	

Mutation	**FGFR3**	**N.**	NOTE
TYPE	SNV	1	associated with PT53, DDR2
	INDEL	0	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	1	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

Mutation	**ERBB2/ERBB4**	**N.**	NOTE
TYPE	SNV	2	
	INDEL	0	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	2	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

Mutation	**MET**	**N.**	NOTE
TYPE	SNV	1	
	INDEL	0	
CLINICAL VAR		1	UNCERTAIN SIGNIFICANCE
VARIANT EFFECT	MISSENSE	1	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

Mutation	**BRAF**	**N.**	NOTE
TYPE	SNV	1	V600E
	INDEL	0	
CLINICAL VAR		1	PATHOGENIC
VARIANT EFFECT	MISSENSE	1	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

Mutation	ALK	N.	NOTE
TYPE	SNV	1	
	INDEL	0	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	1	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

Considering the detected genes, in both cohorts, TP53 and PIK3CA were the most mutated ones. In particular, mutations of TP53 and PIK3CA were detected in 67% and 27% of pCR women versus 43% and 28% of No-pCR patients respectively (p value not significant). In the pCR cohort, both ERBB2/ERBB4 and DDR2 were found mutated in 5% of cases; all the other mutated genes were detected in the 3% of women. Considering the No-pCR group, SMAD4 and FGFR3 were detected in 6% of cases followed by KRAS and MET in 5% of cases (Fig. 14 and Fig. 15).

No difference in the rate of PIK3CA mutation according to hormone receptors status was found (22% HR positive and 27% of HR negative). As expected, TP53 mutation was higher in HR negative BC compared to HR positive ones (595 vs 37%).

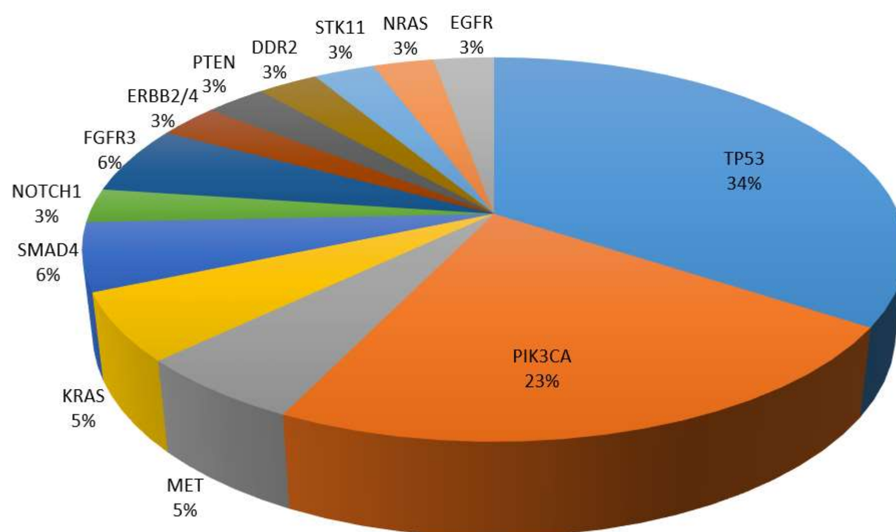


Figure 14 *Detected genes in No-pCR group patients*

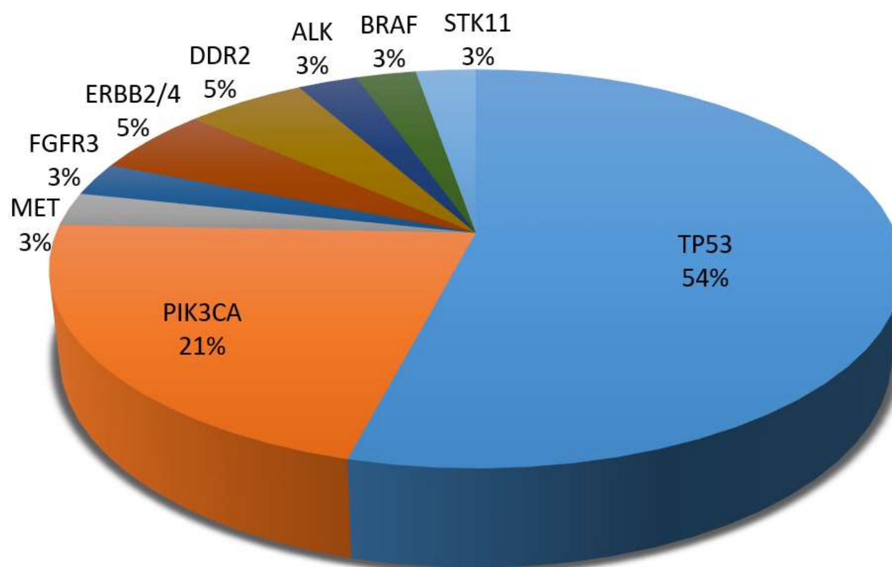


Figure 15 *Detected genes in pCR group patients*

5.3 Comparison between primary tumor and residual disease

In the group of patients with residual disease, twenty-five women had DNA analysis performed on both diagnostic tumor biopsy and matched residual breast cancer. Overall, we found 41 mutations. The total number of the detected mutations was increased from the matched breast cancer biopsy to the surgical specimen (32 vs 41 respectively). In particular, more than half of the patients changed the mutational profile during the neoadjuvant treatment. In 9 cases the number of mutated genes increased in the surgical tissue while in 2 cases the primary mutations were lost. In 2 cases the mutation profile of residual breast cancer was complete different from the primary biopsy one (Fig. 16).

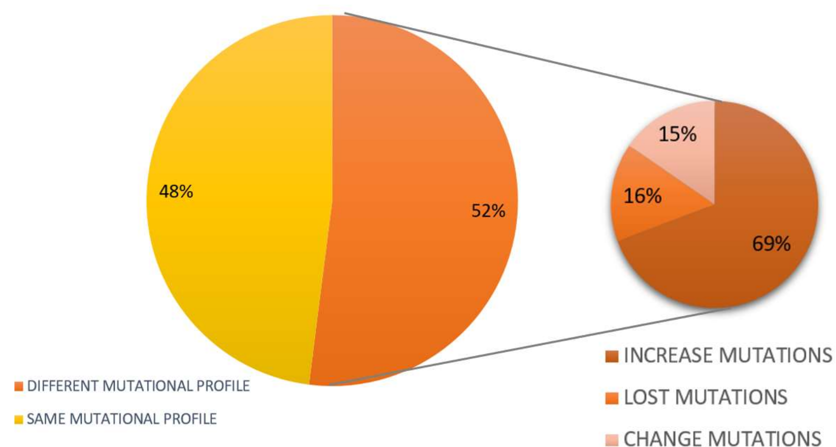


Figure 16 *Different mutational profile among diagnostic breast cancer biopsy and matched residual breast cancer.*

Considering the mutational burden in the residual tumors, only 2 patients had no mutation detected, 10 had one mutation, 9 had two mutations, 3 had three mutations and 1 breast cancer presented four mutations (Table 4 and Fig 17).

Table 4 *Patients mutational burden in breast biopsy versus residual breast cancer disease*

	Biopsy *N. of patients*	**Residual disease** *N. of patients*
No mutations	6 (24%)	2 (8%)
1 mutation	7 (28%)	10 (40%)
2 mutations	11 (44%)	9 (36%)
3 mutations	1 (4%)	3 (12%)
4 mutations	0	1 (4%)

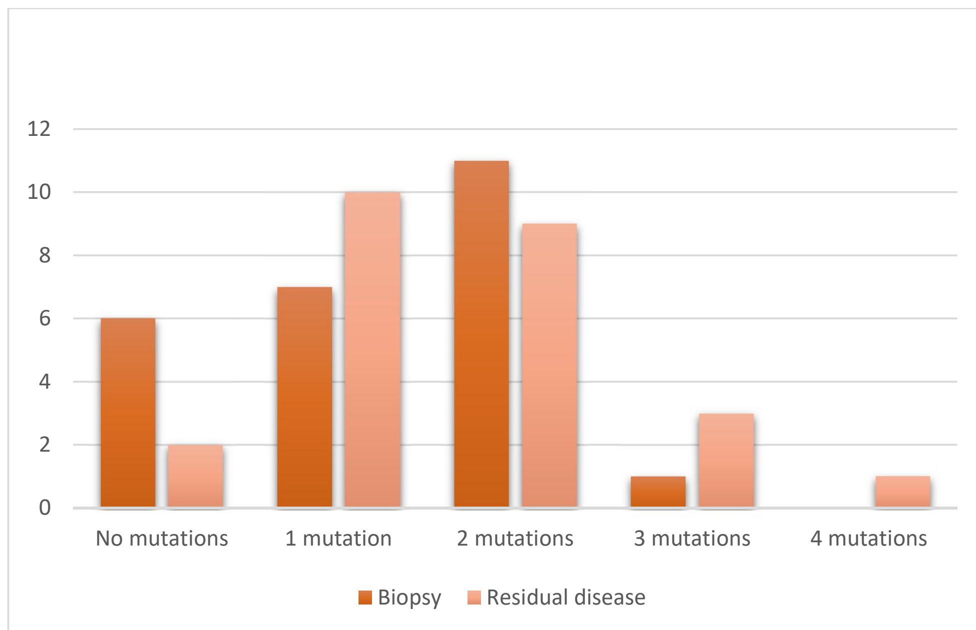


Figure 17 *Mutational burden in breast cancer biopsy compared to residual breast cancer*

Regarding the meaning of the detected mutations, 14 mutations found in the surgery tissue were known to be pathogenic/likely pathogenic (PI3KCA, TP53). The following tables list all the detected mutated genes in residual tumor.

<u>LIST OF DETECTED MUTATIONS IN RESIDUAL BREAST CANCER</u>

MUT	**P53**	**13**	NOTE
TYPE	SNV	6	
	INDEL	7	

CLINICAL VAR		3	PATHOGENIC
VARIANT EFFECT	MISSENSE	4	
	NONSENSE	2	
	FRAMESCHIFT/DEL	7	

MUT	**PI3KCA**	**8**	NOTE
TYPE	SNV	8	H1047R n.4, E548K n.3, T1025A n.1
	INDEL		
CLINICAL VAR	**CLINICAL VAR**	8	PATHOGENIC
VARIANT EFFECT	MISSENSE	8	
	NONSENSE		
	FRAMESCHIFT/DEL		

MUT	**MET**	**N.**	NOTE
TYPE	SNV	3	E168D
	INDEL	0	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	3	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

MUT	**NOTCH1**	**N.**	NOTE
TYPE	SNV	1	
	INDEL	1	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	1	
	NONSENSE	0	
	FRAMESCHIFT/DEL	1	

MUT	**FGFR3**	**4**	NOTE
TYPE	SNV	4	
	INDEL	0	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	4	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

MUT	**ERBB4 e ERBB2**	**N.**	NOTE
TYPE	SNV	2	
	INDEL	0	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	2	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

MUT	**KRAS**	**N.**	NOTE
TYPE	SNV	1	
	INDEL	0	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	1	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

MUT	**SMAD4**	**N.**	NOTE
TYPE	SNV	1	
	INDEL	0	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	0	
	NONSENSE	1	
	FRAMESCHIFT/DEL	0	

MUT	**DDR2**	**N.**	NOTE
TYPE	SNV	1	
	INDEL	0	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	1	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

MUT	**EGFR**	**N.**	NOTE
TYPE	SNV	1	
	INDEL	0	
CLINICAL VAR			
VARIANT EFFECT	MISSENSE	1	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

MUT	**PTEN**	**N.**	NOTE
TYPE	SNV	0	
	INDEL	3	
CLINICAL VAR		1	PATHOGENIC
VARIANT EFFECT	MISSENSE	0	
	NONSENSE	0	
	FRAMESCHIFT/DEL	3	

MUT	**AKT1**	**N.**	NOTE
TYPE	SNV	1	
	INDEL	0	
CLINICAL VAR		1	PATHOGENIC

VARIANT EFFECT	MISSENSE	1	
	NONSENSE	0	
	FRAMESCHIFT/DEL	0	

MUT	**ALK**	**N.**	NOTE
TYPE	SNV	0	
	INDEL	1	
CLINICAL VAR		1	PATHOGENIC
VARIANT EFFECT	MISSENSE	0	
	NONSENSE	0	
	FRAMESCHIFT/DEL	1	

Comparing the detected mutated genes in breast biopsy and matched surgical sample, we found an increase number of PIK3CA, TP53, MET, NOTCH1, FGFR3, and PTEN mutations in the residual breast cancer tissue (Fig. 18).

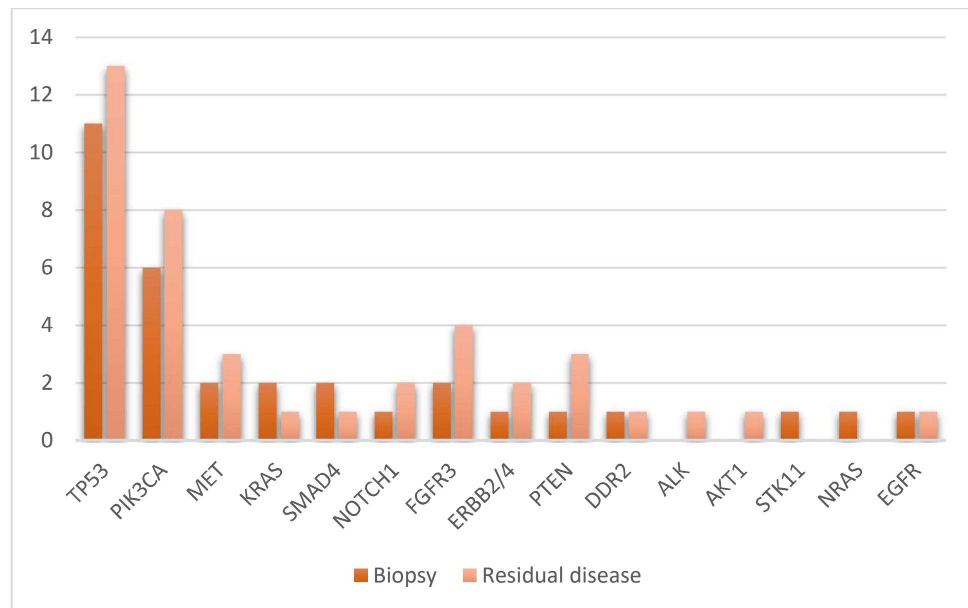


Figure 18 *Biopsy versus surgery tissue detected mutated genes.*

5.4 Prognostic value of detected gene mutations

We performed a survival analysis in term of relapse free survival (RFS) considering the mutational burden of disease, detected genes mutations and the treatment induced mutation in the residual disease. Survival analysis in term of overall survival was not perform because of the few number of accorded deaths at the time of the analysis, all in the subgroup of No pCR patients.

Considering the RSF in No-pCR group versus pCR group, as expected patients who achieved the pCR at the end of the NACT had significantly lower risk of relapse compared to patients with residual disease (p value 0.037 Log-rank test; 25% vs 6%, respectively) (Fig 19).

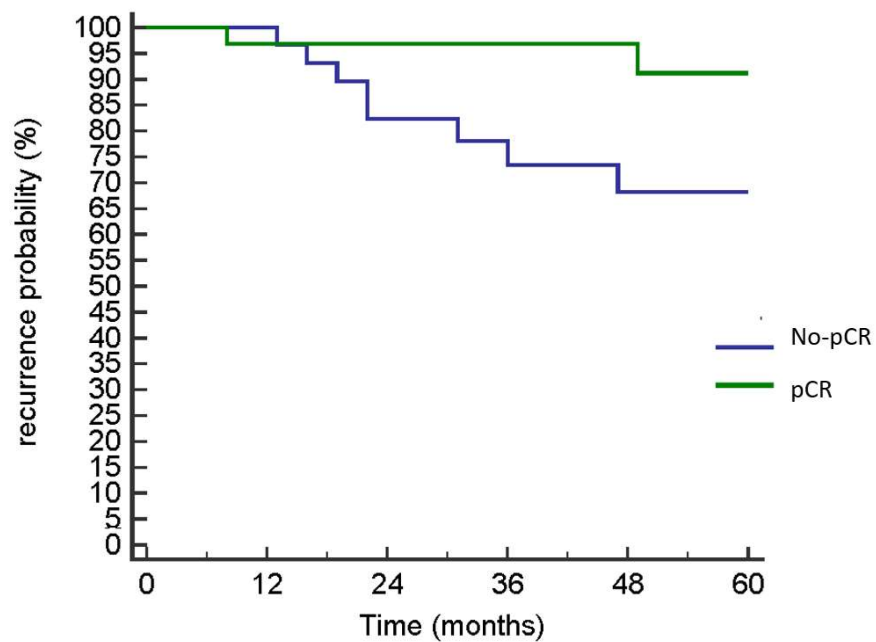


Figure 19 *Kaplan-Meier curves for Relapse Free Survival according to pCR. p value Log-rank test*

No statistically significant difference according to mutational burden of disease detected in the diagnostic biopsy have been found. In particular, there were no differences between patients with no detected mutations and patients with at least one gene mutation (33% versus 10%, respectively, p value 0.119 Log-rank test) (Fig. 20) as well as not significant difference among patients with zero or one mutation and those with two or more mutations (11% versus 18%, respectively, p value 0.35 Log-rank test) (Fig. 21).

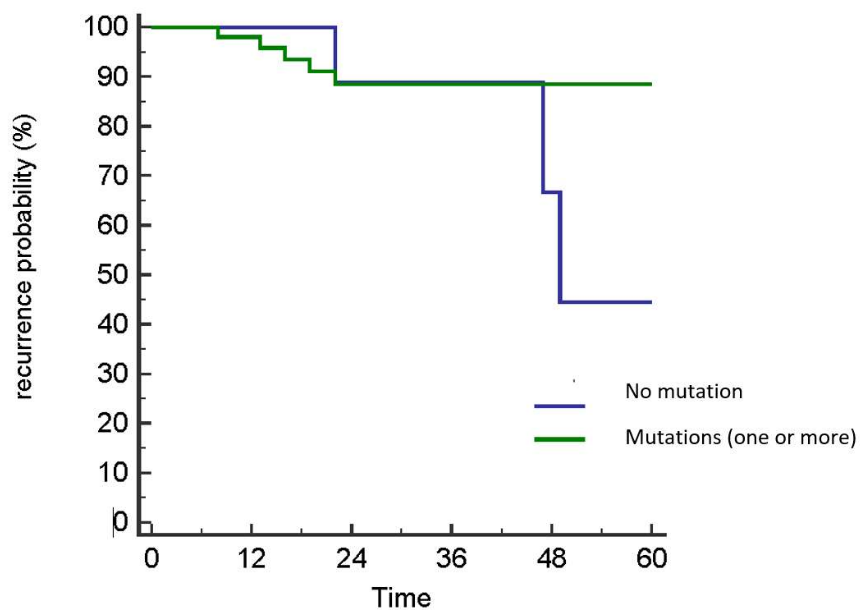


Figure 20 *Kaplan-Meier curves for Relapse Free Survival according to the number of mutations (no mutation vs at least one detected mutation)*

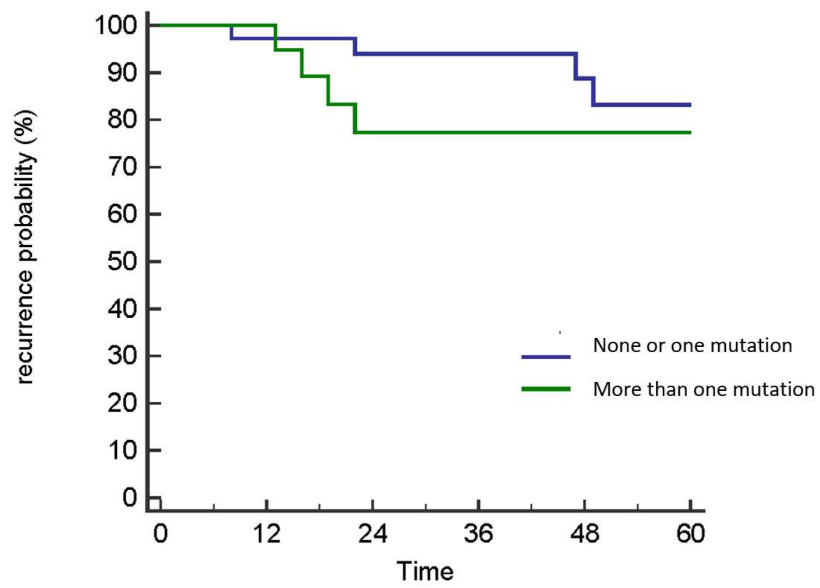


Figure 21 *Kaplan-Meier curves for Relapse Free Survival according to the number of mutations (none or one mutation vs more than one detected mutation)*

No difference in term of RFS among patients with TP53 and/or PIK3CA mutations versus those without these gene mutations on the biopsy tissue were funded (Fig. 22).

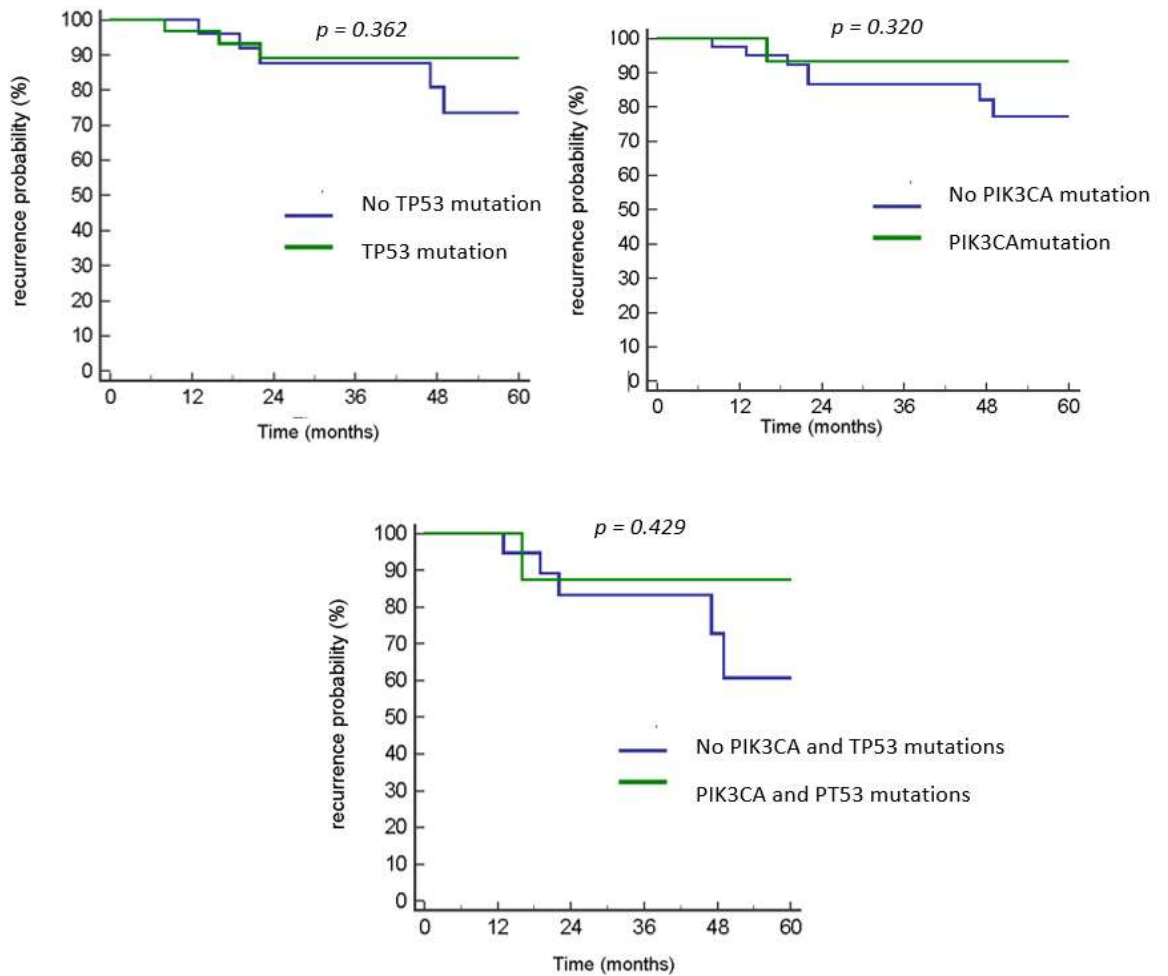


Figure 22 *Kaplan-Meier curves for Relapse Free Survival according to TP53 and PIK3CA status in the breast cancer biopsy, (p value log-rank test)*

Regarding the mutational profile of the residual breast cancer disease, the detection of TP53 or PIK3CA mutations did not significantly influence the risk of relapse (Fig. 23).

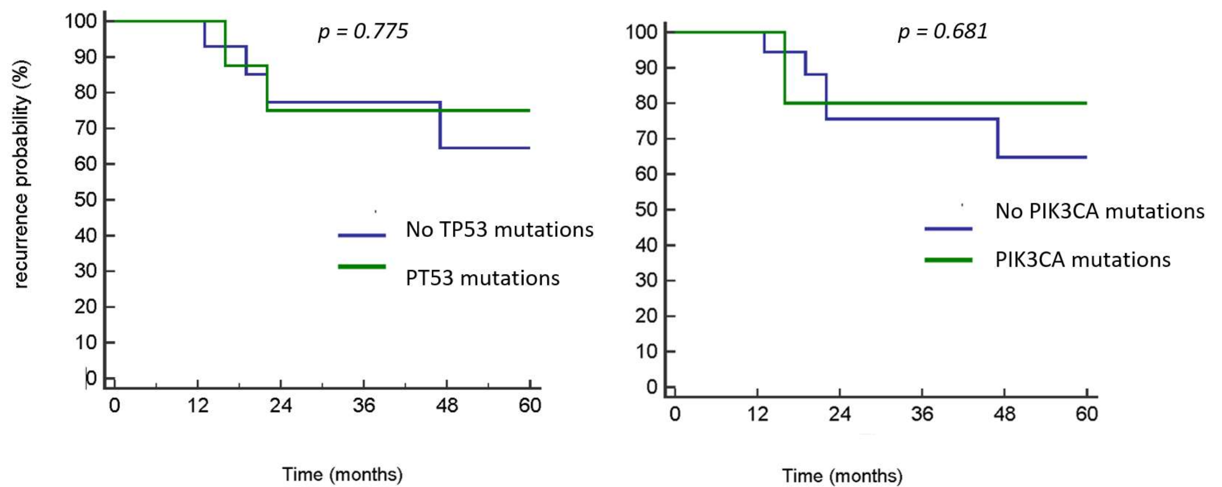


Figure 23 *Kaplan-Meier curves for Relapse Free Survival according to TP53 and PIK3CA status in the residual breast cancer.(p value log-rank test)*

On the contrary, changes in the gene mutational profile during the NACT treatment seems to influence the risk of relapse. In particular, patients with different mutational profile between diagnostic biopsy and matched residual breast cancer had a significantly higher risk of recurrence compared to patients with no treatment induced gene modifications. In fact, all the recurrence occurred in the subgroup of patients with different gene status after NACT. Forty-two per cent of patients with treatment induced gene changes relapsed (p value 0.019 Log-rank test) (Fig. 24). In particular, the increase of the mutational tumor burden seemed to be mostly involved in the risk of relapse (Fig. 25). All the recurrences were in the subgroup of patients with treatment-induced genes mutations.

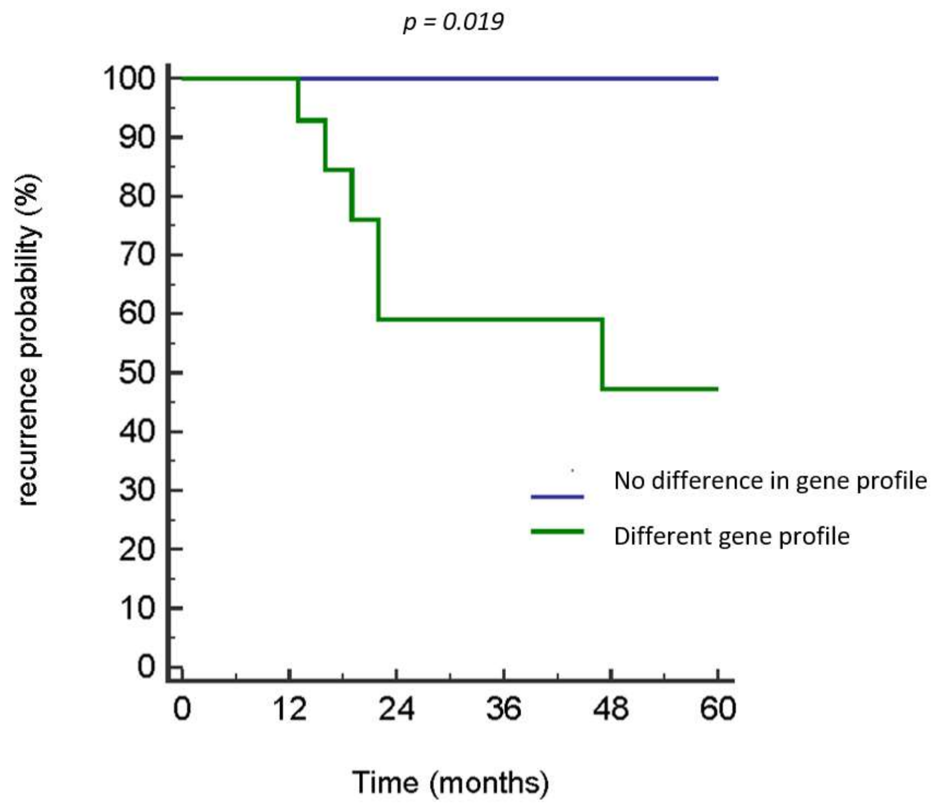


Figure 24 *Kaplan-Meier curves for Relapse Free Survival according to modification in breast cancer mutational profile pre versus post NACT (p value Log-rank test)*

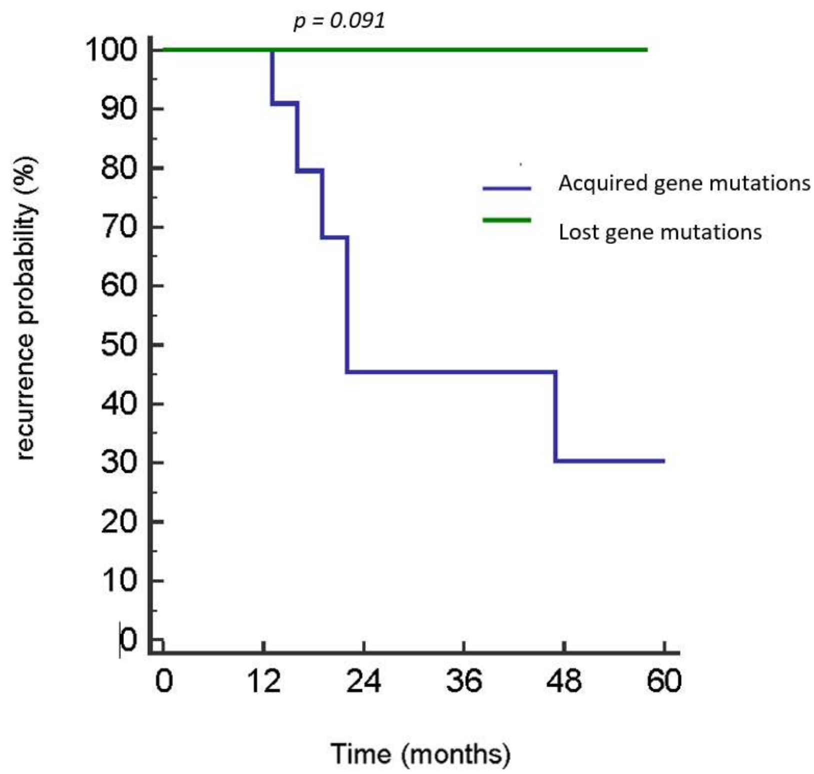


Figure 25 *Kaplan-Meier curves for Relapse Free Survival for subgroup of patients with acquired gene mutations versus lost gene mutations, (p value Log-rank test)*

6 Discussion

Breast cancer is a dynamic and heterogeneous entity. New gene analysis technology showed that breast cancer is not a single entity, but there are several breast cancers with different genomic profiles reflecting in different treatment sensitivity and survival outcomes [72]. Moreover, during cancer progression tumours acquired genomic mutations able to influence treatment sensitivity too [49]. The heterogeneity of the tumors, exacerbated by the selective pressures imposed by the chemo and targeted therapy, confers a major resistance to anti-cancer treatments and radiation therapy [49]. In this context, emerging evidence from NACT trials showed how the residual tumor genetic landscape is largely involved in anticancer sensitivity/resistance mechanisms [73].Therefore, there is an urgent need to understand cancer biology in order to predict resistance to systemic primary therapies as well as to adjuvant treatment in patients with NACT resistant tumors. Proper understanding of the breast cancer mutational landscape may lead to a personalized systemic treatment selection. Moreover, the identification of driver gene mutations may lead to the development of new targeted treatments too. Against this backdrop, we evaluated a panel of 21 genes involved in treatment resistance, comparing cancer samples, selected on the bases of treatment sensitivity (No pCR versus pCR group), and taken from the breast cancer diagnostic biopsy and residual tumor after NACT. The study allowed for the identification of mutated genes on both diagnostic biopsy and residual disease able to predict treatment resistance and survival outcomes.

Considering results from the breast cancer diagnostic biopsy analysis, the detection rate of mutations was 79% in No-pCR group versus 90% in pCR one. The high rate of mutations underlines that the analysed genes were highly involved in breast cancer tumorigenesis. Mutations on these genes appears in the first steps of cancer progressions. The most frequently mutated genes were TP53 and PIK3CA in both groups of patients. The detected mutation rate in our study population was similar to those reported in literature (30% for PIK3CA and 50% for TP53) [22, 67]. In particular, mutations of PIK3CA were present in 27% of patients, similar between pCR and No pCR subgroup (27% vs 28%). On the contrary, TP53 mutations were overall present in 55% of diagnostic breast cancer biopsy, mainly in pCR subgroup (67% vs 43%). This finding confirms a higher rate of pCR in TP53 mutated patients according to other published researches [14]. Even if the TP53 status seems to be a predictor for pCR, survival studies showed worse outcomes in TP53-mutated patients [14]. This evidence may depend by the fact that TP53 induces arrest and senescence instead of apoptosis. Several pre-clinical evidence have shown that senescent cells drive relapse by producing cytokines that promote proliferation, survival, angiogenesis and increase in cancer stem cells population [74]. In our analysis, no correlation between TP53 status and risk of relapse/death have been found likely due to the high rate of TP53 mutated tumors and the low rate of relapse in the study population enrolled. Regarding PIK3CA mutations, preclinical evidence suggest that mutated cancer cells have an abnormal pathway activation, which lead to resistance to trastuzumab [41]. A meta-analysis on the role of PIK3CA mutations and response to NACT published in

2018 confirmed these results [67]. In particular, in unselected HER2 positive breast cancer, PIK3CA mutation seems to play a relevant role in chemo sensitivity, but its role is less clear in patients selected according to hormone receptors status. In fact, the pCR rate was significantly correlated to PIK3CA status in hormone receptors positive - HER2 positive breast cancer but not in hormone receptors negative patients [67]. This association suggests a potential strong interaction between hormone receptors and HER2 pathways. Looking at our pCR subgroup, only 7 women had hormone receptors positive breast cancer. This may justify the lack of significant correlation between PIK3CA mutation and pCR rate in our study population. Moreover, the absence of significant correlation between PIK3CA status and survival outcome may depend to the relatively small sample size of hormone receptors positive tumors with a relatively low recurrence rate too.

Considering residual breast cancer disease, it is well clear that NACT is able to change the tumor mutational profile. Since the presence of residual tumor after NACT confers an increased risk of recurrence, a better characterization of these patients is necessary [64]. Our finding showed a potential prognostic value of treatment induced mutational burden modifications. Changes in the residual breast cancer mutational profile (acquired or lost mutations) were negative prognostic factors in term of relapse free survival in our study population. In particular, patients with a different mutational profile between diagnostic biopsy and residual disease had a significantly higher risk of relapse compared to those without gene modifications. Overall, the total number of detected mutations was increased from the matched breast cancer biopsy to the surgical specimen (32 vs

41 respectively) as well as the number of patients with at least one detected mutations (76% vs 92%). More than half of the patients changed the mutational profile during the neoadjuvant treatment, 69% increased the number of the detected gene mutations in the surgical tissue compared to the matched diagnostic biopsy. In two cases the mutational profile of residual tumor was completed different from the primary breast cancer biopsy one. Both these patients early relapsed. In particular, even if not statistically significant, the acquisition of new mutations compared to the loss seems to increase the risk of relapse. In fact, all the recurrence occurred in the subgroup of patients with an increased mutational burden in the residual tumor compared to the pre-treatment breast cancer sample. This finding can be explain by the selective pressure of the NCACT on breast cancer cells. In resistant cancer cells, therapy induced gene mutations able to confer a more aggressive tumor behaviour, reflecting in worse survival outcomes. The observations of the actual research could be useful in this context, where NACT will be more and more used to select the best post-operative treatment. Our results suggested that patients with a change in mutational profile could be better candidate for an alternative adjuvant treatment, whereas patients maintaining the same molecular profile could stay on the same treatment used before surgery.

Considering single genes status, TP53 and PIK3CA mutations were the most detected in residual breast cancer too. Both mutations did not show a prognostic value. The adjuvant treatment administered after NACT could be responsible for the lack of significance. Nowadays in clinical practice, all the patients underwent

NACT received the same adjuvant treatment (such as trastuzumab) regardless the residual disease and the gene mutational profile. From the clinical point of view, administered adjuvant treatment according to tumor response to chemotherapy showed significant survival outcomes. Recent evidence from the phase III Katherine trial showed how modulating adjuvant treatment according to tumor response gave a reduction in 50% in term of invasive breast cancer recurrence and death [48]. Moreover, the treatment selection based on tumor mutational profile gave promising results too. Data from clinical trials conducted in the metastatic setting showed that mutations in driver genes such as PIK3CA were able to predict treatment response [75]. For example, patients with constitutive activation of PI3K/AKT pathway, via mutations in PIK3CA or through the loss of PTEN, had resistance to trastuzumab, lapatinib, and pertuzumab-containing therapies but not to TDM-1 [35, 46, 50]. Moreover, patient with hyperactivation of PI3K pathway could have a PFS benefit from everolimus in association with trastuzumab and chemotherapy [76]. For that reason, a personalized adjuvant strategy may underline the predictive and prognostic value of the single gene mutation.

Overall, the lack in the identification of a prognostic and/or predictive mutational gene profile may be justified by the complexity of the tumor biology. Knowledge on cancer progression suggested that the carcinogenesis is moved by multiple gene mutations that generate changes in several molecular pathways involved in cell survival [77]. Abnormalities in DNA methylation, microRNA and protein expression increased the molecular architecture of breast cancer too[77].

For that reason, studies on breast cancer biology need not only a single level (single gene mutations) analysis but also a RNA, protein level and epigenetic modification studies. Furthermore, tissue sample from breast cancer biopsy may be different from the liquid biopsy sample one. Liquid biopsy from patient's plasma gives gene information through the analyses of CTCs, ctDNA and exosomes [78]. Studies with both, tissue and blood samples, may better describe the complex biology of breast cancer through the identification of driver gene mutations in all these levels. Knowledge in this field still represent the goal for a personalized treatment strategy. We strongly think that choose a neo/adjuvant treatment based on a multistep gene mutational profile analysis may select patients based on treatment sensitivity, improving survival outcomes. This is a research area in which more studies are clearly needed.

In spite of our finding, this study presents several limitations and must be considered hypothesis-generating. Firstly, this is a retrospective study with a small sample size. Secondary, due to the retrospective nature of our analysis, samples were fixed and processed for storage in different periods and by different technicians, with no purpose of genomic analysis. This variability might have reduced the quality and preservation of some tissues, increasing the rate of variants detected in some of the samples.

7 Conclusion

Survival benefit in early breast cancer patients is strictly linkable to the improvement in genomic breast cancer profile knowledge. Findings from our research confirm that mutations on driver genes are present from the first steps of breast cancer carcinogenesis and/or may arise during cancer treatment. In particular, during NACT breast cancer may acquire gene mutations able to confer resistance to anti-cancer systemic therapy. The selective pressures imposed by chemo and targeted therapies change the mutational profile of the majority of residual breast cancer. Our finding demonstrated that patients with changes in the residual tumor mutational profile, as both gains and losses of mutated genes, have an increased risk of relapse. For that reason, a better characterization of these patients is necessary. The identifications of targetable treatment based on the detected mutated genes may contribute to selected patients for different adjuvant treatment. Profiling breast cancer sample before any intervention and after treatment is the first critical step in the precision medicine era.

REFERENCE

1. Momenimovahed, Z. and H. Salehiniya, *Epidemiological characteristics of and risk factors for breast cancer in the world.* Breast Cancer (Dove Med Press), 2019. **11**: p. 151-164.
2. Benson, J.R. and I. Jatoi, *The global breast cancer burden.* Future Oncol, 2012. **8**(6): p. 697-702.
3. Sun, Y.S., et al., *Risk Factors and Preventions of Breast Cancer.* Int J Biol Sci, 2017. **13**(11): p. 1387-1397.
4. Dall, G.V. and K.L. Britt, *Estrogen Effects on the Mammary Gland in Early and Late Life and Breast Cancer Risk.* Front Oncol, 2017. **7**: p. 110.
5. Horn, J., et al., *Reproductive factors and the risk of breast cancer in old age: a Norwegian cohort study.* Breast Cancer Res Treat, 2013. **139**(1): p. 237-43.
6. Hamajima, N., et al., *Alcohol, tobacco and breast cancer--collaborative reanalysis of individual data from 53 epidemiological studies, including 58,515 women with breast cancer and 95,067 women without the disease.* Br J Cancer, 2002. **87**(11): p. 1234-45.
7. Brownlee, Z., et al., *Late complications of radiation therapy for breast cancer: evolution in techniques and risk over time.* Gland Surg, 2018. **7**(4): p. 371-378.
8. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
9. Lin, N.U. and E.P. Winer, *New targets for therapy in breast cancer: small molecule tyrosine kinase inhibitors.* Breast Cancer Res, 2004. **6**(5): p. 204-10.
10. Moasser, M.M., *Targeting the function of the HER2 oncogene in human cancer therapeutics.* Oncogene, 2007. **26**(46): p. 6577-92.
11. Moasser, M.M., *The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis.* Oncogene, 2007. **26**(45): p. 6469-87.
12. https://clinicaltrials.gov/ct2/show/NCT03944434. *First line ribociclib in combination with a non steroidal aromatase inhibitor in women or men aged 70 years-old or older, with hormone receptor positive/HER2 negative advanced breast cancer NCT03944434*
13. Marino, M., P. Galluzzo, and P. Ascenzi, *Estrogen signaling multiple pathways to impact gene transcription.* Curr Genomics, 2006. **7**(8): p. 497-508.
14. Abubakar, M., et al., *Clinicopathological and epidemiological significance of breast cancer subtype reclassification based on p53 immunohistochemical expression.* NPJ Breast Cancer, 2019. **5**: p. 20.
15. Vivanco, I. and C.L. Sawyers, *The phosphatidylinositol 3-Kinase AKT pathway in human cancer.* Nat Rev Cancer, 2002. **2**(7): p. 489-501.
16. Yang, J., et al., *Targeting PI3K in cancer: mechanisms and advances in clinical trials.* Mol Cancer, 2019. **18**(1): p. 26.
17. Li, S., et al., *Loss of PTEN expression in breast cancer: association with clinicopathological characteristics and prognosis.* Oncotarget, 2017. **8**(19): p. 32043-32054.
18. McCubrey, J.A., et al., *Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance.* Biochim Biophys Acta, 2007. **1773**(8): p. 1263-84.

19. Park, M.T. and S.J. Lee, *Cell cycle and cancer.* J Biochem Mol Biol, 2003. **36**(1): p. 60-5.
20. Sandal, T., *Molecular aspects of the mammalian cell cycle and cancer.* Oncologist, 2002. **7**(1): p. 73-81.
21. Johnson, J., et al., *Targeting the RB-E2F pathway in breast cancer.* Oncogene, 2016. **35**(37): p. 4829-35.
22. Gasco, M., S. Shami, and T. Crook, *The p53 pathway in breast cancer.* Breast Cancer Res, 2002. **4**(2): p. 70-6.
23. Schlessinger, J., *Common and distinct elements in cellular signaling via EGF and FGF receptors.* Science, 2004. **306**(5701): p. 1506-7.
24. Folkman, J., *Role of angiogenesis in tumor growth and metastasis.* Semin Oncol, 2002. **29**(6 Suppl 16): p. 15-8.
25. Torgovnick, A. and B. Schumacher, *DNA repair mechanisms in cancer development and therapy.* Front Genet, 2015. **6**: p. 157.
26. Powell, S.N. and L.A. Kachnic, *Roles of BRCA1 and BRCA2 in homologous recombination, DNA replication fidelity and the cellular response to ionizing radiation.* Oncogene, 2003. **22**(37): p. 5784-91.
27. Thomas, S.J., et al., *The role of JAK/STAT signalling in the pathogenesis, prognosis and treatment of solid tumours.* Br J Cancer, 2015. **113**(3): p. 365-71.
28. Senkus, E., et al., *Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up.* Ann Oncol, 2015. **26 Suppl 5**: p. v8-30.
29. Menard, S., et al., *HER2 as a prognostic factor in breast cancer.* Oncology, 2001. **61 Suppl 2**: p. 67-72.
30. Slamon, D.J., et al., *Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2.* N Engl J Med, 2001. **344**(11): p. 783-92.
31. Slamon, D., et al., *Adjuvant trastuzumab in HER2-positive breast cancer.* N Engl J Med, 2011. **365**(14): p. 1273-83.
32. Swain, S.M., et al., *Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer.* N Engl J Med, 2015. **372**(8): p. 724-34.
33. Park, J.W., et al., *Anti-p185HER2 monoclonal antibodies: biological properties and potential for immunotherapy.* Cancer Treat Res, 1992. **61**: p. 193-211.
34. Vogel, C.L., et al., *Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer.* J Clin Oncol, 2002. **20**(3): p. 719-26.
35. Hudis, C.A., *Trastuzumab--mechanism of action and use in clinical practice.* N Engl J Med, 2007. **357**(1): p. 39-51.
36. Klapper, L.N., et al., *Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2.* Cancer Res, 2000. **60**(13): p. 3384-8.
37. Clynes, R.A., et al., *Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets.* Nat Med, 2000. **6**(4): p. 443-6.
38. Arnould, L., et al., *Trastuzumab-based treatment of HER2-positive breast cancer: an antibody-dependent cellular cytotoxicity mechanism?* Br J Cancer, 2006. **94**(2): p. 259-67.
39. Junttila, T.T., et al., *Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941.* Cancer Cell, 2009. **15**(5): p. 429-40.

40. Nagata, Y., et al., *PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients.* Cancer Cell, 2004. **6**(2): p. 117-27.
41. Vu, T. and F.X. Claret, *Trastuzumab: updated mechanisms of action and resistance in breast cancer.* Front Oncol, 2012. **2**: p. 62.
42. Ishii, K., N. Morii, and H. Yamashiro, *Pertuzumab in the treatment of HER2-positive breast cancer: an evidence-based review of its safety, efficacy, and place in therapy.* Core Evid, 2019. **14**: p. 51-70.
43. von Minckwitz, G., et al., *Adjuvant Pertuzumab and Trastuzumab in Early HER2-Positive Breast Cancer.* N Engl J Med, 2017. **377**(2): p. 122-131.
44. Schneeweiss, A., et al., *Pertuzumab plus trastuzumab in combination with standard neoadjuvant anthracycline-containing and anthracycline-free chemotherapy regimens in patients with HER2-positive early breast cancer: a randomized phase II cardiac safety study (TRYPHAENA).* Ann Oncol, 2013. **24**(9): p. 2278-84.
45. Gianni, L., et al., *Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial.* Lancet Oncol, 2012. **13**(1): p. 25-32.
46. Barok, M., H. Joensuu, and J. Isola, *Trastuzumab emtansine: mechanisms of action and drug resistance.* Breast Cancer Res, 2014. **16**(2): p. 209.
47. Verma, S., et al., *Trastuzumab emtansine for HER2-positive advanced breast cancer.* N Engl J Med, 2012. **367**(19): p. 1783-91.
48. von Minckwitz, G., et al., *Trastuzumab Emtansine for Residual Invasive HER2-Positive Breast Cancer.* N Engl J Med, 2019. **380**(7): p. 617-628.
49. Angus, L., et al., *The genomic landscape of metastatic breast cancer highlights changes in mutation and signature frequencies.* Nat Genet, 2019. **51**(10): p. 1450-1458.
50. D'Amato, V., et al., *Mechanisms of lapatinib resistance in HER2-driven breast cancer.* Cancer Treat Rev, 2015. **41**(10): p. 877-83.
51. Segovia-Mendoza, M., et al., *Efficacy and mechanism of action of the tyrosine kinase inhibitors gefitinib, lapatinib and neratinib in the treatment of HER2-positive breast cancer: preclinical and clinical evidence.* Am J Cancer Res, 2015. **5**(9): p. 2531-61.
52. Geyer, C.E., et al., *Lapatinib plus capecitabine for HER2-positive advanced breast cancer.* N Engl J Med, 2006. **355**(26): p. 2733-43.
53. Pernas, S. and S.M. Tolaney, *HER2-positive breast cancer: new therapeutic frontiers and overcoming resistance.* Ther Adv Med Oncol, 2019. **11**: p. 1758835919833519.
54. Pohlmann, P.R., I.A. Mayer, and R. Mernaugh, *Resistance to Trastuzumab in Breast Cancer.* Clin Cancer Res, 2009. **15**(24): p. 7479-7491.
55. Ferris, R.L., E.M. Jaffee, and S. Ferrone, *Tumor antigen-targeted, monoclonal antibody-based immunotherapy: clinical response, cellular immunity, and immunoescape.* J Clin Oncol, 2010. **28**(28): p. 4390-9.
56. Lee, A.V., X. Cui, and S. Oesterreich, *Cross-talk among estrogen receptor, epidermal growth factor, and insulin-like growth factor signaling in breast cancer.* Clin Cancer Res, 2001. **7**(12 Suppl): p. 4429s-4435s; discussion 4411s-4412s.
57. Scaltriti, M., et al., *Expression of p95HER2, a truncated form of the HER2 receptor, and response to anti-HER2 therapies in breast cancer.* J Natl Cancer Inst, 2007. **99**(8): p. 628-38.

58. Castiglioni, F., et al., *Role of exon-16-deleted HER2 in breast carcinomas.* Endocr Relat Cancer, 2006. **13**(1): p. 221-32.
59. Luque-Cabal, M., et al., *Mechanisms Behind the Resistance to Trastuzumab in HER2-Amplified Breast Cancer and Strategies to Overcome It.* Clin Med Insights Oncol, 2016. **10**(Suppl 1): p. 21-30.
60. Majewski, I.J., et al., *PIK3CA mutations are associated with decreased benefit to neoadjuvant human epidermal growth factor receptor 2-targeted therapies in breast cancer.* J Clin Oncol, 2015. **33**(12): p. 1334-9.
61. Hamel, S., et al., *Both t-Darpp and DARPP-32 can cause resistance to trastuzumab in breast cancer cells and are frequently expressed in primary breast cancers.* Breast Cancer Res Treat, 2010. **120**(1): p. 47-57.
62. Scaltriti, M., et al., *Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2+ breast cancer patients.* Proc Natl Acad Sci U S A, 2011. **108**(9): p. 3761-6.
63. DiGiovanna, M.P., et al., *Relationship of epidermal growth factor receptor expression to ErbB-2 signaling activity and prognosis in breast cancer patients.* J Clin Oncol, 2005. **23**(6): p. 1152-60.
64. Cortazar, P., et al., *Pathological complete response and long-term clinical benefit in breast cancer: the CTNeoBC pooled analysis.* Lancet, 2014. **384**(9938): p. 164-72.
65. Early Breast Cancer Trialists' Collaborative, G., *Long-term outcomes for neoadjuvant versus adjuvant chemotherapy in early breast cancer: meta-analysis of individual patient data from ten randomised trials.* Lancet Oncol, 2018. **19**(1): p. 27-39.
66. Atalay, G., et al., *Novel therapeutic strategies targeting the epidermal growth factor receptor (EGFR) family and its downstream effectors in breast cancer.* Ann Oncol, 2003. **14**(9): p. 1346-63.
67. Sobhani, N., et al., *The prognostic value of PI3K mutational status in breast cancer: A meta-analysis.* J Cell Biochem, 2018. **119**(6): p. 4287-4292.
68. Omarini, C., et al., *Differential molecular pathways expression in HER2 positive early breast cancer according to hormone receptor status.* J Cancer Res Clin Oncol, 2019. **145**(4): p. 821-828.
69. Liu, N., et al., *SMAD4 expression in breast ductal carcinoma correlates with prognosis.* Oncol Lett, 2015. **10**(3): p. 1709-1715.
70. Gao, C., et al., *Exon 3 mutations of CTNNB1 drive tumorigenesis: a review.* Oncotarget, 2018. **9**(4): p. 5492-5508.
71. Yeh, C.H., M. Bellon, and C. Nicot, *FBXW7: a critical tumor suppressor of human cancers.* Mol Cancer, 2018. **17**(1): p. 115.
72. Engstrom, M.J., et al., *Molecular subtypes, histopathological grade and survival in a historic cohort of breast cancer patients.* Breast Cancer Res Treat, 2013. **140**(3): p. 463-73.
73. Toss, A. and M. Cristofanilli, *Molecular characterization and targeted therapeutic approaches in breast cancer.* Breast Cancer Res, 2015. **17**: p. 60.
74. Rao, S.G. and J.G. Jackson, *SASP: Tumor Suppressor or Promoter? Yes!* Trends Cancer, 2016. **2**(11): p. 676-687.
75. Vogelstein, B. and K.W. Kinzler, *Cancer genes and the pathways they control.* Nat Med, 2004. **10**(8): p. 789-99.
76. Baselga, J., et al., *Relationship between Tumor Biomarkers and Efficacy in EMILIA, a Phase III Study of Trastuzumab Emtansine in HER2-Positive Metastatic Breast Cancer.* Clin Cancer Res, 2016. **22**(15): p. 3755-63.

77. Pantel, K. and R.H. Brakenhoff, *Dissecting the metastatic cascade.* Nat Rev Cancer, 2004. **4**(6): p. 448-56.
78. Alimirzaie, S., M. Bagherzadeh, and M.R. Akbari, *Liquid biopsy in breast cancer: A comprehensive review.* Clin Genet, 2019. **95**(6): p. 643-660.