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CICLO XXII

Evaluation of adaptive and innate immune mechanisms of action of Pertuzumab plus Trastuzumab against HER2-positive breast cancer

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1. Riassunto

I linfociti infiltranti il tumore (TILs) sono associati ad una maggiore efficacia terapeutica della terapia neoadiuvante basata su trastuzumab / pertuzumab in pazienti con carcinoma mammario HER2-positivo. Il trastuzumab sottocutaneo (SC) ha dimostrato un'efficacia non inferiore rispetto alla somministrazione endovenosa (EV), con un profilo di sicurezza simile. È interessante notare come il trastuzumab SC è stato osservato essere più immunogeno del trastuzumab EV e agire a diversi livelli immunologici. Pertanto, modificando la modalità di somministrazione di trastuzumab, si potrebbe interferire sul sistema immunitario a diversi livelli ed esercitare un'immunomodulazione favorevole nel tumore mammario HER2-positivo.

In questo studio non comparativo, di fase II, randomizzato, nel setting neoadiuvante, erano eleggibili pazienti con diagnosi di carcinoma mammario HER2-positivo, istologicamente confermato, non precedentemente trattato, in stadio localmente avanzato, infiammatorio o iniziale. Le pazienti sono state inizialmente trattate con chemioterapia a base di 5-fluoruracile, epirubicina e ciclofosfamide (FEC) per 3 cicli. Quindi sono state assegnate in modo casuale in un rapporto 1: 1 a ricevere docetaxel + pertuzumab EV + trastuzumab EV per 4 cicli (braccio A) o docetaxel + pertuzumab EV + trastuzumab SC per 4 cicli (braccio B). Dopo l'intervento chirurgico, tutte le pazienti hanno ricevuto trastuzumab per 14 cicli utilizzando la stessa formulazione (SC o EV) della fase preoperatoria. L'endpoint primario dello studio era il tasso di TILs stromali (sTILs) nella malattia residua dopo intervento chirurgico. I campioni chirurgici della biopsia iniziale sul tumore primitivo e post-trattamento sono stati analizzati per quantificare i TILs. Sono stati inoltre raccolti dei campioni di sangue periferico, a tre punti-tempo predeterminati durante la terapia neoadiuvante, per l'analisi della concentrazione e dell'attività delle cellule linfocitarie tumore-specifiche. Fattibilità, efficacia e sicurezza dello studio sono infine state valutate.

Tra novembre 2016 e settembre 2017, secondo un progetto in due fasi di Simon, abbiamo arruolato 65 pazienti, di cui due ritenute non ammissibili per lo studio. Pertanto sono state valutate complessivamente 63 pazienti (31 nel braccio A e 32 nel braccio B) per l'analisi degli endpoints primari e secondari. I tassi di risposta patologica completa ottenuti dopo trattamento neoadiuvante sono stati del 64,5% nel braccio A e del 59,4% nel braccio B, rispettivamente. Gli eventi avversi più comuni di grado 3 o superiore sono stati neutropenia (15 [48,4%] pazienti nel braccio A e 11 [34,4%] nel braccio B), neurotossicità (1 [3,2%] e 2 [6,2%], rispettivamente) e diarrea (1 [3,2%] e 1 [3,1%], rispettivamente). Non ci sono stati casi di insufficienza cardiaca congestizia. Durante l'intervento chirurgico, 11 pazienti nel braccio A e 13 pazienti nel braccio B sono risultate valutabili per l'analisi dei TILs. Come parametro di cut-off è stato utilizzato il valore mediano dei sTILs (7,5%) riscontrato nelle biopsie tumorali pre-trattamento e sono stati osservati alti livelli di sTILs nel 27,3% e nel 46,1% dei tumori residui dopo trattamento neoadiuvante nei bracci A e B, rispettivamente. È interessante notare come sia emersa una correlazione inversa significativa tra l'espressione di PD-L1 sui sTILs prima del trattamento neoadiuvante e il co-recettore CD3 delle cellule T espresso su sTILs post-trattamento (ρ = -0,70 di Pearson). Questa correlazione è risultata particolarmente evidente nel braccio B (ρ = -0,85).

La terapia neoadiuvante con trastuzumab SC o EV in associazione con pertuzumab e chemioterapia ha dimostrato un effetto significativo sull'espressione di sTILs su tessuto tumorale dopo intervento chirurgico. In particolare il braccio B, a cui è stato somministrato il trastuzumab SC, ha mostrato sia un più rilevante arricchimento di sTILs nel residuo tumorale post-trattamento, sia un più significativo incremento delle cellule CD3 su sangue periferico.

I risultati ottenuti suggeriscono un ruolo della somministrazione sottocutanea di anticorpi anti-HER2 nel determinare variazioni immunofenotipiche favorevoli della risposta immunitaria dell'ospite nelle pazienti affette da tumore mammario HER2-positivo in stadio iniziale con residuo di malattia dopo trattamento neoadiuvante.

1. Abstract

Tumor-infiltrating lymphocytes (TILs) have been reported to be associated with increased therapeutic efficacy of trastuzumab/pertuzumab-based neoadjuvant therapy (NT) in patients (pts) with HER2-positive breast cancer (BC). Subcutaneous (SC) trastuzumab has non-inferior efficacy to intravenous (IV) administration, with a similar safety profile. Interestingly, SC trastuzumab has been observed to be more immunogenic than IV trastuzumab and act at different immunologic levels. Therefore, by modifying the modality of administration of trastuzumab, it could be possible to interfere with different immune pathways and exert a favorable immunomodulation in HER2-positive BC.

In this non-comparative, phase II, neoadjuvant, randomized study, patients were eligible if they had previously untreated, histologically confirmed, locally advanced, inflammatory, or early-stage HER2-positive BC. Patients were treated with 5-fluoruracil, epirubicin, cyclophosphamide (FEC) chemotherapy for 3 cycles. Then they were randomly assigned in a 1:1 ratio to receive docetaxel + pertuzumab IV + trastuzumab IV for 4 cycles (arm A) or docetaxel + pertuzumab IV + trastuzumab SC for 4 cycles (arm B). Post-surgery, all patients received trastuzumab for 14 cycles using the same formulation (SC or IV) of the preoperative phase. The primary endpoint was the rate of stromal TILs (sTILs) on residual disease after surgery. Tumor biopsy and post-treatment surgical samples were centrally analyzed for TILs. Blood samples were also collected during NT for tumor-specific lymphocyte cell activity analysis. Feasibility, efficacy and safety were also evaluated.

Between November 2016 and September 2017, according to an adaptive Simon's two-stage optimal design, we enrolled 65 pts, of whom two were deemed ineligible for the study. Thus, 63 patients (31 in arm A and 32 in arm B) were assessed for the primary and secondary endpoints. The pathologic complete response (pCR; no invasive tumor in breast and axilla) rates were 64.5% in arm A, and 59.4% in arm B. The most common adverse events of grade 3 or higher were neutropenia (15 [48.4%] patients in arm A, and 11 [34.4%]

in arm B), neurotoxicity (1 [3.2%], and 2 [6.2%], respectively), and diarrhea (1 [3.2%], and 1 [3.1%], respectively). There were no events of congestive heart failure. At surgery, 11 patients in arm A and 13 patients in arm B were evaluable for TIL analysis. The median value of sTILs (7.5%) on pre-treatment tumor biopsies was used as the cut-off value, and high sTIL levels were observed in 27.3% and in 46.1% of residual tumors after treatment arm A and B, respectively. Interestingly, a significant inverse correlation was observed between PD-L1 expression on pretreatment sTILs and the T cell co-receptor CD3 expressed on post-treatment sTILs (Pearson's $\rho = -0.70$). This finding was particularly evident in the arm B group ($\rho = -0.85$).

NT with either SC or IV trastuzumab in combination with pertuzumab and chemotherapy had a significant effect on sTIL expression at surgery. In particular, the SC trastuzumab-based arm exerted the most relevant enrichment of sTILS in post-treatment residual tumors and a greater rise of CD3 cells on peripheral good. These findings suggest a role for the SC administration of anti-HER2 mAbs in determining favorable variations of host immune response parameters among patients with HER2-positive early BC who had residual disease after NT.

3. Introduction

3.1 Epidemiologic and histopathologic aspect of breast cancer

3.1.1 Epidemiology

Breast cancer (BC) is the most frequently diagnosed cancer in women and it is the second most common cause of cancer-related death in women worldwide[1]. Approximately 1 in 8 women worldwide have a lifetime risk of developing breast cancer. Over 1.5 million women are diagnosed with breast cancer every year throughout the world[2,3]. In the European Union, there are approximately 361,600 new cases of breast cancer in women each year, and 93,500 deaths (15,5% of cancer-related deaths)[4]. In Italy the new cases are 52,800 and 12,200 deaths[5]. The trend of incidence rate for breast cancer in Italy appears to be slightly increasing (+0.3% per year), while mortality continues to fall significantly (-0.8% per year). This is due to the breast cancer screening programmes (mammography screening) together with the introduction of new and active agents for the treatment of breast cancer have resulted in increasing detection of breast cancer, which is potential curable. Around 79% of breast cancers are potentially operable (stage T13N0/+M0), 7% are locally advanced (T4NxM0), and 6% are metastatic (M1) at diagnosis[6]. In consequence, 5-year survival rates are increasing. The 5-year survival rate of women with breast cancer in Italy is 87%. The 5-year survival rates of breast cancer varied widely in developed and developing countries. The rate is over 80% in the first ones, but below 40% in the latter.

3.1.2 Risk factors

There are numerous risk factors which can increase the possibility of developing breast cancer.

Sex is the most important risk factor, most breast cancer occur in women and the number of cases is 100 times higher in women than in men [7].

The risk of developing breast cancer increases with **age**, with the probability of developing breast cancer being 2.3% up to 49 years (1 in 43 women), 5.4% in the 50-69 age group (1 in 18 women), and 4.5% in the 70-84 age group (1 in 22 women). This correlation with age could be linked to the continuous and progressive endocrine proliferative stimulus that the mammary epithelium undergoes over the years, together with the progressive damage to DNA and the accumulation of epigenetic changes that modifies the balance in the expression of oncogenes and tumor suppressor genes.

The incidence curve increases exponentially until the age of menopause (around 50-55 years), and then slows down reaching a plateau after menopause, to subsequently increase again after 60 years of age. This specific trend is linked both to the endocrinological history of a woman, and the availability and coverage of mammography screening programs.

Other factors of increased risk have been identified:

Reproductive factors: a long fertile period, with early menarche and late menopause, and therefore a longer exposure of the glandular epithelium to the proliferative stimuli of ovarian estrogens; nullity, first full-term pregnancy after 30, no breastfeeding.

Hormonal factors: increased risk in women taking hormone replacement therapy during menopause, especially if based on synthetic estrogen-progestins with androgenic activity; increased risk in women taking oral contraceptives.

Dietary and metabolic factors: high consumption of alcohol and animal fats and low consumption of vegetable fibers seem to be associated with an increased risk of breast

cancer[8]. Diet and behavior leading to obesity and metabolic syndrome are also increasingly important. Obesity is a recognized risk factor, probably linked to the excess of fat tissue that in postmenopausal women is the main source of synthesis of circulating estrogen, resulting in excessive hormone stimulation of the mammary gland. Metabolic syndrome is characterized by the presence of at least three of the following factors: abdominal obesity, altered glucose metabolism (diabetes or prediabetes), high lipid levels (cholesterol and/or triglycerides), and arterial hypertension. Metabolic syndrome increases the risk of cardiovascular disease but also of breast cancer: it is hypothesized that subjects with metabolic syndrome show a resistance to insulin to which the body reacts by increasing the levels of this substance. Insulin acts on the membrane receptor of insulin-like growth factor 1 (IGF-1R), activating the intracellular signal pathways essential for neoplastic growth.

Metabolic syndrome is based on genetic predisposition, but its development is clearly favored by sedentary lifestyles and high-calorie diets rich in fats and simple carbohydrates. Hence, by acting on these modifiable risk factors through regular daily physical activity combined with a balanced diet (as for example the Mediterranean diet), the risk of developing breast cancer could be reduced by improving the metabolic and hormonal balance of the woman.

As already mentioned above, it is possible to modify the risk of breast cancer by acting on predisposing factors, or those considered as such. In the USA, a significant reduction in the incidence of breast cancer, mainly hormone-responsive tumors, was observed in 2003 in women aged ≥50 years. Among various hypotheses, the most accredited is that this reduction is related to a drastic decline in the prescription of hormone replacement therapy after the publication of the results of a large study (Women's Health Initiative) that showed an increased incidence of breast cancer and ischemic heart disease with the use of hormone therapy containing estrogen-progestins.

The increase in risk attributable to the use of preparations containing estrogen and

progestins was found to be related to the duration of the replacement therapy and to be reversible upon its suspension[9-11]. In addition, a recently published study presented a predictive model of absolute risk for Italian women that identifies three modifiable factors (physical activity, alcohol consumption, and body mass index) on which prevention strategies can be based, and specifically regular daily physical activity combined with a balanced (Mediterranean) diet, factors that improve the metabolic and hormonal balance. This study shows how intervening on these factors can reduce the risk over 20 years by 1.6% in menopausal women, and by up to 3.2% in women with a positive family history and 4.1% in women at high risk for other causes (about 10% of the entire population)[12].

Prior radiotherapy (of the chest, and especially before 30 years of age) and **prior** breast **dysplasia or neoplasm**.

Familiarity and heredity: although most breast cancers are sporadic forms, 5%-7% are linked to hereditary factors, 1/4 of which are determined by the mutation of two genes: BRCA-1 and BRCA-2. In women with BRCA-1 mutations, the life-time risk of breast cancer is 65%, and 40% in women with BRCA-2 mutations. BRCA1 and BRCA2 are two anti-oncogenes located on chromosome 17q21 and 13q12, respectively. They both encode tumor suppressor proteins which are essential for the repair of double-strand DNA breaks. Cells lacking BRCA1 or BRCA2 are unable to repair double strand breaks by homologous recombination and therefore these cells may incur mutations during strand repair and often accumulate chromosomal rearrangements during successive rounds of cell division[13].

Genetic examination should be performed in patients diagnosed with breast cancer by the age of 40 whose tumor was characterized by lack of estrogen, progesterone and HER2 receptor overexpression (triple negative patients). Genetic consultation is also recommended to patients with bilateral breast cancer and family history of breast and/or ovarian cancer[13].

Other hereditary factors are represented by:

- Mutations of the ATM gene (Ataxia Telangiectasia Mutated)7 or CHEK2 gene[14]

- Mutation of the PALB210 gene
- Li-Fraumeni Syndrome (p53 mutation)
- Cowden Syndrome (PTEN gene mutation)
- Ataxia-telangiectasia, Peutz-Jeghers syndrome.

3.1.3 Histologic classification

More than 95% of malignant breast cancers are Adenocarcinomas, classified as invasive (infiltrative) or non-invasive (in situ) lesions according to its relation to the basement membrane[15]. In “in situ” carcinoma the cells are restrained inside the mammary ductal-lobular system, whereas in invasive carcinoma the cells spread beyond that structure and penetrate into stroma. From here, the cells can also invade the vessels and then reach the regional and remote lymph nodes[16]. Invasive and in situ carcinomas were also classified as ductal and lobular based on the site from which the tumor originated. However, it is now found that this sort of tumor growth variation is not related to the site or the cell of origin, but there could be differences in tumor cell biology: whether the tumor cells express E-cadherin or not[15].

Ductal carcinoma in situ (DCIS) is a neoplastic proliferation of epithelial cells limited to the ducts or lobules characterized by cellular and nuclear atypia. Myoepithelial cells are conserved, although they may be reduced in number. The risk of developing invasive cancer is directly proportional to the grade of the DCIS. The number of cases of CDIS has rapidly increased from less than 5% to 15-30% of all carcinomas since the introduction of population screening programmes. Most CDIS are identified as being associated with calcifications; less commonly, the periductal fibrosis surrounding the CDIS forms an area of increased mammography density.

Lobular Carcinoma in Situ (LCIS) is an intralobular proliferation of small and fairly uniform cells, originating in the terminal duct lobular unit, with or without pagetoid

involvement of terminal ducts. Cells lack E-Cadherine, the cellular adhesion protein, therefore, their shape is roundish and their degree of cohesion. Long-term follow-up for the women with LCIS concluded that it constitutes a risk factor and a non-obligatory precursor for the development of invasive cancer. It has no distinguishing features on gross examination, in fact, it is not associated either with calcifications or with a stromal reaction that can form thickening and is usually found incidentally in breast specimen or biopsy performed for other reasons.

It is multicentric in about 70% of cases and bilateral in approximately 30%–40% of cases.

Invasive ductal carcinomas (IDC) are breast cancers having malignant ductal proliferation along with stromal invasion in the presence or absence of DCIS, apart from their relative proportion. It is the most common form of invasive breast cancer and It accounts for 55% of breast cancer incidence upon diagnosis[16]. IDCs are a heterogenous group of tumors classified according to cytoarchitectural features, as they have wide scope of morphological variation. Some of them have enough distinctive features and particular behavior to be classified as special subtypes (as tubular, mucinous, and medullary) while the majority, which constitute about 75% of IDC, fail to exhibit sufficient morphological features and are generally designated as IDC not otherwise specified (NOS). Tumor cells are pleomorphic, vary in shape and size, and are usually with prominent nucleoli and numerous mitoses. The areas of necrosis and calcification can be detected in 60% of cases.

Invasive lobular carcinoma (ILC) constitutes 5%–15% of invasive breast cancer and usually affects older age group women affected by conventional IDC. ILC tumor cells are typically round, small, relatively uniform, and non-cohesive and have characteristic growth pattern with single-file infiltration of the stroma. The diagnosis of ILC can be made in the presence of these cytoarchitectural features even in the absence of in situ component. Inactivations of E-cadherin by mutation, loss of heterozygosity, or methylation are

characteristic molecular changes in ILC, particularly the pleomorphic subtype[17].

The immunohistochemical evaluation of the hormone receptor, proliferative activity (Ki67) and HER2 status, allows to identify the 4 phenotypic subgroups of breast cancer that present a “relative” correspondence with the 4 derived from the gene expression profiles.

Immunophenotypic groups of clinical relevance and with important therapeutic implications are:

- Luminal A: are represented by tumors with positive estrogenic receptors, with positive progesterone receptors with a positivity value higher than 20%, with negative HER2 and low Ki67 (cut off 20% and not more than 14%)
- Luminal B / HER2 Negative: positive hormone receptors, negative HER2 and high proliferative activity;
- Luminal B / HER2-positive: positive hormone receptors, HER2 overexpressed (score 3+ of immunohistochemical reactions) or amplified, any value of proliferative activity;
- HER2-positive (non-luminal): HER2 overexpressed (score 3+ of immunohistochemical reactions) or amplified (FISH or other methods) and both negative hormone receptors;
- Triple-negative: absence of hormone receptor expression and HER2 negativity.

3.1.4 Intrinsic molecular subtypes of breast cancer

Breast cancer is recognized as a heterogeneous disease, both on a molecular basis and in terms of clinical behaviour. The molecular profiling studies of primary tumors showed that breast cancers could be divided into biologic (or “intrinsic”) subtypes based on the analyses of gene expression[18]. Four main intrinsic molecular subtypes of breast

cancer have been characterized: Luminal A, Luminal B, HER2-enriched and Basal-like[19]. Luminal A and B subtypes at the RNA and protein level, are largely distinguished by the expression of two main biological processes: proliferation/cell cycle-related and luminal/hormone-regulated pathways. Compared to Luminal A tumours, Luminal B tumours have higher expression of proliferation/cell cycle-related genes or proteins (e.g. MKI67 and AURKA) and lower expression of several luminal-related genes or proteins such as the progesterone receptor (PR), but not the estrogen receptor, which is found similarly expressed between the two luminal subtypes and can only help distinguish luminal from non-luminal disease. Interestingly, a subgroup of Luminal B tumours is found hypermethylated, and a subgroup of Luminal A (6.3–7.8%) and Luminal B (16.4–20.8%) tumours show HER2-amplification/overexpression.

The HER2-enriched subtype is characterized at the RNA and protein level by the over-expression/amplification of HER2-related and proliferation-related genes and proteins (ERBB2/HER2), intermediate expression of luminal-related genes and proteins (e.g. ESR1 and PGR) and low expression of basal-related genes and proteins (e.g. keratin 5). The Basal-like subtype is characterized at the RNA and protein level by the high expression of proliferation-related genes (e.g. MKI67) and keratins typically expressed by the basal layer of the skin (e.g. keratins 5, 14 and 17), intermediate expression of HER2-related genes, and very low expression of luminal-related genes. BRCA1-mutated breast cancer is associated with Basal-like disease. Finally, ERBB2/HER2 overexpression/amplification is found in 2.1–17.4% of tumours with a Basal-like profile[19].

3.1.4.1 Clinical implications within HR+/HER2-negative disease

Within HR+/HER2-negative breast cancer, 90–95% of tumours fall into the Luminal A and B subtypes. Most of the direct evidence of general chemo-sensitivity of the Luminal A and B subtypes comes from the neoadjuvant setting. For example, in a cohort of 208 patients with luminal disease treated with anthracycline/taxane-based chemotherapy and

with pathologic complete response (pCR) data, the pCR rates in patients with the Luminal A and B subtypes were 3% and 16%. Overall, this data suggest that among the 2 luminal subtypes, the Luminal A tumours are less chemosensitive than Luminal B tumours. Even if one assumes that all patients benefit to the same extend from multi-agent chemotherapy[20], intrinsic subtyping together with prognostic factors such as tumour size and nodal status can be used to help decide when adjuvant chemotherapy should not be administered because the risk of relapsing without it is very low. For example, if the risk of relapsing at distant sites of a patient is estimated to be ≤10% without chemotherapy, the absolute benefit from chemotherapy would be ≤3%. Although Luminal A tumours seem to benefit less from multi-agent chemotherapy than Luminal B tumours, this does not preclude that this group of tumours cannot benefit from particular cytotoxic agents or regimens. For example, a retrospective analyses of the GEICAM9906 study (FECx6 vs. FECx4 and weekly paclitaxel) and CALGB9342/9840 (3-weekly paclitaxel vs. weekly paclitaxel) showed that low-proliferative tumours, mostly a subset of Luminal As, benefit substantially from the weekly paclitaxel regimen whereas high proliferative tumours did not[21].

Regarding the benefit from endocrine therapy, both tumour subtypes have shown to derive a similar relative benefit by looking at the proportional fall in the proliferation marker Ki67 upon treatment with an aromatase inhibitor in the neoadjuvant setting. However, since Luminal A tumours have a lower baseline proliferation status than Luminal B tumours, a larger proportion can achieve low post-treatment values. At the adjuvant setting, Luminal A tumours showed a higher benefit from adjuvant tamoxifen than Luminal B tumours. Within HR+/HER2-negative early disease, it is expected to identify a subpopulation of non-luminal subtypes (i.e. HER2-enriched and Basal-like) by gene expression. Data suggest that HER2-enriched and Basal-like diseases might not benefit much from endocrine therapy despite being ER+[21].

This group of patients is considerable and deserves special attention.

One interesting aspect is that, compared to early HR+/HER2-negative BC, the proportion of HER2-E subtype seems increased from 5% to 10% in patients with advanced or metastatic HR+/HER2-negative BC. This increase in the HER2-E subtype in the advanced setting can be due to patient selection, a true shift in tumor biology due to inherent tumor evolution or treatment effects, or a combination of everything. The current evidence does support this last possibility. Overall, the acquisition of a HER2-E profile might reflect the appearance of estrogen-independency in a tumor previously estrogen-dependent (i.e. luminal).

3.1.4.2 Clinical implications within HER2-positive disease

All the intrinsic molecular subtypes (HER2-enriched, Luminal B, Luminal A and Basal-like) can be identified within HER2-positive disease with different proportions. Neoadjuvant clinical trials suggest that all the intrinsic subtypes benefit from anti-HER2 treatment (trastuzumab) in combination with chemotherapy, although HER2-enriched might benefit the most, while the benefit of adding a second anti-HER2, especially in the context of chemotherapy, might reside in the HER2-enriched subtype[19].

3.1.4.3 Clinical implications within triple-negative (TN) disease

Within TN disease, all the intrinsic molecular subtypes can be identified, although the vast majority fall into the Basal-like subtype (86%).

At present, chemotherapy is the standard treatment for early-stage and metastatic TNBC. Polychemotherapy is highly effective in high proportion of patients with TN early breast cancer. The standard polychemotherapy regimen in early breast cancer is based on anthracycline/taxane-based combinations. However, clinical trials suggest that Basal-like breast cancers are more sensitive to interstrand crosslinking agents that damage the DNA such as platinum, because of deficiencies in the BRCA-associated DNA repair mechanism[19].

A study recently tested the impact of adding bevacizumab to standard neoadjuvant

anthracycline/ taxane-based chemotherapy in TN early breast cancer. The results showed that in the general TN population bevacizumab increased the pCR rates in the breast. In a subsequent retrospective analysis, a greater benefit from bevacizumab was observed in basal-like disease, but not in non-basal-like disease. This is an example of how the Basal-like versus not classification can predict sensitivity to a particular treatment strategy. Further studies are needed to determine if Basal-like disease is a biomarker of response or benefit from bevacizumab, especially in the metastatic setting where it is still approved in some countries.

An improved understanding of the immunogenicity of TNBC has led to clinical studies of several immunotherapeutic agents. Early phase I trials with immune checkpoint inhibitors in TNBC report an overall response rate of up to 19% with durable clinical responses and a tolerable safety profile. The hope is that immunotherapy strategies will provide new therapeutic options for TNBC[17].

3.1.5 Prognostic and predictive factors

Prognostic factors are related to the patient's prognosis (survival), while predictive factors are related to the potential effectiveness of an anticancer treatment.

Some prognostic factors that have been shown to be important and useful in the selection of the type of treatment, such as:

- tumor size
- state of axillary lymph nodes
- histological grade
- proliferative activity (Ki67)
- histological type
- vascular invasion
- HER-2 status
- hormone receptor status

- patient's age (<35 years: worse prognosis)
- Intrinsic molecular subtypes

Tumor size: Tumor size correlates with the presence and number of involved axillary lymph nodes and is also an independent prognostic factor, with distant recurrence rates increasing with larger tumor size. Patients with tumors <1 cm had a 5-year OS of close to 99% compared with 89% for tumors between 1 cm and 3 cm and 86% for tumors between 3 cm and 5 cm. For node-negative patients, tumor size is the most powerful prognostic factor and is routinely used to make adjuvant treatment decisions. In general, patients with a tumor size of > 1–2 cm warrant consideration of adjuvant therapy since they may have a distant recurrence risk of ≥20%[22].

Axillary lymph node status: The most significant prognostic indicator for patients with early-stage breast cancer is the presence or absence of axillary lymph node involvement. Furthermore, there is a direct relationship between the number of involved axil-lary nodes and the risk for distant recurrence. The impact of the presence of isolated tumor cells (ITC) or micrometastases in the sentinel lymph node does not seem to be relevant on the prognosis. Most clinical trials stratify patients based on four nodal groups: negative nodes, 1–3 positive nodes, 4–9 positive nodes, and 10 or more positive nodes. The 5-year survival for patients with node-negative disease is 82.8% compared with 73% for 1–3 positive nodes, 45.7% for 4–12 positive nodes, and 28.4% for ≥13 positive nodes. These data demonstrate that the risk of recurrence is significant enough with lymph node-positive disease to warrant adjuvant systemic therapy since, generally, a future risk of distant recurrence of 20% or greater is regarded significant enough to consider the risks of therapy[22]. For lower-risk patients, especially those who are node negative, an individualized assessment utilizing other prognostic factors must be performed.

Histological grade: Tumors can be classified as well differentiated (grade 1), moderately differentiated (grade 2), and poorly differentiated (grade 3) on the base of

mitotic index, differentiation, and pleomorphism of the cells. A high histological grade (G3) is considered an unfavorable prognostic factor.

Proliferative activity: proliferative activity measured with the Ki67 labeling index (percentage of nuclei of tumor cells that stain with the antibody for the Mib1 protein encoded by the Ki67 gene) is a recognized prognostic factor. Currently it is not yet possible to define a single threshold value below or above which the tumor can be defined as having a low or high proliferative activity, in order to predict the effectiveness of chemotherapy or endocrine therapy.

Histological type: The pathologic characteristics of the tumor have prognostic significance. Certain subtypes such as tubular, mucinous, and medullary have a more favorable prognosis than unspecified breast cancer[22].

Vascular invasion: Vascular invasion is not universally accepted as a prognostic factor but has been reported in several studies to be predictive of a worse progression-free survival and overall survival in N- and N+ patients with other risk factors such as histological grade, size of the tumor and hormone receptor status.

HER2 status: HER-2 overexpression by immunohistochemistry or HER2 gene amplification, present in approximately 13%-15% of breast cancers, is a well-established prognostic factor and predictive factor for response to anti-HER2 treatment (eg, trastuzumab, lapatinib, pertuzumab) and likely for hormone therapy resistance (tamoxifen). It is extremely important that testing is carried out in accredited laboratories. The two most commonly used methods are immunohistochemistry, which evaluates the possible overexpression of the HER-2 receptor, and fluorescence in situ hybridization (FISH), which measures gene amplification. A tumor is defined as HER-2 positive if a positive 3+ score is attributed by the immunohistochemical method or if gene amplification is present with the FISH method. In 2+ cases, it is important to evaluate gene amplification.

Hormone receptor status: The presence of estrogen and progesterone receptors (ER, PgR) in an invasive breast cancer is both prognostic and predictive of treatment

efficacy. The new recommendations by the American society of clinical oncology (ASCO) for the immuno-histochemical determination of hormone receptors, consider tumors with at least 1% positive cells to be positive. However, there is relationship between the levels of receptor positivity and the benefits of endocrine treatments, both in metastatic disease and in adjuvant or neoadjuvant settings. Tumors with high levels of receptors have a higher benefit from hormonal therapy, although many other factors may influence the hormone-responsiveness of tumors such as the HER-2 status, histological grade and Ki67.

Intrinsic molecular subtypes: In early breast cancer, Luminal B disease has worse baseline distant recurrence-free survival at 5-and 10-years regardless of adjuvant systemic therapy compared to Luminal A disease. In the vast majority of studies, the three main variables that predict outcome in early breast cancer are nodal status, tumour size and intrinsic subtyping.

A part from predicting baseline prognosis, the Luminal A vs B classification, together with tumour size and nodal status, predicts the residual risk of recurring at a distant site within the 5e10-years of follow-up. In a retrospective analysis of the ABCSG-08 study, late distant relapse-free survival was found significantly different between Luminal A and B subtypes in all patients and in the node-negative subgroup. These results suggest that intrinsic subtype has the ability to inform decisions concerning the length of endocrine therapy (i.e. 5 vs 10 years), being the low-risk Luminal A tumours with low tumour burden (e.g. tumour size 1 cm and node-negative) the group were 5 years of endocrine therapy might be sufficient[19].

Two large studies have evaluated the prognostic value of HR status within HER2-pos-itive breast cancer. In the 4-year follow-up of the N9831 and National Surgical Adju-vant Breast and Bowel Project B-31 adjuvant trials of trastuzumab in HER2-positive and HR-positive disease was found statistically significantly associated with approximately 40% increased disease-free survival and overall survival, compared to hormone receptor-negative disease. This association of hormone receptor status with survival was found to

be independent of the main clinical-pathological variables, including trastuzumab administration. In both studies, HR-negative disease experienced more cancer relapse in the first 5 years than HR-positive. Interestingly, patients with HR-negative tumours were less likely to experience first recurrence in bone and more likely to recur in brain, compared to patients with hormone receptor-positive tumors[19].

Triple negative disease is biologically heterogeneous and although Basal-like disease predominates, there is a small group of non-Basal-like tumours (HER2-enriched). No data is available regarding the prognostic impact of the intrinsic molecular subtypes defined within TN disease. Subtyping within TN will not have a clinical impact based on prognosis-only since no group has such an outstanding outcome that would allow avoiding chemotherapy[19].

***Multifocality*:** multifocality refers to the presence of several cancer foci separated by healthy parenchyma. “Satellite nodes” of the primary node are defined as lesions located less than 5 mm from it and separated by healthy parenchyma. It is good practice to report the number of invasion foci on the diagnostic report. The TNM system indicates that the T score is attributed according to the size of the major focus when several tumors are present in the same breast. It has been shown that multifocality has an impact on lymph node metastases, increased local recurrences and increased risk of cancer-related death. This aspect is controversial. A review found that multifocality/multicentricity are not independent prognostic factors for survival, as they are more frequently associated with larger tumors, grade 3, lymphovascular invasion and lymph node metastases.39 Recently, the term “diffuse carcinoma” has been coined, indicating a tumor that usually shows a lobular growth pattern and spreads to one or more quadrants. Often these tumors are difficult to identify in radiology and ultrasound scans.

3.2 HER 2-positive breast cancer

HER2 (Her-2/neu, c-erbB-2) is a 185-kDa transmembrane tyrosine kinase protein giving higher aggressiveness in breast cancers. In humans, HER2 overexpression occurs in 15-20% of primary breast tumors, and is associated with diminished disease-free (DFS) and overall survival (OS). HER2 controls cellular division and repair in breast cells. The overexpression of HER2, can result in uncontrolled growth and division of breast cells[23].

Typically, HER2 is expressed at a low level on the surface of epithelial cells and is necessary for the normal development of many tissues, including those of the breast, ovary, lung, liver, kidney, and central nervous system. In contrast, in breast cancer cells, immunohistochemical analyses have revealed extremely high levels of HER2, which can reach up to two million receptors per cell. The overexpression of HER2 is of crucial importance since its activation triggers multiple downstream pathways required for the abnormal proliferation of cancer cells.

HER2 is a member of a family receptors that includes the epidermal growth factor receptor (EGFR), also known as HER1, HER3, and HER4.

HER2 is activated by the formation of homodimers or heterodimers with other EGFR proteins. This dimerization results in autophosphorylation and/or transphosphorylation of specific tyrosine residues in EGFR intracellular domains, which in turn leads to the activation of the Ras/Raf/mitogen-activated protein kinase, the phosphoinositide 3-kinase/Akt, and the phospholipase Cγ (PLCγ)/protein kinase C (PKC) pathways. Of note, the HER2-HER3 heterodimer is the most potent stimulator of downstream pathways, particularly the PI3K/Akt, a master regulator of cell growth and survival. Moreover, HER2 dimerization promotes the mislocalization and rapid degradation of cell cycle inhibitor p27Kip1 protein leading to cell cycle progression[24].

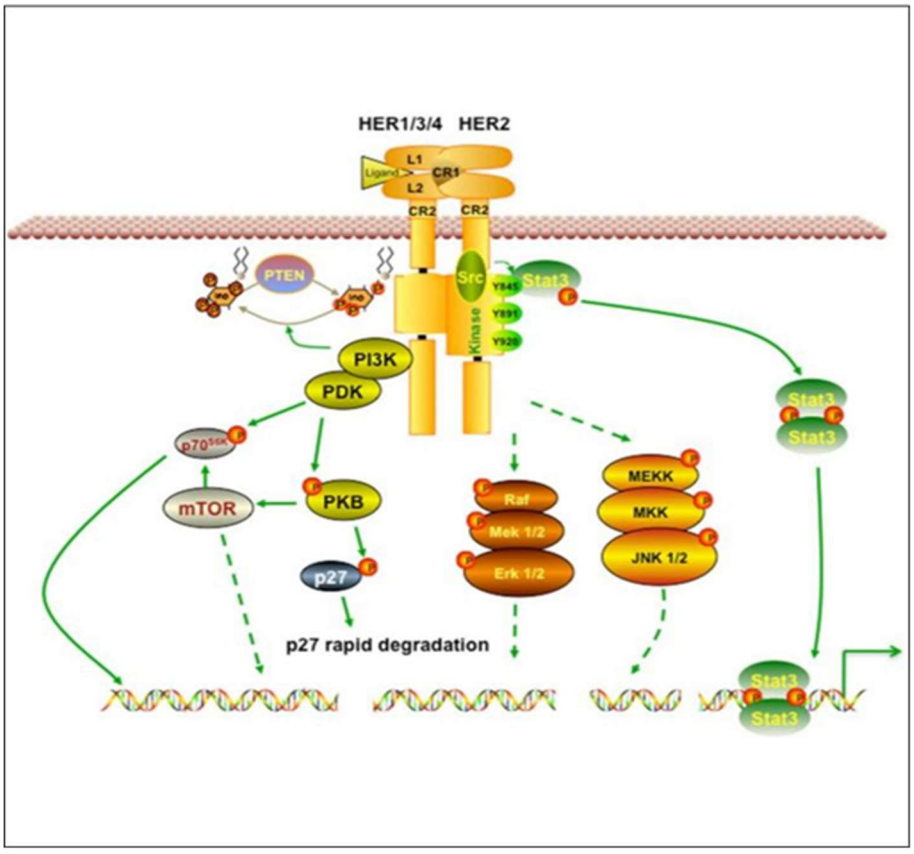


FIG 1. HER2 signaling pathway. *Receptor homodimerization or heterodimerization is the prerequisite step for HER2 activation. Its activation then triggers a broad spectrum of downstream cascades to promote numer-ous effects, including cell growth, proliferation, and survival. PI3K/Akt is one of the most well studied path-ways activated by HER2. Activated PI3K/Akt also triggers mTOR, a master positive regulator of cell metabo-lism. In addition, HER2 activation can activate Ras/Raf and MEK pathways, which favors cancer cells' growth and migration.*

3.2.2 Immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH)

Laboratory testing for HER2 status in breast cancer is performed according to guide-lines developed by the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP). There are currently two approved methods for determining HER2 status in breast cancer: immunohistochemistry (IHC) and in situ hybridization (ISH). Immunohistochemistry is a technique used to determine the presence and level of specific cellular proteins. IHC measures protein expression using specially labeled antibodies that can bind to the proteins of interest. The antibody is mixed with the cellular components of the tumor. After a set amount of time, the mixture is rinsed and only those antibodies

attached to their protein targets will remain. The presence of the antibodies can be detected by viewing the sample under a microscope because areas containing bound antibodies will appear a different color than areas lacking antibodies. Samples with more protein will bind more antibody and therefore appear darker. This allows the test to reveal not only whether a protein is present but also the relative amount of the protein. Test results are based on the strength of the staining and the percent of cells stained.

In situ hybridization indicates the localization of gene expression in their cellular environment. A labeled RNA or DNA probe can be used to hybridize to a known target mRNA or DNA sequence within a sample. This labeled RNA or DNA probe can then be detected by using an antibody to detect the label on the probe. FISH allows the quantification of HER2 gene copy number per cell using fluorescent-labelled probes that recognize and bind to HER2 gene in cell nuclei. Testing (positive, equivocal or negative) is now recommended for primary and metastatic tumors (if speciment available).

Tissue from the primary tumor can be obtained through a core needle biopsy, as well as from an incisional and excisional surgical procedure. Metastases can be biopsied from chest wall, regional lymph nodes, or distant organs[25].

The algorithms for evaluation of HER2 protein expression by IHC and HER2 amplification by single-probe or dual-probe ISH are presented in Figures 1, 2, and 3.

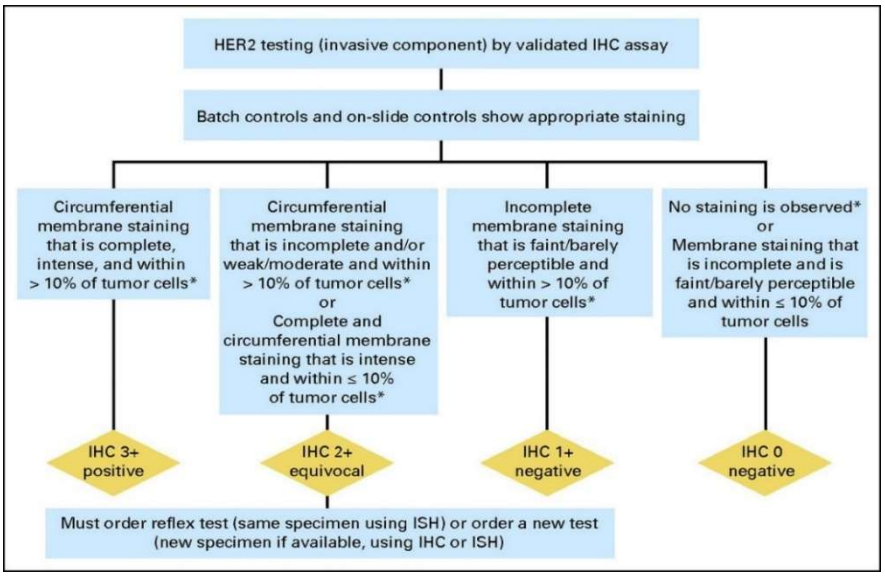


FIG.2 Algorithm for evaluation of human epidermal growth factor receptor 2 (HER2) protein expression by immunohistochemistry (IHC) assay of the invasive component of a breast cancer specimen. *Although categories of HER2 status by IHC can be created that are not covered by these definitions, in practice they are rare and if encountered should be considered IHC 2 equivocal. ISH, in situ hybridization. NOTE: the final reported results assume that there is no apparent histopathologic discordance observed by the pathologist.*

(*) *Readily appreciated using a low-power objective and observed within a homogeneous and contiguous invasive cell population.*

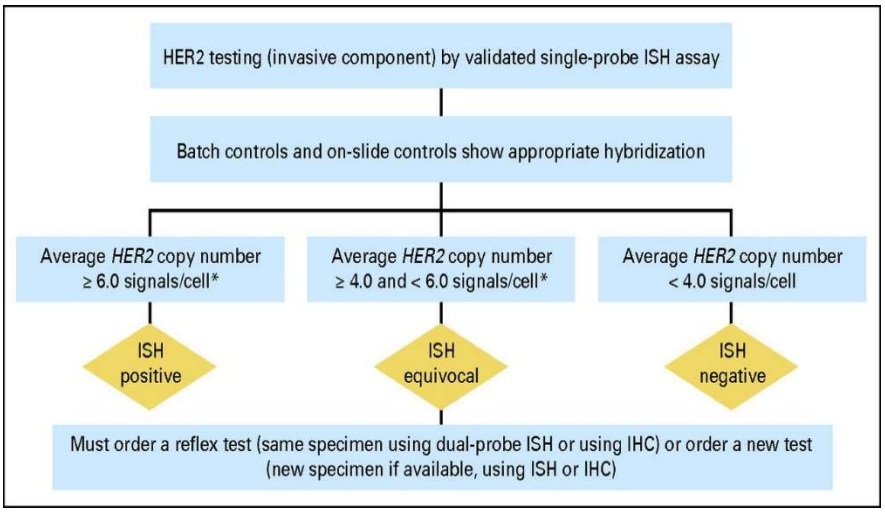


FIG 3. Algorithm for evaluation of human epidermal growth factor receptor 2 (HER2) gene amplification by in situ hybridization (ISH) assay of the invasive component of a breast cancer specimen using a single-signal (HER2 gene) assay (single-probe ISH). *Amplification in a single-probe ISH assay is defined by examining the average HER2 copy number. If there is a second contiguous population of cells with in-creased HER2 signals per cell, and this cell population consists of more than 10% of tumor cells on the slide (defined by image analysis or visual estimation of the ISH or immunohistochemistry [IHC] slide), a separate counting of at least 20 nonoverlapping cells must also be performed within this cell population and also reported. Although categories of HER2 status by ISH can be created that are not covered by these definitions, in practice they are rare and if encountered should be considered ISH equivocal (see Data Supplement 2E). NOTE: the final reported results assume that there is no apparent histopathologic discordance observed by the pathologist.*

(*) Observed in a homogeneous and contiguous population.

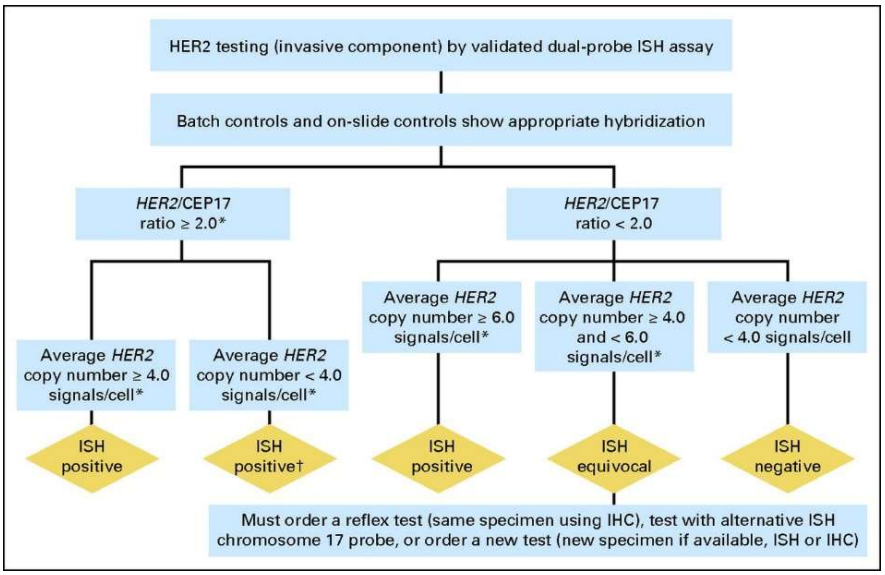


FIG 4. Algorithm for evaluation of human epidermal growth factor receptor 2 (HER2) gene amplification by in situ hybridization (ISH) assay of the invasive component of a breast cancer specimen using a dual-signal (HER2 gene) assay (dual-probe ISH). *Amplification in a dual-probe ISH assay is defined by examining first the HER2/CEP17 ratio followed by the average HER2 copy number (see Data Supplement 2E for more details). If there is a second contiguous population of cells with increased HER2 signals per cell, and this cell population consists of more than 10% of tumor cells on the slide (defined by image analysis or visual estimation of the ISH or immunohistochemistry [IHC] slide), a separate counting of at least 20 nonoverlapping cells must also be performed within this cell population and also reported. Although categories of HER2 status by ISH can be created that are not covered by these definitions, in practice they are rare and if encountered should be considered ISH equivocal (see Data Supplement 2E). NOTE. The final reported results assume that there is no apparent histopathologic discordance observed by the pathologist.*

(*)Observed in a homogeneous and contiguous population. (†) See Data Supplement 2E for more information on these rare scenarios.

HER2 test result as **positive** for HER2 if:

- IHC 3+ based on circumferential membrane staining that is complete, intense.
- ISH positive based on:

- Single-probe average HER2 copy number ≥ 6.0 signals/cell.
- Dual-probe HER2/CEP17 ratio ≥ 2.0; with an average HER2 copy number ≥ 4.0 signals/cell.
- Dual-probe HER2/CEP17 ratio ≥ 2.0; with an average HER2 copy number < 4.0 signals/cell.
- Dual-probe HER2/CEP17 ratio < 2.0; with an average HER2 copy number ≥ 6.0 signals/cell.

HER2 test result as **equivocal** and order reflex test (same specimen using the alternative test) or new test (new specimen, if available, using same or alternative test) if:

- IHC 2+ based on circumferential membrane staining that is incomplete and/or weak/moderate and within > 10% of the invasive tumor cells; or complete and circumferential membrane staining that is intense and within ≤10% of the invasive tumor cells.
- ISH equivocal based on:

- Single-probe ISH average HER2 copy number ≥ 4.0 and < 6.0 signals/cell.
- Dual-probe HER2/CEP17 ratio <2.0 with an average HER2 copy number ≥ 4.0 and < 6.0 signals/cell.

HER2 test result as **negative** if a single test (or both tests) performed show:

- IHC 1 + as defined by incomplete membrane staining that is faint/barely perceptible and within > 10% of the invasive tumor cells.
- IHC 0 as defined by no staining observed or membrane staining that is incomplete and is faint/barely perceptible and within ≤10% of the invasive tumor cells.
- ISH negative based on:

- Single-probe average HER2 copy number < 4.0 signals/cell.
- Dual-probe HER2/CEP17 ratio < 2.0 with an average HER2 copy number < 4.0 signals/cell.

HER2 test result as **indeterminate** if technical issues prevent one or both tests (IHC and ISH) from being reported as positive, negative, or equivocal.

If the test result is clearly positive or clearly negative retesting is not needed. A section of the tumor from the excisional specimen should be tested if the result is negative with an apparent histopathologic discordance. If this result is positive, no further testing is

needed. However, if the test is negative it may be appropriate to repeat the test in a different block from the patient's tumor. If all three tests are negative, no additional testing is recommended. If initial HER2 test result is equivocal, reflex testing should be performed on the same specimen using the alternative test or on an alternative specimen.

If this reflex test (same specimen/same tissue) does not render a positive or negative HER2 test result, the pathologist should review histopathologic features, confer if possible with the oncologist regarding additional HER2 testing. HER2 testing guides decision to pursue HER2-targeted therapy.

3.2.3 HER2-Targeted terapy

Amplification of the HER2 gene occurs in approximately 20% of primary breast cancers and leads to marked overexpression of the HER2 protein on the cell surface—typically more than 1 million copies per cell—and constitutive activation of HER2 signaling. These cancers frequently have a high proliferative rate and are associated with poor clinical outcomes in the absence of systemic therapy. The development of a specific, HER2-targeted therapy led to marked improvements in survival for patients with HER2-positive (HER2+) cancers in the adjuvant and advanced disease settings and confirmed the clinical utility of HER2 as a therapeutic target[26]. Five human epidermal growth factor receptor 2-targeted therapies are currently approved by the Food and Drug Administration (FDA) for the treatment of HER2-positive breast cancers: trastuzumab, pertuzumab, trastuzumab emtansine (T-DM1), lapatinib, and neratinib. These can be divided into three categories: anti-HER2 monoclonal antibodies (trastuzumab and pertuzumab), antibody–drug conjugate (T-DM1), and small-molecule pan-HER tyrosine kinase inhibitors (TKIs; lapatinib and neratinib). A number of other HER-directed TKIs are currently in development, including tucatinib, poziotinib, and pyrotinib[27].

Trastuzumab is a humanised immunoglobulin G1 (IgG1) directed against the

extracellular domain of HER2. Since its initial approval in 1998, trastuzumab has become standard of care for patients with HER2-positive BC and is widely used for its approved indications in both the adjuvant and metastatic settings. The addition of trastuzumab to standard chemotherapy increases disease-free survival (DFS), and improves overall survival (OS). Neoadjuvant treatment with a sequential anthracycline-taxane-based chemotherapy in combination with trastuzumab (Herceptin) is currently a preferred therapy for patients with HER2-positive breast cancer[28].

Trastuzumab has been proposed to trigger HER2 internalization and degradation through promoting the activity of tyrosine kinase – ubiquitin ligase c-Cbl. It was observed that the binding of trastuzumab to HER2 recruits c-Cbl to its docking site, Tyr1112 where c-Cbl ubiquitinates HER2 and leads to its degradation.

As an antibody, one of the major mechanisms of trastuzumab is to attract immune cells to tumor sites that overexpress HER2, by a mechanism called antibody-dependent cellular cytotoxicity (ADCC). The structure of an IgG antibody is comprised of two antigen-binding fragments (Fabs) linked to a single crystalline fragment (Fc) domain via the hinge region. This structural arrangement allows antibodies to link bound antigen with humoral and cellular components of the immune system. Fc gamma receptors (FcyRs) are expressed on a number of cells in the immune system including phagocytes like macrophages and monocytes, granulocytes like neutrophils and eosinophils, and lymphocytes of the innate immune system (NK cells) or adaptative immune system. Intracellular signalling through the activating FcyRs leads to immune effector functions such as antibody-dependent cell-mediated cytotoxicity[23].

The most well known effect of trastuzumab is the inhibition of the MAPK and PI3K/Akt pathways, which leads to an increase in cell cycle arrest, and the suppression of cell growth and proliferation. It is widely accepted that by interfering with the dimerization of HER2, trastuzumab inhibits HER2 activation and suppresses Akt phosphorylation[29].

Clinical benefits are greatest in patients with tumours strongly overexpressing HER2,

graded 3+ by immunohistochemistry (IHC), and/or with HER2 gene amplification. However, following adjuvant trastuzumab, relapse can occur up to 25.4% of patients at 10 years of follow-up. What is more, only 25-30% of HER2-positive mBC patients will respond to trastuzumab and most of them will experience disease progression during the first year of treatment. All these findings are consistent with the occurrence of intrinsic or acquired drug resistance in the majority of patients[23].

This has resulted in the emergence of several novel HER2-targeting agents, including the monoclonal antibody pertuzumab, which inhibits HER2 dimerization, the antibody-drug T-DM1, and the tyrosine kinase inhibitors (TKIs) lapatinib and neratinib, which target the signal transduction pathway downstream from HER2.

Pertuzumab is a fully humanised monoclonal antibody based on the human IgG1(κ) framework sequences and consisting of two heavy chains (449 residues) and two light chains (214 residues). Similar to trastuzumab, pertuzumab is directed against the extracellular domain of HER2; however, it differs from trastuzumab in the epitope-binding regions of the light chain (12 amino acid differences) and heavy chain (29 amino acid differences). As a result, pertuzumab binds to an epitope within what is known as sub-domain 2 of HER2, while the epitope for trastuzumab is localised to sub-domain 4[30].

Pertuzumab acts by blocking the dimerisation of HER2 with other HER family members, including HER1, HER3, and HER4. As a result, pertuzumab inhibits ligand-initiated intracellular signalling through two major signal pathways, MAP-kinase and PI3-kinase. Inhibition of these signalling pathways can result in growth arrest and apoptosis, respectively. Clinical data suggest that a more comprehensive blockade of HER2 through interruption of heterodimerisation may provide clinical benefit[30].

As pertuzumab and trastuzumab are not competing for the same binding epitope on HER2, their combination may lead to higher antibody load on tumor cells resulting in increased ADCC. Compared to monotherapy, combination of the two mAbs enhanced the recruitment of NK cells responsible for ADCC.

In patients with HER2-positive metastatic breast cancer, pertuzumab added to trastuzumab and docetaxel has been shown to significantly prolong both progression-free survival and overall survival.

The combination of pertuzumab and trastuzumab with chemotherapy is approved as first-line treatment of metastatic breast cancer[31].

Trastuzumab emtansine (T-DM1) is a novel antibody–drug conjugate (ADC) developed to treat HER2+ cancers. T-DM1 consists of the cytotoxic agent DM1 (derivative of maytansine) linked to trastuzumab. DM1 is a microtubule polymerization inhibitor that was selected for use in T-DM1 because of its high potency. Once T-DM1 binds to the extracellular domain of HER2, the complex is internalized into the cell, where the antibody is degraded by proteases, releasing the active metabolite, lysine-Nε-MCC-DM1, into the cytoplasm. Because this metabolite is a charged molecule, it is relatively membrane impermeable, reducing the possibility that the DM1 could enter a neighboring cell, thus further limiting the potential for nonspecific toxicity. In addition to its ability to deliver DM1 selectively to tumor cells, T-DM1 retains the effector functions of trastuzumab, including inhibition of HER2-mediated signal transduction and activation of antibody-dependent cell-mediated cytotoxicity[32].

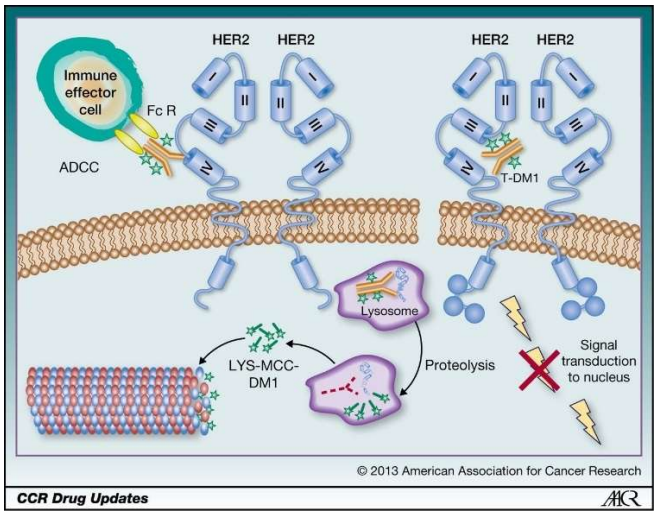


FIG 5. Mechanism of action of T-DM1*. T-DM1 binds to the extracellular domain of HER2, followed by internalization of the HER2/T-DM1 complex into lysosomes. The complex is then proteolytically degraded, releasing lysine-MCC-DM1 into the cytoplasm, where it inhibits microtubule polymerization. T-DM1 also retains effector functions of trastuzumab, including Fcγ receptor–mediated activation of ADCC and inhibition of HER2 mediated signal transduction, which may contribute to the efficacy of T-DM1.*

In patients with metastatic breast cancer who were resistant to trastuzumab, T-DM1 significantly prolonged time to progression and resulted in an improvement in OS of 4 months compared to lapatinib and capecitabine (29.9 months vs 25.9 months; HR 0.75). T-DM1 is now ap-proved as a treatment for metastatic breast cancer that has progressed on first-line trastuzumab-based therapy.

While T-DM1 has demonstrated efficacy as a rescue therapy for patients with meta-static breast cancer who become resistant to trastuzumab, it is not more efffective than trastuzumab and chemotherapy as a first-line treatment. In the phase III MARIANNE tri-al, neither T-DM1 alone nor in combination with pertuzumab improved PFS compared with trastuzumab and chemotherapy.

In recent years, several tyrosine kinase inhibitors (TKIs) have been developed that target the signal transduction pathway downstream from HER2, including lapatinib and neratinib. **Lapatinib**, a reversible epidermal growth factor receptor (EGFR/ERBB1/HER1) and HER2 TKI, is indicated for use with capecitabine in patients with metastatic HER2+ breast cancer whose disease progressed on trastuzumab; with letrozole in the treatment of patients with HER2+, ER+ breast cancer; for use in combination with: capecitabine for patients with advanced or metastatic disease with progression following prior therapy, which must have included anthracyclines and taxanes, and therapy with trastuzumab in the metastatic setting; trastuzumab in patients with hormone receptor-negative metastatic breast cancer that progressed on prior trastuzumab in combination with chemotherapy; and an aromatase inhibitor for postmenopausal women with hormone receptor-positive metastatic disease, not currently intended for chemotherapy. However, in preclinical studies most HER2 somatic mutations were resistant to lapatinib[27].

Neratinib has emerged as a potent inhibitor of HER2 activity. Neratinib is an irreversible pan-inhibitor of HER2/4 and EGFR that has been shown to be more efffective than lapatinib at blocking HER2 activation. In preclinical trials in tumor cell lines expressing HER2 somatic mutations, neratinib led to potent inhibition of intracellular signaling, cell

proliferation, and colony formation[24]. Neratinib is indicated for the extended adjuvant treatment of adult patients with early-stage HER2-overexpressed/amplified breast cancer, following adjuvant trastuzumab-based therapy. Neratinib was granted marketing authorization by the European Commission in 2018 and is indicated for extended adjuvant treatment of adult patients with early-stage hormone receptor-positive HER2-overexpressed/amplified breast cancer who are less than 1 year from the completion of prior adjuvant trastuzumab-based therapy[27].

3.3 Monoclonal antibodies in HER 2+ breast cancer

3.3.1 Trastuzumab IV

3.3.1.1 Therapeutic indications

3.3.1.1.1 Metastatic breast cancer

Trastuzumab (Herceptin) is indicated for the treatment of adult patients with HER2-positive metastatic breast cancer:

- as monotherapy for the treatment of those patients who have received at least two chemotherapy regimens for their metastatic disease. Prior chemotherapy must have included at least an anthracycline and a taxane unless patients are unsuitable for these treatments. Hormone receptor-positive patients must also have failed hormonal therapy, unless patients are unsuitable for these treatments.
- in combination with paclitaxel for the treatment of those patients who have not received chemotherapy for their metastatic disease and for whom an anthracycline is not suitable.
- in combination with docetaxel for the treatment of those patients who have not received chemotherapy for their metastatic disease.

- in combination with an aromatase inhibitor for the treatment of postmenopausal patients with hormone-receptor positive MBC, not previously treated with trastuzumab.

3.3.1.1.2 Early breast cancer

Herceptin is indicated for the treatment of adult patients with HER2-positive early breast cancer:

- following surgery, chemotherapy (neoadjuvant or adjuvant) and radiotherapy (if applicable).
- following adjuvant chemotherapy with doxorubicin and cyclophosphamide, in combination with paclitaxel or docetaxel.
- in combination with adjuvant chemotherapy consisting of docetaxel and carboplatin.
- in combination with neoadjuvant chemotherapy followed by adjuvant Herceptin therapy, for locally advanced (including inflammatory) disease or tumours > 2 cm in diameter.

3.3.1.2 Efficacy of trastuzumab IV in Early Breast Cancer

3.3.1.2.1 Adjuvant Setting

Six phase III multi-centre randomized controlled trials investigated the efficacy and safety of adjuvant trastuzumab IV in combination with or after standard adjuvant chemotherapy in the treatment of early breast cancer [o sullivan]:

- Herceptin Adjuvant (HERA, BO16348) trial
- North Central Cancer Treatment Group trial (NCCTG) N9831 trial
- National Surgical Adjuvant Breast and Bowel Project (NSABP) trial B-31
- Breast Cancer International Research Group (BCIRG-006) study
- Protocol Adjuvant dans le Cancer du Sein (PACS04) trial
- Finland Herceptin (FinHer) trial (Joensuu et al. 2009)

Together, these trials accrued more than 15,000 women with node-positive or high-risk node-negative breast cancer and used a variety of cytotoxic agents in various combinations, doses, and orders of administration. Four of these trials (HERA, N9831, B31 and BCIRG-006) are considered pivotal.

The HERA trial was an international, multicenter, randomized, controlled trial comparing 1 year or 2 years of trastuzumab given every 3 weeks in patients with HER-2/ neu-positive early breast cancer who had completed locoregional therapy and at least four cycles of neoadjuvant or adjuvant chemotherapy with or without radioterapy[33]. The NSABP B-31 trial compared four cycles of doxorubicin and cyclophosphamide (AC) followed by four cycles of every-3-week paclitaxel(P) with AC+P plus 52 weeks of trastuzumab (T) beginning with the first cycle of P [33].

The N9831 trial was a three-arm study that compared four cycles of AC followed by 12 weekly doses of paclitaxel (AC+P, arm A) with AC+P followed by 52 weeks of trastuzumab (T) beginning after P (arm B) and with AC+P plus 52 weeks of T beginning with the first P cycle (arm C)[33].

In the global, multicenter, randomized BCIRG 006 trial, treatment with AC followed by docetaxel was compared with AC followed by docetaxel plus trastuzumab, and with docetaxel in combination with carboplatin and trastuzumab[33]. (see table 1)

All four pivotal randomized controlled trials (HERA, N9831, B31 and BCIRG-006) demonstrated significantly improved DFS, and three (HERA, B31 and BCIRG-006) demonstrated significantly improved OS (see Table 1). The DFS benefits were observed regardless of age, nodal status, hormonal status, or tumour size in all trials. Importantly, the most recent follow-up data from the HERA trial and the combined analysis of the NCCTG N9831 and NASBP B-31 trials both demonstrate consistent DFS and OS advantages of adjuvant trastuzumab over a median follow-up of 4 years[34]. Further, the significant benefits in DFS and OS were maintained over a median follow-up of approximately 10 years in the BCIRG-006 study, which is the longest follow-up reported

to date[35]. The longterm clinical benefits of one-year trastuzumab treatment clearly continue to outweigh the risks of adverse effects and the regimen is considered standard of care with support from all major treatment guidelines.

Of the four pivotal randomized trials, the N9831 study was the only one to directly compare the concurrent and sequential use of trastuzumab. This study identified a strong trend for a 25% reduction in the risk of an outcome event when trastuzumab is started concurrently as compared to sequentially after paclitaxel[36]. Therefore, based on a positive risk/benefit ratio, the authors recommended that trastuzumab be incorporated in a concurrent fashion when administered with paclitaxel, which also resulted in the approval of the concurrent use of trastuzumab and chemotherapy.

3.3.1.2.2 Neo-adjuvant Setting

The combination of an anthracycline and a taxane can be considered the standard neo-adjuvant chemotherapy due to the high rate of pCR[37]. In the neoadjuvant setting Smith et al. reported an enhanced response to anthracyclines by addiction of docetaxel[38]. The NSABP-B27 trial, designed to determine the effect of adding docetaxel to preoperative doxorubicin and cyclophosfamide, failed to show an improvement of DFS and OS while pathological complete response rate was doubled by addition of preoperative docetaxel and improved the incidence of local recurrence[39]. The pCR rate remained a significant predictor of OS regardless of treatment, as was the pathological nodal status after chemotherapy. The ABCSG-14 trial compared 3 versus 6 cycles of neoadjuvant epirubicin plus docetaxel. Six cycles resulted in a significantly higher pCR rate, a higher percentage of patients with negative axillary status and a trend towards more breast-conserving surgery respect 3 cycles of chemotherapy. Rates of adverse events were similar[40]. In patients with operable disease, a non-cross-resistant regimen containing anthracycline and taxane was well tolerated and produced high response rates (78%) and rates of breast conserving surgery (63%), with a frequency of symptomatic cardiac dysfunction < 0.5%. The phase III randomized GeparTrio Study investigated the relationship between extended

chemotherapy and pathological complete response at surgery. Patients considered responsive to 2 cycles of docetaxel, doxorubicin and cyclophosphamide were randomly assigned to receive four or six more cycles of TAC, for a total of six or eight TAC cycles. Patients receiving eight TAC cycles had statistically significantly higher sonographic response rates but not pathological complete response rates than those receiving six TAC cycles. However, they also had more toxic effects[41].

Some clinical data indicated a great benefit from combining a drug targeting the HER2 receptor with chemotherapy in the neoadjuvant setting, with an unexpected high rate of pathological complete response. Stage II and III HER2-positive breast cancer patients achieved a high rate of PCR with trastuzumab given concurrently with paclitaxel and FEC75 chemotherapy[42]. No severe cardiac events were observed with the regimen. The regimen adopted by MD Anderson (paclitaxel followed by FEC) is well tolerated and yielded a high pCR rate when combined with Trastuzumab in HER2-positive patients[42]. Moreover, the NOAH trial was designed to assess event-free survival in patients HER2-positive locally advanced or inflammatory breast cancer receiving neoadjuvant chemotherapy (regimen consisted of doxorubicin, paclitaxel, cyclophosphamide, methotrexate, and fluorouracil) with or without 1 year of trastuzumab[43]. Trastuzumab significantly improved event-free survival in patients with HER2-positive breast cancer, was well tolerated and, despite concurrent administration with doxorubicin, only 2% of patients developed symptomatic cardiac failure that responded to cardiac drugs. This result indicates that the addition of neoadjuvant and adjuvant trastuzumab to neoadjuvant chemotherapy should be considered for women with HER2-positive locally advanced or inflammatory breast cancer to improve event-free survival, survival, and clinical and pathological tumor responses[43].

Neoadjuvant treatment with a sequential anthracycline-taxane-based chemotherapy in combination with trastuzumab is currently a preferred therapy for patients with HER2-positive breast cancer. This approach is based on the higher Pcr of 40% seen with the

addition of trastuzumab, compared with a 17% pCR with chemotherapy alone[44]. The pCR can be increased to 75% with dual HER2-receptor blockade and chemotherapy[45]. Higher pCR rates are found in hormone-receptor-negative tumors. Patients with a pCR after chemotherapy and trastuzumab showed a significantly better outcome compared with those who did not have a pCR. The need for additional or alternate treatment options is great in patients who do not achieve a pCR[46].

A randomized trial investigated the effect of the timing of trastuzumab administration with anthracycline and taxane neoadjuvant chemotherapy[47]. Women with operable HER2-positive invasive breast cancer were randomly assigned (1:1) to sequential treatment received fluorouracil 500 mg/m2, epirubicin 75 mg/m2, and cyclophosphamide 500 mg/m2 (FEC-75) on day 1 of a 21-day cycle for four cycles followed by paclitaxel 80 mg/m2 and trastuzumab 2 mg/kg (after a 4 mg/kg loading dose) once per week for 12 weeks, while those randomly assigned to the concurrent treatment group received paclitaxel and trastuzumab once per week for 12 weeks followed by four cycles of FEC-75 (on day 1 of each 21-day cycle) and once-weekly trastuzumab, in the same doses as the sequential group. Surgery, including evaluation of the axilla, was done within 6 weeks of completion of neoadjuvant treatment. The primary outcome was the percentage of patients who had a pathological complete response in the intention-to-treat population. From Sept 15, 2007, to Dec 15, 2011, two hundred eighty-two women were enrolled (140 in the sequential group, 142 in the concurrent group). 78 of 138 (56.5%, 95% CI 47.8–64.9) patients who received sequential treatment had a pathological complete response in the breast versus 77 of 142 (54.2%, 95% CI 45.7–62.6) who received concurrent treatment (difference 2.3%, 95% CI–9.3 to 13.9). No treatment-related deaths occurred. The most common severe toxic effects were neutropenia (35 [25.3%] of 138 patients in the sequential group *vs* 45 [31.7%] of 142 patients in the concurrent group) and fatigue (six [4.3%] *vs* 12 [8.5%]). Left ventricular ejection fraction dropped below the institutional lower limit of normal at week 12 in one (0.8%) of 130 patients who received sequential treatment and four (2.9%) of 137

patients who received concurrent treatment; by week 24, it had dropped below this limit in nine (7.1%) of 126 patients and in six (4.6%) of 130 patients, respectively. In conclusion, Concurrent administration of trastuzumab with anthracyclines offers no additional benefit and is not warranted[46].

3.3.1.3 Adverse Events of trastuzumab IV

3.3.1.3.1 Cardiac Safety of trastuzumab IV

The most clinically relevant AE associated with trastuzumab IV is left ventricular cardiac dysfunction (eg, CHF). In patients with HER2-positive eBC enrolled in pivotal clinical studies, trastuzumab treatment for one year (administered concurrently or sequentially with chemotherapy) appeared to be associated with a decrease in LVEF, an increase in the incidence of CHF (where specified, this was severe [New York Heart Association or NYHA] class III or IV or grade 3 or 4 or symptomatic CHF) and discontinuation of treatment as a result of cardiac AEs[48]. Cardiac toxicity described as NYHA class III/IV CHF occurred in 0%–0.9% of patients in the control arms and in 0%–3.8% of patients in the trastuzumab-containing arms of the 4 pivotal studies (HERA, N9831, B31 and BCIRG-006). However, the cardiotoxicity observed with concurrent or sequential trastuzumab treatment appeared to be mostly reversible following trastuzumab discontinuation, and no significant increase in cardiac death was reported[48]. An overview of cardiac safety data from selected studies of trastuzumab in combination with a taxane after anthracyclines for HER2-overexpressing eBC shows rates of symptomatic or severe CHF of < 4% and asymptomatic declines in LVEFs of > 10 points in ≤ 30% of patients. However, inter-study comparisons of chemotherapy-induced cardiac dysfunction are difficult because of the use of different definitions of cardiac dysfunction and different parameters for assessing cardiac safety[49]. These levels were considered below safety cut-off points set by the individual studies' independent data monitoring committees[50].

The NSABP B-31 study determined the 5-year cumulative cardiac event rate (NYHA

class III or IV CHF or cardiac death) to be 3.8% in patients randomly assigned to trastuzumab versus 0.9% in patients who received chemotherapy alone [8]. In the NCCTG N9831 study, the incidence of CHF was 0% in the chemotherapy-alone arm, 2.2% in patients who received sequential chemotherapy and trastuzumab, and 3.3% in patients who received concurrent chemotherapy and trastuzumab. An independent adjudication of the cardiac events occurring in studies B-31 and N9831 determined that the incidence of symptomatic heart failure events was 2.0% in trastuzumab-treated patients compared with 0.45% in the chemotherapy-alone arm, and that and the majority (86%) of these patients recovered with appropriate treatment[51].

The long-term incidence of cardiac AEs in patients with eBC who were treated with trastuzumab IV for one year after completion of neoadjuvant or adjuvant chemotherapy was also evaluated in the HERA study. Of the 1,698 patients randomly assigned to observation and 1,703 randomly assigned to one year of trastuzumab treatment, 94% had been treated with anthracyclines. The incidence of discontinuation of trastuzumab because of cardiac disorders was low (5.1%). At a median follow-up of 3.6 years, the incidence of cardiac endpoints remained low, though it was higher in the trastuzumab group than in the observation group (severe CHF, 0.8% vs. 0.0%, respectively; confirmed significant LVEF decreases, 3.6% vs. 0.6%, respectively). In the trastuzumab group, 59 of 73 patients with a cardiac endpoint reached acute recovery[52].

3.3.1.3.2 Post-marketing safety of trastuzumab IV

As of September 2012, over one million patients were estimated to have been treated with trastuzumab IV (Herceptin®/trastuzumab Periodic Safety Update Report No. 1048093). The most common (occurring in ≥ 1 out of 10 treated patients) adverse reactions are infusion-associated symptoms such as fever and chills, usually following the first infusion of trastuzumab IV. These symptoms are usually mild to moderate in severity and occur infrequently with subsequent trastuzumab IV infusions in up to 40% of patients. Other very common (≥ 1/10 patients) adverse reactions include febrile neutropenia, tremor,

dizziness, headache, blood pressure changes (increase or decrease), irregular heartbeat, palpitation, cardiac flutter, decreased ejection fraction, dyspnoea, wheezing, diarrhoea, vomiting, nausea, lip swelling, abdominal pain, erythema, rash, swelling of the face, arthralgia, muscle tightness, myalgia, asthenia, chest pain, fatigue, influenza-like symptoms, infusion-related reaction, and pain.

Some adverse reactions to trastuzumab IV infusion can be serious and include dyspnoea, hypotension, elevated blood pressure, wheezing, bronchospasm, tachycardia, reduced oxygen saturation, and respiratory distress. In the post-marketing setting, very rare (<1/10,000) occurrences of severe infusion reactions leading to a fatal outcome have been associated with the use of trastuzumab IV.

Severe pulmonary events leading to death have been reported with the use of trastuzumab IV in the post-marketing setting (4 out of 10,000 treated patients). Signs, symptoms, and clinical findings included interstitial lung disease including pulmonary infiltrates, acute respiratory distress syndrome, pneumonia, pneumonitis, pleural effusion, respiratory distress, acute pulmonary oedema, and pulmonary insufficiency. These events may or may not occur as sequelae of infusion reactions. Patients with symptomatic intrinsic lung disease or with extensive tumour involvement of the lungs, resulting in dyspnoea at rest, may be at greater risk of severe reactions. Other risk factors associated with interstitial lung disease include previous or concomitant therapy with other anti-neoplastic therapies such as taxanes, gemcitabine, vinorelbine, and radiation therapy.

In addition, severe hypersensitivity reactions have been infrequently reported in patients treated with trastuzumab IV (the exact incidence of these events is unknown). Signs and symptoms include anaphylaxis, urticaria, bronchospasm, angioedema, and/or hypotension. In some cases, the reactions have been fatal. Symptom onset generally occurred during an infusion, but onset after the completion of an infusion has also been reported. Reactions were most commonly reported in association with the initial infusion.

The immunogenicity of trastuzumab IV has been investigated in clinical studies that

included 903 mBC patients. Human anti–human antibodies to trastuzumab were detected in one patient, who had no allergic manifestations.

See the current trastuzumab (RO 45-2317, Herceptin®) IB for further details on the full safety profile of trastuzumab IV.

3.3.2 Trastuzumab SC

Trastuzumab for subcutaneous (SC) administration has been developed to address the known limitations of IV administration (eg, infusion-related reactions, long administration times, requirement for hospital facilities, treatment barrier for patients with poor venous access, continued use of port-a-cath systems). Administration of trastuzumab SC takes significantly less time (up to 5 minutes) compared to IV infusion (30 to 90 minutes) and this is expected to improve treatment convenience and compliance. These attributes are particularly important for patients treated over prolonged periods of time, such as in the adjuvant setting. In clinical studies conducted to date, administration of trastuzumab SC is associated with a reduced frequency and intensity of administration-related reactions.

The therapeutic indications for the subcutaneous formulation of trastuzumab are the same as for the intravenous formulation.

In the HannaH study (a Phase III randomised, open-label, international study of the SC formulation of trastuzumab in HER2-positive eBC patients), the safety profile of trastuzumab SC was comparable to that of trastuzumab IV[53]. Subcutaneous injection of trastuzumab SC formulation was generally well tolerated with a low incidence of injection site reactions (grades 1 and 2). These findings support the potential of trastuzumab SC to provide improved convenience for patients compared to the existing IV formulation[53]. In addition, SC administration also offers the potential for administration of trastuzumab outside a hospital/outpatient clinic setting in the future, further improving convenience and compliance.

The feasibility and patient acceptability of SC administration of any drug is dependent on the volume that must be administered. A key excipient in the SC formulation is the enzyme hyaluronidase, which enables larger volumes to be administered without a decrease in patient acceptability. Hyaluronidase transiently hydrolyses hyaluronan, a matrix component of the subcutaneous matrix. The hydrolysis leads to decreased viscosity of the subcutaneous matrix and, thus, to an improved delivery of subcutaneously administered drugs to the systemic circulation. The decreased viscosity is also expected to facilitate SC administration of larger volumes of fluid. More recently, preparations of recombinant humanised hyaluronidase (rHuPH20) have become available.

3.3.2.1 Recombinant human hyaluronidase (rHuPH20)

3.3.2.1.1 Nonclinical studies with rHuPH20

After IV administration in the dose range 0.3 to 30 mg/kg, rHuPH20 demonstrated nonlinear PK, rapid clearance and a half-life of around 5 minutes at the lowest dose tested. The bioavailability of rHuPH20 following SC administration was extremely low (not determinable at low doses, 6% to 8% in the dose range 3 to 30 mg/kg). Treatment of various species with rHuPH20 (IV or SC) was generally well tolerated and no major abnormalities were noted in any toxicology studies.

3.3.2.1.2 Clinical studies with rHuPH20

The safety and efficacy of hyaluronidase products have been widely established. The most significant safety risk identified is hypersensitivity/allergenicity, which is thought to be related to the lack of purity of the animal-derived preparations[54]. The purity, and hence the safety risks, of hyaluronidase preparations have been further improved by the development of the humanised recombinant enzyme rHuPH20. Clinical data are available from 4 studies with rHuPH20. Overall, the results of these studies have shown that rHuPH20 is generally well tolerated, with no SAEs reported. Reported AEs were mild or moderate in severity and most were injection site reactions.

3.3.2.2 Nonclinical Studies with trastuzumab SC

Overall, these studies showed that rHuPH20 enabled more rapid absorption of trastuzumab SC, and that SC administration of trastuzumab formulated with rHuPH20 was well tolerated locally and systemically.

3.3.2.3 Clinical Studies with trastuzumab SC

3.3.2.3.1 Study BP22023

Study BP22023 was a dose-finding study of trastuzumab SC, conducted in healthy male volunteers and patients with HER2-positive eBC[55]. Part 1 of the study was de-signed to select a trastuzumab SC dose (formulated with rHuPH20) that resulted in comparable exposure and trough levels at least as high as those achieved with trastuzumab IV at a dose of 6 mg/kg. Part 2 of the study was designed to confirm the SC dose selected from part 1. A total of 24 healthy male subjects and 42 female patients with HER2-positive eBC received single doses of either trastuzumab IV or SC.

In Cohorts A and B, there was no apparent dose-related increase in AEs and SC ad-ministration was generally well tolerated. The most commonly observed AEs in these patients were headache (27 events; 18 mild, 8 moderate, 1 severe), diarrhoea (8 events; 6 mild, 2 moderate), lethargy (6 events; 4 mild, 2 moderate), and injection site erythema (6 events; all mild).

Immunogenicity Results from Study BP22023

Blood samples were taken at screening, Days 15, 85 and follow up (5 months post dose) to allow for testing of antibodies to either trastuzumab or rHuPH20.

Nine of 58 (15.5%) subjects who had been administered trastuzumab SC were deemed positive for antibodies to rHuPH20 after confirmatory assay analysis. Five of these subjects had a positive confirmatory assay at all time-points including screening, one sub-ject was positive at screening, Day 15 and follow up and negative at Day 85, one subject was positive at screening, Day 15 and Day 85 but negative at follow up, another one was

positive at Day 85 but negative at follow up and the remaining subject was positive at Day 85 and follow up. All samples were negative in the neutralizing antibody assay. A total of 263 samples were assayed in 58 subjects for the occurrence of anti trastuzumab antibodies. Eleven samples in 8/58 (14%) subjects receiving trastuzumab SC were positive for antibodies to trastuzumab after the confirmatory assay. All samples from the screening visits were negative for anti-trastuzumab antibodies. In 6 subjects out of the 8 above, the follow-up sample was negative after one positive sample. No anti-trastuzumab antibodies were detected in subjects receiving trastuzumab IV. Neutralizing antibody tests for trastuzumab were not performed as the assay was not available at the time. Samples have been discarded in the interim. Likewise, results from titering assays are not available.

The presence of a positive confirmatory assay for trastuzumab or rHuPH20 was not associated with a difference in safety or trastuzumab PK profile. This also held true for the 3 patients who developed anti-drug antibodies (ADA) to both proteins.

3.3.2.3.2 Study BO22227

Study BO22227 (HannaH) compared the PK profile, efficacy, and safety of the SC and IV formulations in patients with HER2-positive eBC[53]. The HannaH study was a Phase III, randomised, international, open-label, study in the (neo)adjuvant setting.

Patients with HER2-positive, operable, locally advanced or inflammatory BC were randomly assigned to 8 cycles of neoadjuvant chemotherapy administered concurrently with trastuzumab every 3 weeks either IV (8 mg/kg loading dose, 6 mg/kg maintenance dose) or SC (fixed dose of 600 mg); 1:1 ratio. Chemotherapy consisted of 4 cycles of docetaxel (75 mg/m²) followed by 4 cycles of fluorouracil (500 mg/m²), epirubicin (75 mg/m²), and cyclophosphamide (500 mg/m²), every 3 weeks. After surgery, patients continued trastuzumab to complete 1 year of treatment. Co-primary endpoints were serum trough concentration (Ctrough) at predose cycle 8 before surgery (non-inferiority margin for the ratio between groups of 0.80) and pCR (non-inferiority margin for the difference between groups of –12·5%), analysed in the per-protocol population. In this study, 299

patients were randomly assigned to receive trastuzumab IV and 297 to receive trastuzumab SC. This study showed that trastuzumab SC was non-inferior to trastuzumab IV for both co-primary endpoints. The most common AEs were neutropenia (99/298 [33.2%] vs 86/297 [29.0%]), leucopoenia (17/298 [5.7%] vs 12/297 [4.0%]), and febrile neutropenia (10/298 [3.4%] vs 17/297 [5.7%]) in the IV versus SC treatment groups, respectively. However, more patients experienced SAEs in the SC group (62/297 [21%] patients) than in the IV group (37/298 [12%] patients); the difference was mainly attributable to infections and infestations (24/297 [8.1%] in the SC group vs 13/298 [4.4%] in the IV group). Four AEs led to death (1 in the IV group and 3 in the SC group), all of which occurred during the neoadjuvant phase. Of these 4 AEs, 2 (both in the SC group) were deemed to be treatment related. Trastuzumab SC, administered over about 5 min, has a PK profile and efficacy non-inferior to standard IV administration, with a similar safety profile to trastuzumab IV, and, therefore, offers a valid treatment alternative[53-56].

3.3.2.3.3 Study BO25532

Study BO25532 (CP3) was a randomised, open-label, parallel, two-arm, multicentre Phase I study to investigate the comparability of PK of trastuzumab administered SC using either the SID or a conventional syringe and hypodermic needle. The study also assessed the performance of the SID and evaluated the immunogenicity of trastuzumab and rHuPH20. Enrolment was completed in September 2011, with a total of 119 healthy male subjects randomised 1:1 to receive a single 600 mg subcutaneous injection by either administration method. Trastuzumab was well tolerated after single-dose administration by both methods and no apparent differences related to the injection method were observed[56].

3.3.2.3.4 Study MO22982

MO22982 (PrefHer) was a Phase II international, randomised, open-label, two-cohort, two-arm crossover study to evaluate patient's preference and HCP satisfaction with

SC versus IV administration of trastuzumab in HER2-positive eBC, following surgery and completion of chemotherapy (neoadjuvant or adjuvant)[57]. Patients in Arm A received trastuzumab SC (4 cycles) followed by trastuzumab IV (4 cycles). Patients randomised to Arm B received trastuzumab IV (4 cycles) followed by trastuzumab SC (4 cycles). Patients enrolled into Cohort 1 received trastuzumab SC administered via a subcutaneous single use injection device (SID), and patients enrolled into Cohort 2 received trastuzumab SC administered from a vial with a hand-held syringe. A total of 245 patients were randomized. Patient preference, healthcare professional satisfaction, and safety data pooled from Cohort 1 and also Cohort 2, where s.c. Trastuzumab was delivered via hand-held syringe, were reported. PrefHer revealed compelling and consistent patient preferences for sc. over IV trastuzumab, regardless of SID or hand-held syringe delivery. S.c. was well tolerated and safety was consistent with previous reports, including the HannaH study (NCT00950300). No new safety signals were identified compared with the known IV profile in EBC[53, 58].

PrefHer and HannaH confirmed that s.c. Trastuzumab is a validated and preferred option over IV for improving patients' care in HER2-positive breast cancer. Administration of trastuzumab SC to patients could help reduce time required at clinic visits, increase compliance, and provide them with more free time for their daily activities.

3.3.2.3.5 Study MO28048

MO28048 (SafeHer) is a Phase III, prospective, two-cohort, non-randomised, multicentre, international, open-label study to assess the safety of assisted and self-administered trastuzumab SC as adjuvant therapy in patients with operable HER2-positive eBC. Approximately 2,500 patients with HER2-positive eBC whose tumour has been excised have been enrolled into the study. The trial is ongoing, but not recruiting participants.

3.3.3 Pertuzumab

3.3.3.1 Non-Clinical studies with pertuzumab

In addition to the complementary mechanisms implicated by the two antibodies, increased ADCC may account for their synergistic effect in vivo. ADCC is generally dependent on the number of antibodies bound to target cells. As pertuzumab and trastuzumab are not competing for the same binding epitope on HER2, their combination may lead to higher antibody load on tumor cells resulting in increased ADCC. This effect is not achieved by simply increasing the dose of trastuzumab.

3.3.3.2 Clinical studies and indication with pertuzumab

Pertuzumab (Perjeta) was approved by the FDA in 2012 for the treatment of HER2-positive mBC in combination with trastuzumab and docetaxel in patients who have not received previous anti-HER2 therapy or chemotherapy for metastatic disease.

3.3.3.2.1 CLEOPATRA

The randomised, double-blind, placebo-controlled, Phase III Clinical Evaluation of pertuzumab and trastuzumab (CLEOPATRA) study assessed the efficacy and safety of the combination of pertuzumab and trastuzumab with docetaxel as first-line treatment for patients with HER2-positive mBC[59]. This study established that targeting HER2-positive tumours with two anti-HER2 monoclonal antibodies that have complementary mechanisms of action along with chemotherapy, as compared with placebo plus trastuzumab plus docetaxel, resulted in a significant increase in the objective response rate, a reduction in the risk of progression or death (hazard ratio [HR], 0.62), an increase of 6.1 months in median PFS and a significant improvement in ORR as well as a significant increase in OS (HR, 0.66; median for pertuzumab arm not reached at time of analysis)[59]. The dose of docetaxel was 75 mg/m2 IV every 3 weeks (could be adjusted to 55 mg/m2 or 100 mg/m2 depending on tolerance) for at least 6 cycles. The combination therapy with pertuzumab

did not increase the rates of symptomatic or asymptomatic cardiac dysfunction. The AEs (any grade) of diarrhoea, rash, mucosal inflammation, febrile neutropenia, and dry skin were reported more frequently in the pertuzumab group than in the control group.

3.3.3.2.2 NEOSPHERE

The NeoSphere trial investigated the combination of pertuzumab or trastuzumab, or both, with docetaxel and the combination of pertuzumab and trastuzumab with-out chemotherapy in the neoadjuvant setting[60]. Patients were randomly assigned (1:1:1:1) to receive four neoadjuvant cycles of: trastuzumab (8 mg/kg loading dose, fol-lowed by 6 mg/kg every 3 weeks) plus docetaxel (75 mg/m(2), escalating, if tolerated, to 100 mg/m(2) every 3 weeks; group A) or pertuzumab (loading dose 840 mg, followed by 420 mg every 3 weeks) and trastuzumab plus docetaxel (group B) or pertuzumab and trastuzumab (group C) or pertuzumab plus docetaxel (group D). Of 417 eligible patients, patients given pertuzumab and trastuzumab plus docetaxel (group B) had a significantly improved pathological complete response rate (49 of 107 patients; 45·8% [95% CI 36·1-55·7]) compared with those given trastuzumab plus docetaxel (group A; 31 of 107; 29·0% [20·6-38·5]; p = 0·0141). 23 of 96 (24·0% [15·8-33·7]) women given pertuzumab plus docetaxel (group D) had a pathological complete response, as did 18 of 107 (16·8% [10·3-25·3]) given pertuzumab and trastuzumab (group C). The most common adverse events of grade 3 or higher were neutropenia (61 of 107 women in group A, 48 of 107 in group B, one of 108 in group C, and 52 of 94 in group D), febrile neutropenia (eight, nine, none, and seven, respectively), and leucopenia (13, five, none, and seven, respectively). The number of serious adverse events was similar in groups A, B, and D (15-20 serious adverse events per group in 10-17% of patients) but lower in group C (four serious adverse events in 4% of patients). Overall, patients who achieved pCR had a reduced risk of a PFS or DFS event at 5-year study analysis[60].

3.3.3.2.3 TRYPHAENA

TRYPHAENA is a multicentre, randomised phase II clinical trial conducted in 225 adult female patients with HER2-positive locally advanced, operable, or inflammatory breast cancer (T2-4d; primary tumour > 2cm in diameter) who had not received prior trastuzumab, chemotherapy or radiotherapy[61]. Patients with metastases, bilateral breast cancer, clinically important cardiac risk factors or LVEF < 55% were not included. The majority of patients were less than 65 years old. Patients were randomised to receive one of three neoadjuvant regimens prior to surgery as follows:

- 3 cycles of FEC followed by 3 cycles of docetaxel, all given concurrently with perjeta and trastuzumab
- 3 cycles of FEC alone followed by 3 cycles of docetaxel, with trastuzumab and perjeta given concurrently
- 6 cycles of TCH in combination with perjeta.

Randomisation was stratified by breast cancer type (operable, locally advanced, or inflammatory) and ER and /or PgR positivity. Perjeta was given intravenously at an initial dose of 840 mg, followed by 420 mg every three weeks. Trastuzumab was given intravenously at an initial dose of 8 mg/kg, followed by 6 mg/kg every three weeks. FEC (5-fluorouracil [500 mg/m2], epirubicin [100 mg/m2], cyclophosphamide [600 mg/m2]) were given intravenously every three weeks for 3 cycles. Docetaxel was given as an initial dose of 75 mg/m2 IV infusion every three weeks with the option to escalate to 100 mg/m2 at the investigator's discretion if the initial dose was well tolerated. However, in the group treated with perjeta in combination with TCH, docetaxel was given intravenously at 75 mg/m2 (no escalation was permitted) and carboplatin (AUC 6) was given intravenously every three weeks. Following surgery all patients received trastuzumab to complete one year of therapy. The primary endpoint of this study was cardiac safety during the neoadjuvant

treatment period of the study. Secondary efficacy endpoints were pCR rate in the breast (ypT0/is), DFS, PFS and OS.

Demographics were well balanced between arms (median age was 49-50 years, the majority were Caucasian [77%]) and all patients were female. Overall 6% of patients had inflammatory breast cancer, 25% had locally advanced breast cancer and 69% had operable breast cancer. Approximately half the patients in each treatment group had ER-positive and/or PgR-positive disease. Compared with published data for similar regimens without pertuzumab, high pCR rates were observed in all 3 treatment arms. A consistent pattern of results was observed regardless of pCR definition used. The pCR rates were lower in the subgroup of patients with hormone receptor positive tumours (range 46.2% to 50.0%) than in patients with hormone receptor-negative tumours (range 65.0% to 83.8%). pCR rates were similar in patients with operable and locally advanced disease. There were too few patients with inflammatory breast cancer to draw any firm conclusions[61].

Based on the clinical results reported, pertuzumab is indicated for use in combination with trastuzumab and chemotherapy for the neoadjuvant treatment of adult patients with HER2-positive, locally advanced, inflammatory, or early stage breast cancer at high risk of recurrence.

3.3.3.3 Pertuzumab Safety Data

In the neoadjuvant trial NEOSPHERE, the most common ADRs (≥50%) seen with perjeta in combination with trastuzumab and docetaxel were alopecia and neutropenia. The most common NCICTCAE v.3 Grade 3-4 ADR (≥10%) was neutropenia.

In the neoadjuvant trial TRYPHAENA, when perjeta was administered in combination with trastuzumab and FEC (5-fluorouracil, epirubicin, cyclophosphamide) for 3 cycles followed by 3 cycles of perjeta, trastuzumab and docetaxel, the most common ADRs (≥50%) were neutropenia, diarrhea and nausea. The most common NCI-CTCAE v.3 Grade 3-4 ADRs (≥10%) were neutropenia, febrile neutropenia and leucopenia. When perjeta

was administered in combination with trastuzumab and docetaxel for 3 cycles following 3 cycles of FEC (5-fluorouracil, epirubicin, cyclophosphamide), the most common ADRs (≥50%) were diarrhoea, nausea and alopecia. The most common NCI-CTCAE v.3 Grade 3-4 ADRs (≥10%) were neutropenia and leucopenia. Similarly, when perjeta was administered in combination with TCH (docetaxel, carboplatin and trastuzumab) for 6 cycles, the most common ADRs (≥50%) were diarrhoea and alopecia. The most common NCI-CTCAE v.3 Grade 3-4 ADRs (≥10%) were neutropenia, febrile neutropenia, anaemia, leucopenia and diarrhoea. The safety of perjeta administered for more than 6 cycles in the neoadjuvant setting has not been established.

3.4 Immunological aspects of monoclonal antibodies

3.4.1 Rationale for Assessment of Host Immunity for Treatment with pertuzumab and trastuzumab

Pre-clinical studies have suggested that both trastuzumab and pertuzumab works at different levels by blocking dimerization of HER2 with inhibition of intracellular signaling pathways, inducing apoptosis, or activating immune response by antibody dependent cell-mediated cytotoxicity (ADCC)[62]. With the exception of HER2 status, no validated predictive factors of either response or resistance to anti-HER2 monoclonal antibodies (mAbs) have been identified to date. This is part due to the not yet fully elucidated mechanism of action of mAbs in vivo[62].

Innate and adaptive immune responses are components of an integrated system of anti-tumor host defense in which numerous cells and molecules function cooperatively. Natural killer cells, monocytes and neutrophils recognize and kill tumor cells in an antigen-

indipendent manner. Breast cancer antigens, including HER2, have been identified, and T and B lymphocytes specific for these antigens may recognize and destroy tumor cells (adaptative immunity)[62].

Evasion of host immunity is thought to be critical for cancer growth and progression. The clinical relevance of the host immune system in breast cancer has long been unexplored. Studies developed over the past decade have highlighted the biological heterogeneity of breast cancer, prompting researchers to investigate whether the role of the immune system in this malignancy is similar across different molecular subtypes of the disease. The presence of high levels of lymphocytic infiltration has been consistently associated with a more-favourable prognosis in patients with early stage triple-negative and HER2-positive breast cancer[63,64]. These infiltrates seem to reflect favourable host antitumour immune responses, suggesting that immune activation is important for improving survival outcomes[65].

Increasing evidence suggests a significant contribution of innate and adaptive immunity to clinical efficacy of anti-HER2 monoclonal antibodies (mAbs) such as trastuzumab and pertuzumab[66]. Several mutually nonexclusive immune mechanisms have been proposed to explain the broad spectrum of immune-mediate action of anti-HER2 mAbs: anti-idiotype regulation, modifications in cytokine production, complement activation, killing of target cells by antibody-dependent cytotoxicity (ADCC), Fc gamma receptor (FcγR)-mediated activation of both regulatory T cells (Treg) and dendritic cells, and the blockade of cell-cell interaction[67,68].

Programmed cell death protein-1 (PD-1) is an immune checkpoint receptor that prevents overstimulation of adaptive immune responses and contributes to the maintenance of immune tolerance to self-antigens[67]. Cancer cell upregulate PD-L1/PD-L2 to escape from immune surveillance. Several checkpoint inhibitors, in particular anti-PD-1 and anti-PD-L1 mAbs, have been approved to treat a wide spectrum of tumors. Anti-HER2 M Abs may induce PD-L1 upregulation. These findings justify the attemps of combining anti-

HER2 therapies with either anti-PD-1 or anti-PD-L1 mAbs[64]. Inhibition of PD-1 on CD8+ tumor-infiltrating lymphocytes (TILs) within solid tumors is known to restore cytokine secretion, T-cell proliferation and lymphocyte-dependent anti-tumor activity[68]. In breast cancer, PD-1 is expressed in 20% of cases, suggesting PD-1 as a therapeutic target especially in HER2-positive and triple-negative phenotypes[69]. Two phase 1b clinical trials with anti PD-1/PD-L1 antibodies in patients with heavily pretreated MBC have achieved clinical benefit rates by 33 to 52%[70,71].

Genetic evidence in humans supports the need of FcγR-medi-ated ADCC for the efficacy in vivo of both trastuzumab and pertuzumab[62,72,73]. However, the known requirement for CD8+ T cellular cytotoxicity for the efficacy of antitumor mAbs suggests that in addition to ADCC, FcγR–mediated enhancement of antigen presentation may also contribute to adaptive tumor immunity[64,66]. The efficient targeting of mAb-HER2 complexes to Fcγ receptors on APCs accomplishes combined activation of Th1 CD4 and CD8 effector responses, which are down-regulated by the PD-1 checkpoint[74]. It is, thus, possible that co-administration of trastuzumab and pertuzumab may synergically enhance both innate and adaptive anti-HER2 immunity in HER2-positive breast cancer. Moreover, the massive tumor cell death secondary to concomitant chemotherapy administration, and the resulting liberation of large amounts of tumor antigens being presented to APCs, may contribute to the creation a favorable immune microenvironment for anti-HER2 mAbs activity[64,66,69].

3.4.2 Rationale for Assessment of Host Immunity for Treatment with trastuzumab SC

Trastuzumab SC has a pharmacokinetic profile and efficacy non-inferior to standard intravenous administration, with a similar safety profile to trastuzumab IV in HER2-positive early BC[58]. Interestingly, in the phase 3 randomised Hannah trial, trastuzumab SC has been observed to be more immunogenic than trastuzumab IV: 6.8% of the patients in the SC group had anti-trastuzumab antibodies in comparison with an immunogenicity rate of 3.4% observed in the IV group[58]. Infusion-related reactions suggest an immune response including immunoglobulin IgE-to-IgG class switching with trastuzumab SC administration [75].

Unlike the intravenous, subcutaneous administration of trastuzumab does not provide a direct drug absorption into the intravascular compartment[63-65]. After sub-cutaneous administration, Herceptin undergoes several steps through the peripheral lymphatic system and central lymph nodes and only then is poured into the blood stream. mAbs administered subcutaneously may therefore experience an “early contact” with immune cells (B and T lymphocytes) in the lymph nodes (recognised sites of encounters between lymphocytes and antigens)[76].

Since the kinetics and distribution of trastuzumab SC is different from that of trastuzumab IV[55,57], it is possible that trastuzumab SC acts at different immunologic levels. It is thus possible that trastuzumab administrated by the IV route could affect in particular the “humoral” component of the pathological immune response [77,78]. On the contrary, trastuzumab given by the SC route could act on the “cellular” component thus affecting the response of memory CD4+ T cells or other cellular players such as dendritic cells[77,78]. In this case, the onset of immune effects of trastuzumab can be delayed but prolonged in time[75,78]. Therefore, by modifying the modality of administration of trastuzumab, it would be possible to interfere with different pathways of the immune system and to exert a

beneficial/favorable immunomodulation in HER2-positive breast cancer.

3.4.3 TILs in Early Breast Cancer

In contrast to mucosal tissues, such as the intestine, normal breast tissue does not contain large aggregates of immune cells [65]. Some immune-cell populations exist in non malignant breast tissue. For example, macrophages have an important role in the development of the normal breast lobular anatomy and, during lactation, mucosa-associated B cells produce immunoglobulins that are secreted in breast milk[79].

Low numbers of CD8+ and CD4+ T cells can be purified from bulk normal adult breast tissue, but they are rarely detected on histological examination and their functional significance is uncertain[80]. In contrast to normal breast tissue, breast tumours and their adjacent stroma display higher levels of immune-cell infiltrates[81].

Tumor-infiltrating lymphocytes (TILs) are more commonly found in TNBC and HER2-positive breast cancers[82]. In one of the largest studied cohorts of women with lymph-node-positive disease, the median percentage of stromal tissue infiltrated with TILs was 10% in oestrogen receptor (ER)positive and HER2negative samples, 15% in HER2-positive samples, and 20% in ERnegative and HER2negative disease[82].

TILs have been shown to provide prognostic and potentially predictive value, particularly in triple-negative and human epidermal growth factor receptor 2-overexpress-ing BC [63,64].

TILs are commonly detected in H&E stained histological slides via light microscopy, wherein they are distinguished by the typical features of lymphocytes or by additional IHC staining for lymphocyte markers[55]. When evaluating H&E stained slides, TILs are divided into stromal-compartment TILs and intratumoural-compartment TILs[63,64]. Stromal TILs infiltrate the stromal tissue adjacent to the tumour cells, as this fibrous stroma is part of the malignant tumour, stromal immune cells are considered to be true tumour-infiltrating cells.

Intratumoural TILs actively infiltrate tumour-cell 'nests' where they have direct contact with tumour cells[79,82]. At any given point in time, the majority of TILs in breast cancers are typically located in the tumour stroma[65]. Histological specimens are not able to reveal the spatiotemporal dynamics of the immune microenvironment, although the simple classification of TILs into stromal compartment-TILs and intratumoural compartment-TILs in H&Estained sections remains useful. Both stromal and intratumoural TILs comprise a complex mixture of different lymphocyte subtypes, dominated by T cells, with lesser proportions of B cells, NK cells, and macrophages[83]. Once identified, TILs populations are quantified by determining how much of a demarcated area of stroma or tumour present on a slide is infiltrated by immune cells. Intratumoural and stromal TILs populations are highly correlated, although stromal TILs are typically observed in higher numbers and display much larger variability between different tumours[84]. Whereas some tumours have no stromal TILs, others have more than 10,000 immune cells per square millimetre of stromal tissue. Stromal TILs are thus more reliable biomarkers than intratumoural TILs in predicting response to therapy and patient outcome, when assessed via light microscopy of H&E-stained tissue slides[82,84]. The use of IHC staining permits more-accurate identification of intratumoural TILs, as they can be more easily distinguished from epithelial tumour cells by this method.

The distribution of TILs in a tumour can cause difficulties in consistent evaluation on H&Estained sections. Stromal TILs occur in a variety of patterns, including a diffuse but even distribution, a focal infiltrate, or multifocal 'sprinkling' across the tumour bed. Sometimes a gradient exists between zones of high and low TILs numbers within the stroma of a single tumour, and confluence with lymphocytes adjacent to normal lobules or around foci of DCIS. In addition, specific tumour-growth patterns can influence the disposition of TILs in a manner that might compromise comparison across different tumours. A diffuse and solid tumour-growth pattern, consisting of dense tumour-cell nests with only limited intervening stroma, is rapidly evaluated as having a high level of TILs

because the stromal compartment is small. On the other hand, tumours with a more infiltrative and dissociative growth pattern and larger areas of stroma will generally be scored as having lower TILs[84].

Furthermore, the scoring of TILs can also be complicated by the existence of different histological subtypes of breast cancer. In the classic subtype of invasive lobular adenocarcinoma, distinguishing infiltrating tumour cells from TILs in the stroma is not always straightforward. Further difficulties can arise from interpreting the remarkable perivascular abundance of TILs in some tumours with minimal stromal infiltration, and distinguishing clearly intratumoural TILs from tumour cells when IHC staining is not used. Finally, the clinical significance of the spatial heterogeneity in TILs is undetermined and the best approach to scoring tumours in this scenario remains uncertain[65]. As with any biomarker, the utility of TIL assessment relies on the development of a standardized and reproducible scoring methodology.

The richness and diversity of the antitumour immune response is, of course, severely underestimated by assessment with H&E or a few IHC markers. More-comprehensive techniques, such as flow cytometry and multicolour IHC staining with multispectral imaging, enable the quantification and localization of different lymphocyte subpopulations[85]. In a complementary fashion, gene-expression analysis (measured using quantitative polymerase chain reaction (qpCR), RNA microarrays or RNA sequencing (RNASeq), also provide information about relevant lymphocyte subpopulations[86]. As a result of the before mentioned techniques, it has now been established that TILs in breast tumours comprise lymphocyte subpopulations with differing functional significance. This detailed decomposition of TIL subpopulations is desirable, but such an analysis is not generally feasible in studies involving large cohorts of patients, because tissue samples collected in clinical trials are not routinely suitable for flow cytometry, and the amount of archived tissue available is usually quite limited. In this situation, the interpretation of the immune microenvironment often relies on the simple evaluation of standard H&E stained

sections. Indeed, in a patient cohort of a randomized trial of neoadjuvant chemotherapy, the predictive value of evaluating TILs in H&E stained samples has been shown to be very similar to that derived from the analysis of immune gene-expression profiles[79,82].

3.4.3.1 TILs as a Prognostic Biomarker

TILs have been evaluated in nearly 16,000 patients in prospective studies with clinical follow-up data available, which highlights a remarkably rapid accumulation of evidence[79,82]. The conclusions regarding prognosis have been relatively consistent. Patients with TNBC display a robust linear relationship between an increase in TILs numbers over time and improved recurrence-free survival (RFS) end points, as noted in retrospective–prospective analyses performed on the Breast International Group (BIG) 2–98 trial, Finland Herceptin Trial (FinHER), Eastern Cooperative Oncology Group (ECOG) 2197 and ECOG 1199 trials, and the National Epirubicin Adjuvant Trial (NEAT)/BR9601 trial[79,82,87,88]. A similar relationship between RFS and TIL population was seen for patients with HER2-positive tumours in the NeoALLTO trial and the HER2-positive patient cohorts reported by Ali et al[83]. Interpretation of the data for HER2-positive tumours is complicated by the varied use of adjuvant trastuzumab in early study cohorts and the fact that trastuzumab benefit seems to be greater in patients with higher TILs numbers, as seen in the FinHER data[88]. Finally, no significant prognostic value has been found for TILs in ERpositive HER2negative disease[79].

3.4.3.2 TILs as a Predictive Biomarker

Results from several studies using adjuvant therapy suggest that TILs might also serve as a predictive biomarker of benefit from cytotoxic chemotherapy. In the BIG 02–98 trial18, evidence suggested that increased levels of TILs were associated with greater benefit from anthracycline chemotherapy in patients with HER2-positive disease. This association was only noted for anthracycline alone and not for the combination of anthracycline and docetaxel[82]. In the randomized NEAT/BR9601 study19, it was ob-

served that the presence of CD8+ Tcells was associated with greater benefit from epirubicin, with no differences between histological subgroups[79]. This finding is based on preclinical studies, which have shown that anthracycline-induced tumour-cell death is particularly immunogenic[79,82].

The presence of TILs is also associated with increased rates of pCR. Initial evidence from a randomized controlled trial of the association between increased TILs and pCR rates was reported by Denkert and coauthors[89]. The GeparDuo and Gepar-Trio clinical trials of neoadjuvant anthracycline and taxane chemotherapy enrolled a total of 1,058 patients with samples that could be assessed for TILs[65,79]. All histological subtypes were included, and trastuzumab was not used. TILs were analysed as a continuous variable, and also as a dichotomous variable with a cut off of 60% (with lymphocyte predominant breast cancer (LPBC) defined as the presence of ≥60% TILs). The pCR rate was roughly 30% higher in tumours with TILs ≥60% compared with tumours with ≤60% in GeparDuo[65,79], and this finding was also observed in the GeparTrio trial[65,79]. The data from more than 2,000 patients in prospective studies have now been analysed, with the consistent result that increased TILs are associated with higher rates of pCR after neoadjuvant chemotherapy and targeted therapy. In the most-recent study to be published, an analysis of GeparSixto[79], patients with LPBC who were treated with carboplatin in addition to anthracycline and taxane had impressive pCR rates (74% and 78% in TNBC and HER2-positive disease, respectively) compared with the pCR rate of patients with LPBC not treated with carboplatin (43% and 50% in TNBC and HER2-positive disease, respectively). This difference was highly significant ($P < 0.005$). Most of the patients in the neoadjuvant studies mentioned above had TNBC or HER2-positive disease, although GeparDuo, GeparTrio, and GeparQuinto included small numbers of patients with ERpositive tumours. Exploratory analyses in this subgroup revealed that patients with LPBC had a statistically significant higher rate of pCR compared with patients with non-LPBC.

3.4.3.3 TILs as Biomarkers of Residual Disease

Residual disease following neoadjuvant chemotherapy in TNBC is considered a very poor prognostic marker. Neoadjuvant chemotherapy (NACT) plus anti-HER2 treatment can result in the accumulation of TILs in the residual tumour[63,64], and depletion of immunosuppressive regulatory T cells, which is expected to portend a favourable outcome. However, NACT might also promote tumour infiltration by macrophages, which are associated with an increased chance of recurrent disease. Several studies have shown that the presence of TILs in patients with residual disease, nevertheless, confers a good prognosis in an otherwise poor prognostic group[63,64]. The attainment of a pCR, while strongly associated with a good prognosis, is not a validated surrogate for improvement in more-robust survival end points, such as event-free survival and overall survival. In the NeoALTTO study, in which both pCR and event-free survival endpoints were assessed, TILs provided prognostic information independent of pCR rates[63]. In particular, those patients with high TILs had the most-favourable outcomes, even if they did not achieve a pCR. By contrast, those patients who did not achieve a pCR and had low levels of TILs had a poor event-free survival (EFS) at 3 years (67%)[63]. These results suggest the possibility that a group of HER2-positive patients with high TILs have an excellent outcome independent of achieving a pCR and only require trastuzumab as single antiHER2 therapy with their chemotherapy, rather than more expensive or toxic combination therapies.

TILs in residual disease could therefore be used to gain further insight into the role of adaptive immune system to the response to anti-HER2 neoadjuvant treatment.

3.4.4 Natural Killer (NK) cell activity and anti-tumor CD4+/CD8+ T cell response

3.4.4.1 Origin of the breast-cancer immune response

Studies performed in melanoma show that CD4+ and CD8+ T cells that are reactive to peptides arising from cancer-specific mutations (termed 'neoantigens') can be identified in tumour infiltrates[90]. Furthermore, in a patient with advanced-stage cholangiocarcinoma, regression of metastatic lesions was induced by purifying, expanding, and reinfusing clonal T cells specific to an epitope arising from a single mutation found in the tumour[91]. In patients with breast cancer, it has been reported that cytotoxic T cells extracted from tumours are specific to antigens present in the tumour from which they were derived, although these antigens were not defined[92]. In addition, in a study across multiple tumour types using The Cancer Genome Atlas (TCGA) data, investigators found a variable correlation between increasing tumour mutational burden and expression of Tcell effector function, although this correlation was only weakly positive in breast cancers[79]. Given these findings, it seems likely that an important stimulus for immune recognition of breast tumours is the repertoire of mutant peptides produced by the tumour. Extensive studies of this process in model systems have refined the general concept of 'immunoediting'[79, 93]. Initially, the immune system efficiently removes immune-naive tumour cells, but some tumour-cell clones might survive. This is followed by an equilibrium or dormant phase, during which the host immunity controls the cancer but does not eliminate it completely, permitting eventual evolution and outgrowth of tumour-cell clones that eventually escape immune control through various mechanisms, including the diminution of antigenic stimuli and the generation of an immunosuppressive tumour milieu. In the breast-cancer setting, some questions about this concept remain unanswered. Compared with other solid tumours, breast tumours have a low mutation rate, but are nevertheless predicted to

overexpress a median of around 50 ‘private’ potentially immunogenic peptides[90,93]. The mutation rate varies considerably between disease subtypes, with TNBCs reported to accumulate mutations 13 times faster than ERpositive tumours[79]. The accurate prediction of which mutations give rise to immunogenic peptides recognized by effector T cells, is currently impossible without highly involved experimental validation[90,93]. In melanoma, the characterization of mutations that give rise to reactive Tcell clones in tumours of patients has revealed that only relatively few mutations seem to be immunogenic[89]. Nevertheless, considerable technical limitations are present in purifying rare Tcell clones and detecting mutations that might be present in a small fraction of tumour cells, such that the neoantigen repertoire of a tumour is likely to be underestimated by these methods[79,92]. Of note, the most-commonly mutated gene in breast cancer, PIK3CA61, does not seem to affect the level of TILs in the HER2-positive subtype[79].

The Tcell repertoire found in a tumour is presumed to arise from the clonal expansion of suitably stimulated naive Tcell clones, as is the case for the immune response to infection[80,83]. In addition to antigen affinity, the proliferative capacity of each T cell broadly depends on costimulatory signals, such as CD28 expressed on anti-gen-presenting cells, and proinflammatory cytokines, such as IL2 and IL12[80,83]. Colorectal cancers with deletions of genes encoding proinflammatory cytokines display a reduced density of tumour infiltrating T cells and B cells[80,83]. Some cytokines can promote an immunosuppressive microenvironment, and this capacity has been demonstrated in animal models of breast cancer, whereby recruitment of TREG cells to the tumour was driven by tumour-derived CXCL10[68]. Priming of T cells by dendritic cells is another crucial step in generating CD8+-Tcell-mediated antitumour immunity, which also requires type I interferons[68]. Interferon production and recruitment of dendritic cells forms part of innate antitumour immunity, with work suggesting that a key mediator of Tcell priming by dendritic cells is the stimulator of interferon genes complex (STING), which detects tumour DNA taken-up by antigen-presenting cells[80,83].

Aside from mutant peptides, the presentation of damaged intracellular proteins in an irregular context can generate tumour antigens[68]. Different modes of tumour cell death, either spontaneous or induced by chemotherapy or radiotherapy, are also know to lead to immunogenic or immunosuppressive signals in the immune microenvironment[82]. Anthracyclines are notable for causing 'immunogenic' tumour-cell death by promoting phagocytosis of tumour cells and costimulatory responses by dendritic cells[83]. Indeed, the innate and adaptive immune systems in the tumour milieu are co-dependent, as illustrated by the requirement for functional cytotoxic Tcells to achieve more-efficient tumour elimination in response to anthracycline treatment[80,83]. Of interest, germline loss of function of alleles, such as the toll-like receptor 4 (TLR4), impair the innate immune response to tumour-cell death, and are associated with higher rates of relapse after chemotherapy and radiotherapy given with curative intent in patient with breast cancer[68].

3.4.4.2 Lymphocyte subpopulations—functional impact

The presence of TILs confers a favourable prognosis, but it ultimately represents an inadequate immune response when viable tumour deposits remain. As discussed earlier, constant interplay exists between tumours and the immune system via the process of immunoediting, which begins long before a tumour is clinically evident[92]. Another relevant feature is that the immune microenvironment is shaped by competing costimulatory and coinhibitory signals. For example, although the presence of activated CD8+ Tcells in the tumour microenvironment is associated with favourable outcomes, they also induce counter-immune mechanisms, such as expression of PDL1 on tumours, as part of the physiological mechanisms to restrain T cell activity[79]. Similarly, the tumour-specific CD8+ T cells infiltrating melanomas have been shown to express receptors that mediate immunosuppression[90]. Thus, both stimulatory and suppressive signalling in the immune microenvironment are correlated with the presence of an antitumour immune response. Results from several groups, including ours, indicate that TILs predominantly represent a

type I interferon immune response[79]. Strong correlations at the mRNA level between TILs and many genes representing Tcell activation have been observed, but cases with the highest TILs also show the highest expression of Tcell checkpoint receptors, indoleamine 2,3dioxygenase 1 (IDO1), and TREG-cell markers[79]. These data indicate that the TILs present in breast tumours consist of tumour-specific T cells that are chronically exposed to antigen, rather than merely being nonspecific 'passengers' or resident memory lymphocytes that have persisted after the elimination of antigenic tumour cells.

3.4.4.3 Circulating NK and CD8+ in HER2-positive BCs treated with neoadjuvant chemotherapy

Innate (ADCC-mediated) and adaptive (T cell-mediated) immune mechanisms are emerging as key players in the modulation of the activity of HER2-targeted drugs, such as the mAb trastuzumab[79]. Indeed, higher efficiency of Antibody Dependent Cell Cytotoxicity (ADCC) and Natural Killer (NK) cell lysis were reported in clinical responders to trastuzumab if compared with non-responders[92,93]. Interestingly, the efficacy of trastuzumab treatment was associated with the enhanced in situ infiltration of interferon-γ producing CD8+ T cells and CD4+ T helper (Th) lymphocytes[92,93], and decreased numbers of circulating regulatory T cells (Treg)/CD4+ and reduced Treg/ inflammatory Th17 ratios[92,93].

In agreement with these findings, a recent characterization of the immune profile of 61 locally advanced BC patients eligible for a neoadjuvant chemotherapy (NAC) schedule demonstrated that, at diagnosis, patients with HER2-overexpressing cancers had a retained immune proficiency and higher CD8+ T cell responses against several tumor-associated antigens (TAAs) if compared to HER2-negative cases, whose general immune background, on the contrary, appeared compromised[79]. Phenotypic and functional characterization of circulating immune cells in the same cohort of BC patients was characterized at diagnosis and throughout NAC (Paclitaxel and trastuzumab). The

percentages of circulating immune cell subsets including T and B lymphocytes, Natural Killer (NK) cells, regulatory T cells, T helper lymphocytes, were quantified by multiparametric flow cytometry. NK cells functional activity was evaluated through the analysis of NF-kB nuclear translocation by multispectral flow cytometry, and with the in vitro monitoring of trastuzumab-mediated ADCC. CD8+ T cell responses against six different tumor-associated antigens (TAA) were characterized by IFN-γ ELISPOT and IFN-γ/IL-2 DualSpot assays[79]. After NAC, HER2-positive patients showed a significant increase in the number of NK cells and regulatory T cells irrespective of the pathological response, whereas patients under-going a pCR disclosed higher percentages of T helper cells. Notably, a significant increase in the number of activated NK cells was observed only in HER2-positive patients achieving a pCR. Characterization of anti-tumor T cell responses highlighted sustained levels of CD8+ T cells specific for survivin and mammaglobin-A throughout NAC in patients undergoing a pCR[79,92,93]. Moreover, HER2-positive patients achieving a pCR were characterized by a multiepitopic and polyfunctional anti-tumor T cell response, markedly reduced in case of partial response.

All these findings indicate that maintenance of functional T cell responses against selected antigens and improvement of NK cell proficiency during NAC are probably critical requirements for pCR induction, especially in HER2-positive BC patients.

3.4.5 FcγR Polymorphisms

There is increasing evidence that the Fc portion of anti-tumor monoclonal antibodies is a major component of their therapeutic activity, through binding to Fcγ receptors expressed by effector cells present in the tumor microenvironment. ADCC is a well-recognized immune effector mechanism in which antigen-specific antibodies direct immune effector cells (such as NK cells and monocytes/macrophages) of the innate immunity to the killing of the antigen-expressing cancer cells [62]. In a pilot study, breast

cancer patients who responded to trastuzumab with complete or partial remission were found to have a higher capability to mediate in vitro ADCC with trastuzumab than were non-responders[62]. ADCC depends on the bifunctional structure of immunoglobulin G (IgG) molecules and comprises an antigen-binding fragment (Fab) that engages the tumor cell and a crystalline fragment (Fc) that binds a Fc gamma receptor (FcγR) on an effector cell such as NK, monocyte or macrophage[62,94]. The concomitant binding of Fab to tumor cell antigen and of Fc to an effector cell through its FcγR brings to the activation of immune cells with consequent destruction of the tumour cell[54,87]. Some common single-nucleotide polymorphisms (SNPs) in the coding regions of the FcγR genes have been associated with differential antibody-binding affinities and functional outcomes. A coding polymorphism, valine (V) 158 phenylalanine (F), located in the extracellular domain of the FcγRIIIa and in an area that directly interacts with the lower hinge region of IgG1, is known to affect binding[62]. The histidine (H) allelic variant at amino acid position 131 (H131) in the FcγRIIa extracellular domain confers higher binding affinity than the arginine (R131) variant[62,72,73]. In vitro studies of these polymorphisms demonstrated that peripheral blood mononuclear cells (PBMCs) homozygous for H131 or V158 induced significantly higher trastuzumab-mediated ADCC than PBMCs with other genotypes with different NK cell activation[62,94].

Some retrospective studies have shown a correlation between FcγRIIIa and FcγRIIa polymorphisms and clinical outcome of trastuzumab[62]. Furthermore, the FcγRIIb I232T polymorphic variant, which is associated with loss of function of the inhibitory FcγRIIb[62], has been shown to be predictive of adjuvant trastuzumab benefit[72]. Evidence for a FcγRIIIa V allele-restricted pCR benefit from dual anti-HER2 neoadjuvant therapy in HER2-positive breast cancer has also recently been reported[95].

4. Objective of the study

4.1 Primary objective

To evaluate variations of host immune response parameters to either trastuzumab SC or trastuzumab IV given in combination with pertuzumab and chemotherapy as neoadjuvant treatment of patients with T2-4d primary HER2-positive breast cancer.

4.2 Secondary objectives

- To evaluate immune biomarkers that may be related with variations of host immune response and clinical efficacy of each treatment regimen
- To evaluate safety and tolerability of each treatment regimen, including pre-operative (neoadjuvant) and post-operative (adjuvant) treatment.
- To evaluate HRQOL during study treatment based on FACT-B.
- To evaluate clinical efficacy of each treatment regimen based on:
    - complete pathological response rate
    - clinical response rate
    - breast conserving surgery rate
    - time to clinical response
    - disease-free survival
    - progression-free survival

5. Materials and methods

5.1 study design

5.1.1 Overview of Study Design

This is a non-comparative phase II open-label, randomized, multicenter, neoadjuvant trial to evaluate variations of host immune response parameters to either trastuzumab SC or trastuzumab IV given in combination with pertuzumab and chemotherapy in chemo naive patients with early stage HER2-positive breast cancer whose primary tumors are > 2 cm and who were scheduled to receive neoadjuvant therapy. In addition, both efficacy and safety of the study treatment regimen were evaluated.

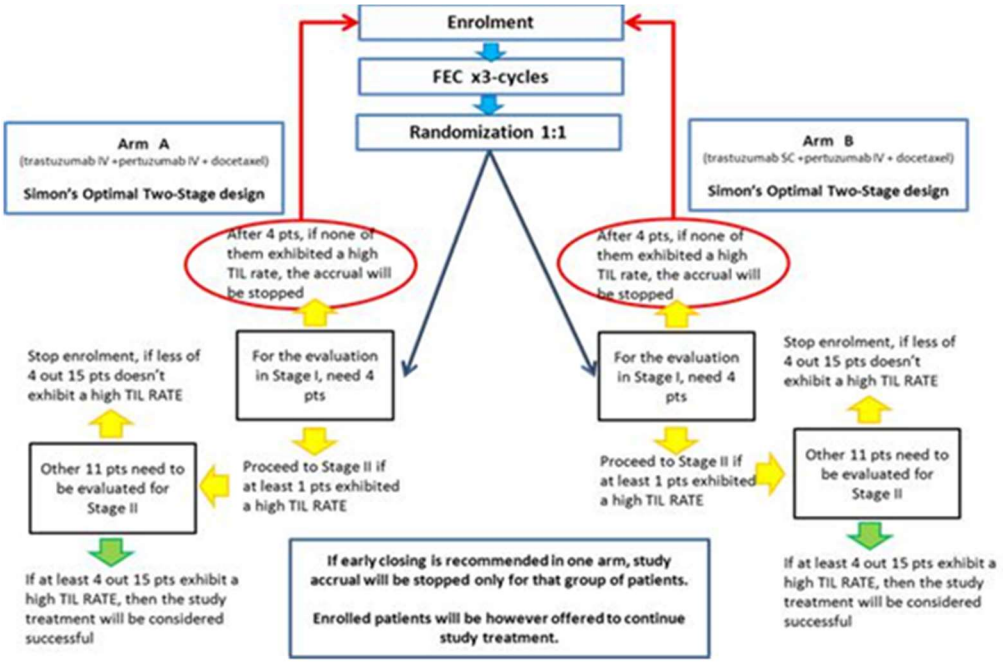


FIG 6. Study Design Diagram

This is a double-blind trial. In fact, the operator who made the biological evaluations is not the same person who followed the patient during neo-adjuvant treatment. The anonymization and the encryption of the arm allocation, guaranteed the blinding for the statistician too.

To participate in the trial, a patient had to fulfill all inclusion/exclusion criteria and need to consent to allow the storage of blood and tumor tissue samples for immune-biomarker analyses as specified in Section 5.4.2.

Enrolled subjects received run-in chemotherapy consisting of 5-fluorouracil, epirubicin and cyclophosphamide (FEC) every 3 weeks (Q3W) for 3 cycles. Once run-in chemotherapy was completed, subjects were randomized to either Arm A to receive trastuzumab IV, pertuzumab and docetaxel Q3W for 4 cycles, or Arm B to receive trastuzumab SC, pertuzumab and docetaxel Q3W for 4 cycles in the neoadjuvant setting (treatment to be administered in that order). Surgery (breast and sentinel node [SN] or axillary lymph node resection) was completed from 3 to 7 weeks after the last administration of the neoadjuvant treatment; and pCR was analyzed. The primary endpoint of the presence of TILs in residual disease was assessed.

Post surgery all patients received trastuzumab for 14 cycles using the same formulation (SC or IV) of the preoperative phase. After completion of post-operative chemotherapy, patients received radiotherapy as per local clinical standard and those patients whose tumors were estrogen-receptor positive received hormone manipulation as per local clinical standard. In summary, all patients received equivalent cumulative doses of the chemotherapeutic agents and pertuzumab. Cumulative doses of trastuzumab were different by treatment arm because of the different formulations used (SC or IV). Patients whose neoadjuvant study treatment was discontinued prior to surgery were managed as per local practice. Approximately 28 days after the last dose of medication, patients were asked to perform a final safety assessment (called Final Visit/Safety Follow-up Visit). After completion of the study treatment, patients were followed up for disease free survival (DFS) until disease progression or until five years after randomization of the last patient, whichever is earlier. In case of withdrawal from the trial due to cardiac toxicity, the patient had to be followed up for cardiac outcome. Blood samples for immune biomarker analysis were collected on all enrolled subjects.

5.1.2 Study Duration

The expected study duration was approximately 3.5 years (42 months) from enrolment of the first patient into the study to the last study assessment of the last patient (including an approximate 24-month recruitment period). Because this was a phase II study with 2 non-comparative arms, Simon's optimal 2-stage design was used for each of the 2 study arms. Interim primary analysis was conducted when the last subject of the first study stage, evaluable for primary endpoint, had surgery following neo-adjuvant chemotherapy. Based on the results of the first-stage, if early closing was recommended, patients on treatment on that date were however offered to continue study treatment until conclusion of their adjuvant phase. Clinical and molecular data for primary and secondary analyses were collected.

Patients were followed up until 5 years since enrollment, or earlier, in the case of withdrawal of consent, loss to follow-up, death, or study closure.

5.1.3 End of Study

The end of study was five years after randomization of the last patient or progressive disease relapse experienced in all patients, whichever is earlier.

5.1.4 Number of Subjects/Assignment to Treatment Groups

Because this is a phase II study with 2 non-comparative arms, Simon's optimal 2-stage design will be used for each of the 2 study arms. A total of 60 patients had to be recruited (first stage: 16 patients), to ensure the desired sample size of at least 30 patients, necessary for the primary analysis. After run-in chemotherapy phase, patients were

randomly assigned using dynamic allocation to Arm A or B.

5.1.5 Centers

This was a multicenter clinical trial. 21 Italian Centres affiliated to the Gruppo Oncologico Italiano di Ricerca Clinica (GOIRC) participated in this study. The coordinating centre was the Medical Oncology Unit, University Hospital of Parma.

5.2 Study Population

5.2.1 Overview

Female patients with early stage HER2-positive breast cancer whose primary tumors were > 2 cm with no metastases.

5.2.2 Inclusion Criteria

5.2.2.1 Disease Specific Inclusion Criteria

- Patients with locally advanced, inflammatory or early stage, unilateral and histologically confirmed invasive breast cancer.
- Primary tumor > 2 cm in diameter.
- HER2-positive breast cancer. Tumors had to be HER2+++ by IHC or FISH/CISH+ (FISH/CISH mandatory for HER2 ++ tumors) according to the Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: ASCO/CAP Clinical Practice Guideline Update.
- Availability of FFPE tissue for biologic and molecular examination before starting primary treatment.

5.2.2.2 General Inclusion Criteria

- Age ≥ 18 years.
- Baseline LVEF ≥ 50% (measured by echocardiography or MUGA).
- Performance status ECOG ≤ 1.
- At least 4 weeks since major unrelated surgery, with full recovery.
- A negative pregnancy test had to be available for pre-menopausal women and for women less than 2 years after the onset of menopause.
- Signed informed consent.

5.2.3 Exclusion Criteria

5.2.3.1 Cancer related Exclusion Criteria

- Metastatic disease (Stage IV) or bilateral breast cancer.
- Previous anticancer therapy or radiotherapy for any malignancy.
- History of other malignancy which could affect compliance with the protocol or interpretation of results. Patients with curatively treated carcinoma in situ of the cervix or basal cell carcinoma, and patients with other curatively treated malignancies who had been disease-free for at least 3 years, were eligible. Patients with previous DCIS of the breast were also eligible for the study.

5.2.3.2 Hematological, biochemical and organ function

- Inadequate bone marrow function (e.g. Absolute Neutrophil Count (ANC) < 1.5 x 109/L, Platelet count < 100 x 109/L and Hb < 9 g/dL).
- Total bilirubin greater than the upper limit of normal (with the exception of Gilbert's syndrome), AST (SGOT), ALT (SGPT) > 2.5 x ULN.
- AST (SGOT) or ALT (SGPT) > 1.5 × ULN with concurrent serum alkaline phosphatase > 2.5 × ULN. Serum alkaline phosphatase may be > 2.5 × ULN only if AST (SGOT) and ALT (SGPT) are < 1.5 × ULN.

- Inadequate renal function, serum creatinine > 1.5 x ULN.
- Uncontrolled hypertension (systolic > 150 and/or diastolic > 100), unstable angina, CHF of any NYHA classification, serious cardiac arrhythmia requiring treatment (exception, atrial fibrillation, paroxysmal supraventricular tachycardia), history of myocardial infarction within 6 months of enrollment, or LVEF < 55%.
- Dyspnea at rest or other diseases which require continuous oxygen therapy.

5.2.3.3 Other Study Drug Related Exclusion Criteria

General Exclusion Criteria

- Severe uncontrolled concomitant disease that contraindicated the use of any of the drugs used in this study or that could put the patient at high risk for treatment-related complications, such as uncontrolled systemic disease (e.g., pulmonary [including interstitial lung disease]) or metabolic disease, wound healing disorders, ulcers, or bone fractures).
- Pregnant and/or lactating women.
- Subjects with reproductive potential not willing to use highly effective non-hormonal method of contraception or two effective forms of non-hormonal contraception. Contraception use had to continue for the duration of study treatment and for at least 7 months post discontinuation of study treatment.
- Received any investigational treatment within 4 weeks of study start.
- Subjects with known infection with HIV, HBV, HCV.
- Known hypersensitivity to any of the study drugs or excipients.
- Subjects assessed by the investigator to be unable or unwilling to comply with the requirements of the protocol.

5.2.4 Concomitant Medication and Treatment

5.2.4.1 Allowed Therapies

In general, all medications taken by the patient for concomitant diseases had to continue during the study treatment period and be recorded on the eCRF. The following list of allowed medications was provided as a guidance, treatment prescribed to the patients had to be adapted according to local standard practice.

5.2.4.2 Excluded Therapies

The following therapies were excluded during the study:

- Anti-cancer therapies other than those administered in this study, including cytotoxic chemotherapy, radiotherapy, (except for adjuvant radiotherapy for breast cancer after completion of chemotherapy) immunotherapy, and biological anti-cancer therapy.
- Any targeted therapy.
- Treatment with steroids except for thyroid hormone replacement therapy and short term corticosteroid, in order to treat or prevent allergic or infusion reactions.
- High doses of systemic corticosteroids. High dose was considered as > 20 mg of dexamethasone a day (or equivalent) for > 7 consecutive days.
- Any investigational agent, except for those used for this study.
- Initiation of herbal remedies. Herbal remedies initiated prior to study entry and continuing during the study were permitted and had to be reported on the appropriate eCRF.
- The following treatments were avoided because of the risk of immunosuppression:
    - Chronic or high-dose oral corticosteroid therapy.

- Tumor necrosis factor-α inhibitors.
- Anti-T-cell antibodies.
- Any oral, injected or implanted hormonal methods of contraception.

5.2.5 Criteria for Premature Withdrawal

Subjects had the right to withdraw from the study at any time for any reason. The investigator also had the right to withdraw subjects from the study in the event of intercurrent illness, adverse events, treatment failure after a prescribed procedure, protocol violation, cure, administrative reasons or for other reasons. An excessive rate of withdrawals could render the study uninterpretable; therefore, unnecessary withdrawal of subjects had been avoided.

5.2.6 Replacement Policy

5.2.6.1 For Subjects

Subjects who withdrew from the study were not replaced.

On achieving the planned study accrual of 60 patients, if less than 50% of cases were expected to exhibit residual tumor disease after neoadjuvant treatment, 3 more subjects were enrolled (total: 63 patients) to ensure the predefined sample size necessary for primary analysis.

5.2.6.2 For Centers

A center may be replaced for the following administrative reasons:

- Excessively slow recruitment.
- Poor protocol adherence.

5.3 Schedule of assessment and procedures

The complete schedule of assessments is presented in Table 9 (see next pages). Subsequent sections provide some specifications and clarifications but do not contain the full list of assessments and procedures.

Table 9 outlines the schedule of assessments during the treatment period, and the 28 day follow up period. The follow-up period for a single patient, started when she has completed study treatment, or stopped study treatment for reasons other than disease relapse.

5.3.1 Screening Examination and Eligibility Screening Form

All subjects had to provide written informed consent before any study specific assessments or procedures are performed. A screening examination was performed between -14 and -1 days before the first study dose (Day 1 of the run-in chemotherapy phase). Patients who fulfilled all the inclusion and none of the exclusion criteria were accepted into the study.

An Eligibility Screening Form (ESF) documenting the investigator's assessment of each screened subject with regard to the protocol's inclusion and exclusion criteria was completed by the investigator.

A screen failure log was maintained by the investigator.

5.3.2 Procedures for Enrollment of Eligible Patients and Assignement to Treatment

All subjects who entered into the screening period for the study received a unique

subject identification number before any study procedures are performed. This number was used to identify the subject throughout the clinical study and on all study documentation related to that subject. The subject identification number remained constant throughout the entire clinical study; it was not changed at the time of rescreening, enrollment, or randomization. This number had not necessarily be the same as the randomization number assigned for the study.

Randomization was administered by a central randomization center via interactive voice and web system (IXRS). Upon completion of run-in chemotherapy, the site contacted the IXRS to receive a unique subject randomization number in order to randomize the subject centrally to receive either trastuzumab IV (Arm A) or trastuzumab SC (Arm B) in a 1:1 manner. As confirmation, the investigator provided a written verification of each patient's registration. Trastuzumab-containing treatment occurred within 5 working days of the patient being randomized into the study. No patient began trastuzumab-containing treatment prior to randomization and assignment of a randomization number. After surgery, the site contacted the IXRS to register the subject into the adjuvant treatment phase.

The sponsor notified the investigators if the study was placed on administrative hold, and when the study was completed or closed to further patient enrollment. Because this is a phase II study with 2 non-comparative arms, Simon's optimal 2-stage design will be used for each of the 2 study arms. Interim primary analysis was conducted when the last subject of the first study stage, evaluable for primary endpoint, had surgery following neoadjuvant chemotherapy. Based on the results of the first-stage, if early closing was recommended, patients on treatment on that date were however be offered to continue study treatment until conclusion of their adjuvant phase. Clinical and molecular data for primary and secondary analyses were collected. The investigator or designee used the electronic Case Report Form to create a new patient with the assigned subject identification number. The patient randomization numbers were to be allocated dynamically in the order in which the

patients are randomized. A Patient Enrollment and Identification Code List was maintained by the investigator. The pass-word-protected and/or encrypted electronic Master Randomization List was kept in a central repository by the Biometrics and Drug Safety Departments.

5.3.3 Clinical Assessments and Procedures

5.3.3.1 Diagnosis of Breast Cancer and Surgical Assessment

Diagnosis of primary breast cancer was performed as per local standard of care. The site surgeon assessed the breast tumor during screening and identified the planned surgical procedure required after completion of neoadjuvant treatment. Following surgery the actual surgery procedure performed was reported.

5.3.3.2 Core Biopsy

It is recommended to use a 14 gauge needle by means of an automatic device fired 3-4 times into the lesion to collect sufficient amount of tumor tissue. At least 3-4 cores had to be collected, to allow for routine pathological examinations, immunohistochemical studies and for molecular analysis.

5.3.3.3 Definition of Pathological Complete Response

Complete absence of infiltrating tumor cells in the breast and in lymph nodes at microscopic examination of the tumor remnants after surgery following primary systemic therapy.

5.3.3.4 Tumor Response Criteria

The baseline breast tumor had to be > 2 cm and measured by mammogram and clinical breast examination. Any additional conventional methods employed as per local medical practice, such as breast ultrasound (US) and MRI was collected. At baseline, Liver

US and Chest X-ray are mandatory. Baseline bone scan was performed if clinically indicated or in case of positive axillary nodes. CT and/or F18 PET scans are acceptable forms of tumor assessment for the presence of distant metastases.

During pre-operative treatment, tumor response was measured using clinical breast examination (CBE) (including breast/axilla/supraclavicular fossa) and/or mammography or other conventional methods as per local medical practice. Before starting treatment, the tumor had to be marked using the method which is standard locally (for example skin tattoo or surgical clip) so that the appropriate excision could be made should the patient experience complete clinical regression of the tumor during therapy. After completion of neoadjuvant treatment and prior to surgery a further tumor response assessment was required including a CBE and mammogram.

Consistency of consecutive mammograms, CBE, or MRIs had to be ensured during all assessments for each patient, with the same technique being used for evaluating the target lesion throughout the treatment period. Tumor measurements had to be made by the same investigator/radiologist for each patient during the study to the extent that this is feasible. In case of clinically measurable superficial (such as skin) lesions, repeated photographs had to be used to document tumor response. These photos had to include a ruler for documentation purposes.

If the lesion showed clear signs of progression, the patient was removed from study treatment and provided with the local standard of care. Discovery of contralateral ductal carcinoma in situ during neoadjuvant treatment was not considered progressive disease. However, invasive contralateral breast carcinoma was classified as progressive disease.

The complete pathological response rate was assessed at a local level following surgery as previously described (Provenzano E, et al. Mod Pathol 2015;28:1185–201). Any residual or necrotic tumor and surrounding tissue specimens was removed and formalin fixed, paraffin embedded and sent to the reference laboratory for TILs analysis, which determines the primary end-point of the study. All pathological response rates were

assessed locally and not independently reviewed.

5.3.3.5 Scheduling of Tumor Assessments

Baseline total tumor burden had to be assessed within a maximum of 2 weeks before first dose of study drug treatment.

Post-baseline tumor assessments were done:

- after completion of run-in FEC chemotherapy and up to 1 day prior to randomization (Arm A vs. Arm B).
- after completion of trastuzumab-containing neoadjuvant therapy (up to 7 days prior to surgery).

If there was suspicion of disease progression based on clinical or laboratory findings before the next scheduled assessment, an unscheduled assessment had to be performed. If a patient inadvertently missed a prescribed tumor assessment or a technical error prevented the evaluation, the patient could continue treatment until the next scheduled assessment, unless signs of clinical progression were present.

5.3.3.6 Performance Status

Performance Status (PS) was measured using the ECOG Performance Status Scale. It is recommended, where possible, that a patient's PS was assessed by the same person throughout the study. PS was assessed at each visit and at final visit.

5.3.3.7 Health-related quality of life (HRQOL)

Health-related quality-of-life (HRQoL) is a multidimensional concept encompassing physical, social, emotional, cognitive and role-related well-being, along with the impact of disease-related symptoms, therapy-induced side effects, and even the financial impact of illness. In women with breast cancer, HRQoL may be adversely affected by general cancer-related factors, such as fatigue, pain and concerns about the illness, along with breast cancer-specific considerations, such as perceived attractiveness or sense of

femininity. It is particularly important to demonstrate that any clinical benefits of a novel therapy are not compromised by reductions in HRQoL arising from adverse events or other treatment-related factors.

Enrolled patients completed validated local-language versions of the Functional Assessment of Cancer Therapy-Breast (FACT-B) questionnaire (FACIT.org) at baseline (within ≤ 2 weeks before first dose of study drug treatment); and:

- after completion of run-in FEC chemotherapy (up to 1 day prior to randomization);
- after completion of trastuzumab-containing neoadjuvant therapy (up to 7 days prior to surgery);
- every 3 months during adjuvant treatment;
- at the Safety Follow-up Visit.

FACT-B (FACIT.org) is a 37-item HRQoL questionnaire comprising the FACT-General (FACT-G) generic cancer instrument and the disease-specific Breast Cancer Subscale (BCS). FACT-G evaluates HRQoL across four domains: physical well-being (PWB), social/family well-being, emotional well-being, and functional well-being (FWB). The BCS consists of 10 items addressing symptoms and issues specifically relevant to patients with breast cancer. Respondents rate each item on a four-point scale, from 0 (‘not at all’) to 4 (‘very much’), with higher scores representing better HRQoL.

5.3.3.8 Clinical Safety Assessments

The NCI Common Terminology Criteria for Adverse Events (CTCAE; version 4) was used to evaluate the clinical safety of the treatment in this study. Patients were assessed for adverse events at each clinical visit and as necessary throughout the study.

A complete medical history (including demographics) was performed at screening. Complete and limited physical examination was performed at each visit. Height at

screening and weight were measured at each cycle to allow calculation of body surface area (BSA) and treatment dose.

5.3.3.8.1 Cardiac Safety: ECG

A twelve lead ECG was performed at:

- Screening.
- Prior (within ≤ 7 days) to trastuzumab-containing treatment.
- After completion of trastuzumab-containing treatment (up to 7 days prior to surgery).
- Every 3 months during adjuvant treatment and at the Safety Follow-up Visit (within ≤ 7days).
- As clinically indicated.

5.3.3.8.2 Cardiac Safety: Echocardiography

Echocardiography is the preferred method to evaluate cardiac function and was conducted as specified in the schedule of assessments and as medically indicated.

It is recommended that the same echocardiographist performs the cardiac evaluations on a patient throughout the study.

The assessment had to be performed as within ≤ 2 weeks prior to the first administration of study medication and the LVEF had to be ≥ 50% before a subject can be enrolled in the study.

The same method of assessment was used throughout the study for each patient, e.g. if ECHO was used as baseline, all subsequent evaluations had to use ECHOs.

LVEF was performed at:

- Screening.
- After completion of run-in FEC chemotherapy and up to 1 day prior to trastuzumab-containing treatment.
- After completion of trastuzumab-containing treatment (up to 7 days

prior to surgery).

- Every 3 months during adjuvant treatment and at the Safety Follow-up Visit (within ≤ 7days).
- As clinically indicated.

5.3.3.8.3 Cardiac Safety: MUGA

If a MUGA was chosen, the same method of assessment had to be used throughout the study. Investigators had to be aware that there may be local guidelines which govern how many MUGA scans (or amount of radiation) a patient may have in a year, and had to ensure that patients were able to adhere to the cardiac assessment schedule.

5.3.4 Laboratory Assessments

The laboratory assessments and procedures described below were performed according to the schedule of assessment.

Protection of patient confidentiality extended to any data generated from the assaying of these samples.

Biological samples taken from all patients could be infectious and were classified as UN3373 Biological Substance, Category B for shipping purposes.

The procedures for the collection, handling and shipping of Immune Biomarker Research Samples to the reference laboratory are specified in the Sample Handling manual.

5.3.4.1 Safety Laboratory Assessments

Hematology and biochemistry was done within a maximum of 2 weeks before first dose of study drug treatment and as part of regular safety assessments. During the study (neoadjuvant phase), a complete blood count and biochemistry analyses were performed prior to administration of study medication (from day -1 to day 1 of each cycle). Complete blood counts was performed on Day 8 of first cycle where docetaxel was administered to monitor any hematological toxicity, particularly neutropenia; additional monitoring was

performed as clinically indicated. Specifically:

- Hematology: complete blood count, including differential and platelets.
- Biochemistry: glucose, urea/BUN, creatinine, uric acid, total protein, albumin, alkaline phosphatase, AST, ALT, LDH. Serum electrolytes (P-, Ca2+, Na+, K+, Cl-), bilirubin, total, direct and indirect. Bilirubin fractions, direct and indirect, had to be measured only if total bilirubin is ULN.
- Coagulation parameters at entry and post surgery: INR and aPTT.
- Urinalysis: was measured with a dipstick at entry and post surgery.
- Pregnancy test in women of child-bearing potential and for all women < 2 years after the onset of menopause (postmenopausal is defined as ≥ 12 months of amenorrhea) within 2 weeks prior to the first dose of either neoadjuvant or adjuvant treatment. In addition to those time points, the serum pregnancy test was repeated only if clinically indicated. For all other women, documentation had to be present in medical history confirming that the patient was not of childbearing potential. Women who have undergone surgical sterilization were exempt from this assessment.

During adjuvant phase (trastuzumab only).

- Hematology: complete blood count, including differential and platelets and as per local medical practice.

5.3.4.2 Immune Biomarker Research Samples

5.3.4.2.1 Tumour Tissue Samples (Mandatory)

Mandatory tumor samples from the primary tumor (diagnostic biopsy) and tumor samples at definitive surgery were collected in the reference laboratory (Department of Biomedical, Biotechnological and Translational Sciences, Pathological Anatomy and Histology Unit, Faculty of Medicine, University of Parma, Via Antonio Gramsci, 14, 43126,

Parma, Italy) for primary endpoint analysis.

Since uncontrolled oxidation processes on the slides may affect the assessment of immune biomarkers, it is strongly recommended sending tumor blocks. The remaining part of the tumor block (roughly 50%) was returned to the institution. Slides sent to the central laboratory were not returned. In case that slides were sent to the central lab, a minimum of 10 unstained, cut slides of 4 µm each, on positively charged glasses, were required.

Leftovers from the predefined biomarker assessments (e.g. aliquots of tumor cell RNA or DNA) were stored for up to 7 years after database closure at which time they was destroyed. For sampling procedures and shipment, see instructions in the sampling handling manual.

Evaluation of TILs

The pathological analysis included standard diagnostic variables and assessment of the tumor inflammatory reaction (TILs) according to current recommendations [77]. Briefly, stromal lymphocytes were scored quantitatively on H&E stained whole-tumor slides as a continuous variable expressed as stromal percentage area within the tumor boundaries. For tumors with heterogeneous TILs, median values were calculated from multiple counts from different tumor areas. Intra-epithelial TILs were also recorded as well as tertiary lymphoid structures. Tumor regression was scored based on recommended criteria.

The composition of the lymphoid infiltrate was characterized using a series of immunostain markers, as follows:

- LCA (clones, 2B11 e PD7/26)
- CD20 (L26)
- CD3 (2GLV6)
- CD4 (SP35)
- CD8 (SP57)

- CD56 (MRQ-42)
- CD25 (4C9)
- PD-1 (NAT105)
- PD-L1/CD274 (SP142)

Antibodies certified for in vitro diagnostic use (CE-IVD) were purchased from Roche Diagnostic. Immunostains were performed on slides cut from FFPE specimens, processed according to standard procedure on a BenchMark (Roche) automatic immunostainer. Immunostains were developed using polymeric systems, Ultraview Detection Kit e Optiview Detection Kit (Roche).

Single markers were scored quantitatively after image digitalization as percentage stromal area, both individually and as ratios (e.g. CD8+/Foxp3+, CD20/CD3, etc). PD-L1 were evaluated on both tumor and lymphoid cells and expressed as combined score of percentage cell positivity and staining intensity.

5.3.4.2.2 Blood Samples (Mandatory)

Mandatory blood samples (20 mL per sample) for immune biomarker assessment were collected at 3 predefined time points:

- before initiation (within ≤ 2 weeks) of the run-in chemotherapy phase;
- before initiation (within ≤ 1 week) of the trastuzumab-containing treatment;
- after completion of trastuzumab-containing neoadjuvant therapy (up to 7 days prior to surgery).

The sample was centralized at the reference laboratory: Laboratory of Viral Immunopathology, Unit of Infectious Diseases and Hepatology, University Hospital of Parma, Via Antonio Gramsci, 14, 43126, Parma, Italy.

Leftovers from the predefined biomarker assessments (e.g. aliquots of tumor cell RNA or DNA) were stored for up to 7 years after database closure at which time they were destroyed.

Tumor-specific lymphocyte cell activity (TLA)

Immune monitoring for adaptive response was performed on Her-2/neu-specific T-cell responses by ex-vivo detection of IFN-gamma producing cells by Elispot. In the assay T-cell stimulation was evaluated by HLA-class II and HLA-A2 restricted peptides representing previously published epitopes[83]. Additionally, a panel of 15-mer overlapping peptides representing the extracellular domain was used to monitor tumor-specific T-cell response. Patients were typed for HLA-A2 that is known to be expressed in 40-50% of Caucasian population while HLA-class II restricted peptides can bind different HLA class II molecules not requiring typing for this restriction.

A thorough NK-cell analysis was conducted by phenotypic and functional characterization of circulating cells. Phenotype defined frequency and intensity of activating and inhibitory NK-cell receptors and intra cytoplasmic expression of molecules like perforins and granzyme providing cytotoxic potential. These parameters can be affected by treatment and combined with proliferation markers can provide insights for recruitment or in-vivo expansion of effector NK-cells. NK-cell cytokine production and cytotoxicity measured functional activation and its association with clinical benefit. Finally, regulatory T-cells frequency was measured in order to understand if counter active immunoregulatory mechanisms were operative during treatment with possible clinical consequences.

Tumor-specific T-cell frequency by Elispot

We studied tumor-specific T cell activity of patients pre- and post-chemotherapy and post-target therapy. We assessed responses to a number of tumor antigens (HLA-class I and class II restricted Her-2/neu epitopes and overlapping synthetic peptides derived from Her-2/neu) using ELIspot. Peripheral blood mononuclear cells (PBMCs) were stimulated with these antigens and with control antigens (CMV, EBV, Flu, Tet. Toxoid) to trigger cytokine production. Frequency of specific-spots were counted at 3 time points.

NK-cell analysis

- Phenotypic characterization of NK-cells derived from PBMCs

Combinations of different monoclonal antibodies identifying activating or inhibitory receptors as well as death receptors and ligands expressed by the NK cell population characterized peripheral NK cells. The following mAb will be used: anti-CD3, CD16, CD56, NKG2D, NKG2A, NKp30, NKp46, NKp44, 2B4. Moreover, an intracellular staining was performed for the evaluation of Perforin and Granzyme-B content, Ki-67 proliferation cell marker within NK cells and CD3 zeta chain expression. Cells were analyzed on a FACSCantoII flow cytometer by using the FACSDiva Software (Becton Dickinson).

Functional analysis of NK-cells

IFN-γ and TNF-α production by NK cells. NK cells were stimulated with or without IL-12 and IL-18 and after surface staining with anti-CD3, CD56, CD16, cells were fixed and permeabilized using fixation and permeabilization media NK-cells were then stained with anti-IFN-γ and TNF-α mAbs for the detection of intracellular cytokines and analyzed by flow cytometry.

Evaluation of cytotoxic function: CD107a degranulation

Peripheral NK-cells were incubated at 37 °C in the presence or absence of IL-15 (1ng/ ml). After 14 hours incubation the activated NK effector cells were cultured with or without K562 target cells in the presence of anti-CD107a mAb. Brefeldin A (10 µg/ml). The percentage of degranulating (CD107a positive) NK cells within the sample incubated with K562 was quantified by subtracting the percentage of CD107a positive cells detected within the corresponding samples cultured without K562 target cells.

Regulatory T-cells

Frequency of circulating Tregs was defined by multiparametric FACS analysis on lymphomononuclear cell population. Staining for Tregs will be for: CD4+, CD25high,

FoxP3+, CD127neg.

Absolute count of circulating NK-cells and T-cell subsets

Whole blood was stained for CD45, CD3, CD4, CD8, CD19 and CD56 for ex-vivo determination of frequency and count of circulating lymphocyte subsets.

FcγR polymorphisms

At baseline, a 3 mL whole blood sample was taken for DNA extraction from every patient. The DNA will be used to determine polymorphic alleles of the FcγRIIIa, FcγRIIa and FcγRIIB genes.

Genomic DNA was purified from peripheral-blood mononuclear cells (PBMCs) using DNA extraction kit (QIAmp DNA blood mini kit, Qiagen, Valencia, CA). FcγRIIa genotyping, as well as FcγRIIIa genotyping, was performed on genomic DNA by polymerase chain reaction (pCR) followed by direct sequencing in both forward and reverse directions, using previously described methods[51].

Total RNA was purified from peripheral-blood mononuclear cells with the Chomczynski and Sacchi method[62], using TRI Reagent (Sigma-Aldrich, UK) and reverse tran-scribed into cDNA with the GeneAMP RNA pCR Control Kit (Applera, Foster City, CA, USA). FcγRIIb analysis was performed on cDNA using a nested pCR approach[62]. The resulting pCR product was genotyped by direct sequencing in both forward and reverse direction.

Molecular data were independently interpreted by two molecular biologists (N.N. and B.B.) who were unaware of the clinical outcome of study patients. All pCR conditions were available on request.

5.3.4.2.3 Biomarker Sample Repository (BSR) Research Samples

Identification of new biomarkers that correlate with disease activity and the efficacy or safety of study treatment is rapidly developing, and several markers of tumor genesis pathways or mechanisms of either response or toxicity to anti-HER2 therapies remain to be determined.

Based on these considerations, patients were asked to give their consent to use left-overs from the predefined biomarker assessments (e.g. aliquots of tumor cell RNA or DNA) for future, unspecified, molecular analyses. Whenever new biological data became available, appropriate informed consent, exhaustively specifying the molecular biomarkers had to be analyzed, was requested.

5.3.5 Clinical Assessments at the Safety Follow-up visit

Patients who completed the study treatment or discontinued from the study early (because of disease progression or unacceptable toxicity) were asked to return to the clinic 28 days after the last dose of study treatment for a Safety Follow up visit.

The clinical and laboratory assessments to be performed at the Safety Follow-up visit were identical to the assessments during the treatment period except that no study drug was administered. See Table 9 for the schedule of assessments performed at the Safety Follow-up visit.

5.3.6 Survival Follow-up Assessments

Patients were followed up until 5 years since enrollment, or earlier, in the case of withdrawal of consent, loss to follow-up, death, or study closure. Beginning 3 months after the Safety Follow-up visit, regular visits were performed every 4-6 months in the first 2 years, and every 6 months from years 3–5. Annual ipsilateral (after BCS) and/or contralateral mammography +/- ultrasound was performed every 1 year. Routine laboratory and/or imaging tests were indicated at the physician's discretion.

During the survival follow-up period only medication applicable for long-term reporting had to be reported, including: BC treatments (eg, hormonal therapy), new anti-cancer treatments given to treat disease recurrence, or medications related to the

treatment of SAEs that are applicable for long-term reporting (eg, treatment of heart failure). After 5 years from enrollment it was required annual assessment of the patient status (dead or alive; with or without recurrence or second primary tumor).

5.4 Study medicinal product

5.4.1 Dose and Schedule

5.4.1.1 Neoadjuvant (Pre-Operative) Treatment

5.4.1.1.1 Run-in Chemotherapy

5-fluorouracil, epirubicin and cyclophosphamide (FEC) were administered in accordance with their summaries of product characteristics (SmPCs) and/or standard practice. FEC was administered every three weeks depending on hematological toxicity and side effect profile.

5-Fluorouracil:

5-Fluorouracil was administered at 500 mg/m2 on Day 1 of Cycles 1-3. It was given as an IV bolus or infusion in accordance with local policy. Patients with body surface area > 2 m2 had to be dose capped at 1000 mg. Dose delays and reduction for toxicity were permitted. 5-Fluorouracil was administered every three weeks for three cycles (see above and Table 9).

Epirubicin:

Epirubicin was administered at 75 mg/m2 on Day 1 of Cycles 1-3. It could be given as an IV bolus over 3-5 minutes or as an infusion over 30 minutes. Dose delays and reduction for toxicity were permitted. Epirubicin was administered every three weeks for three cycles (see above and Table 9).

Cyclophosphamide:

Cyclophosphamide was administered at 500 mg/m2 on Day 1 of Cycles 1-3. It should be given as an IV bolus over 3-5 minutes or as an infusion, in accordance with local policy. Patients with body surface area > 2 m2 had to be dose capped at 1000 mg. Dose delays and reduction for toxicity were permitted. Cyclophosphamide was administered every three weeks for three cycles (see above and Table 9).

5.4.1.1.2 Post-randomization Neoadjuvant Treatment (Trastuzumab-containing Regimen)

Arm A

Trastuzumab IV, pertuzumab and docetaxel were administered Q3W for 4 cycles. The medicinal products had to be administered sequentially on Day 1 of each cycle as follows: trastuzumab IV first, then pertuzumab IV, and then docetaxel IV.

Trastuzumab IV:

Trastuzumab was administered on Day 1 of each cycle at the required loading dose of 8 mg/kg, as an IV infusion. On Day 22 (three weeks after the first dose, and every three weeks thereafter, trastuzumab was administered at a dose of 6 mg/kg as an IV infusion. The initial dose of trastuzumab was administered over 90 (±10) minutes. Trastuzumab IV was administered by a healthcare provider prepared to manage anaphylaxis and an emergency kit was available. Patients were observed for at least six hours after the start of the infusion for symptoms like fever, chills and other infusion-related symptoms. Interruption or slowing of the infusion might help control such symptoms and might be resumed when symptoms abate. If the infusion was well tolerated, subsequent infusions might be administered over 30 (±10) minutes and patients were observed for two hours after the start of infusion. All infusion-related symptoms had to be resolved before study

treatment was given or the patient was discharged. Patients who experience infusion-related symptoms might be pre-medicated with paracetamol and antihistamines for subsequent infusions. Dose reductions for toxicity was not permitted. Trastuzumab IV was administered every three weeks for four cycles.

Pertuzumab IV:

Pertuzumab was administered on Day 1 of the first cycle at the required loading dose of 840 mg as an IV infusion. On Day 22 (three weeks after the first dose), and every three weeks thereafter, pertuzumab was administered at a dose of 420 mg as an IV infusion. Pertuzumab IV had to be administered 60 minutes after the end of trastuzumab IV administration. The initial dose of pertuzumab was administered over 60 (±10) minutes and patients were observed for a further 60 minutes. The infusion had to be slowed or interrupted if the patient experiences fever, chills and other infusion-related symptoms. If the infusion was well tolerated, subsequent doses might be administered over 30 (±10) minutes and patients were observed for a further 30 minutes for symptoms like fever, chills and other infusion-related symptoms. All infusion-related symptoms had to be resolved before chemotherapy was given or the patient was discharged. Patients who experience infusion-related symptoms might be premedicated with paracetamol and antihistamines for subsequent infusions. Dose reductions for toxicity was not permitted. Pertuzumab was administered every three weeks for four cycles. The pertuzumab IV dose was a fixed dose and did not need to be adjusted for body weight.

Docetaxel IV:

Docetaxel was administered in line with the respective product information and/or recognized clinical practice guidelines. It was administered after trastuzumab IV and pertuzumab IV. The recommended initial dose of docetaxel was 75 mg/m2, administered thereafter on a 3-week cycle. The dose of docetaxel might be escalated to 100 mg/m2 at

the investigator's discretion on subsequent cycles if the initial dose was well tolerated. Patients were closely observed from the start of the infusion for hypersensitivity reactions which might occur within minutes. Severe hypotension, bronchospasm or generalized rash/erythema requires immediate discontinuation of docetaxel and appropriate treatment. The infusion might be slowed for minor symptoms like flushing or local cutaneous reactions. Patients experiencing severe hypersensitivity reactions have to be discontinued from the study. Premedication consisting of an oral corticosteroid, such as, dexamethasone 16 mg per day in divided doses for 3 days starting 1 day prior to docetaxel administration, unless contraindicated, might be used. Similarly, prophylactic G-CSF might be used to mitigate the risk of hematological toxicities. Dose delays and reductions were permitted and prophylactic GCSF to maintain the dosing schedule. Docetaxel was administered every three weeks for four cycles.

Arm B

Trastuzumab SC, pertuzumab and docetaxel were administered Q3W for 4 cycles. The medicinal products had to be administered sequentially on Day 1 of each cycle as follows: trastuzumab SC first, then pertuzumab IV, and then docetaxel IV.

Trastuzumab SC:

A fixed dose of 600 mg/5 mL Herceptin SC, irrespective of the patient's weight, was administered throughout the treatment phase. All doses of Herceptin SC were administered as an SC injection into the thigh by a trained healthcare professional over a period of 2−5 minutes. New injections had to be given at least 2.5 cm from the old injection site(s) and never into areas where the skin was red, bruised, tender or hard. During the course of treatment with Herceptin SC, other medicinal products for SC administration had to be preferably injected at different sites. Patients were observed for 6 hours after the first

injection and for 2 hours after subsequent injections for signs or symptoms of adverse reactions.

Significant injection-related symptoms had eventually been resolved before any subsequent study treatment administration. Patients who experience injection-related symptoms might be premedicated with paracetamol and antihistamines for subsequent injections. Dose reductions for toxicity were not permitted. Trastuzumab SC administration might be delayed to assess or treat adverse events, such as cardiac adverse events, myelosuppression or other events. Trastuzumab SC was administered every three weeks for four cycles. The trastuzumab SC dose was a fixed dose and did not need to be adjusted for body weight. No loading dose was required.

Pertuzumab IV:

Pertuzumab was administered on Day 1 of the first cycle at the required loading dose of 840 mg as an IV infusion. On Day 22 (three weeks after the first dose), and every three weeks thereafter, pertuzumab was administered at a dose of 420 mg as an IV infusion. Pertuzumab IV had to be administered 60 minutes after the end of trastuzumab SC administration. The initial dose of pertuzumab was administered over 60 (±10) minutes and patients were observed for a further 60 minutes. The infusion had to be slowed or interrupted if the patient experienced fever, chills and other infusion-related symptoms. If the infusion was well tolerated, subsequent doses might be administered over 30 (±10) minutes and patients were be observed for a further 30 minutes for symptoms like fever, chills and other infusion-related symptoms. All infusion-related symptoms have to be resolved before chemotherapy was given or the patient was discharged. Patients who experience infusion-related symptoms might be pre-medicated with paracetamol and antihistamines for subsequent infusions. Dose reductions for toxicity was not permitted. Pertuzumab was administered every three weeks for four cycles. The pertuzumab IV dose was a fixed dose and did not need to be adjusted for body weight.

Docetaxel IV:

Docetaxel was administered in line with the respective product information and/or recognized clinical practice guidelines. It was administered after trastuzumab SC and pertuzumab IV. The recommended initial dose of docetaxel was 75 mg/m2, administered thereafter on a 3-week cycle. The dose of docetaxel might be escalated to 100 mg/m2 at the investigator's discretion on subsequent cycles if the initial dose was well tolerated. Patients were closely observed from the start of the infusion for hypersensitivity reactions which may occur within minutes. Severe hypotension, bronchospasm or generalized rash/erythema required immediate discontinuation of docetaxel and appropriate treatment. The infusion might be slowed for minor symptoms like flushing or local cutaneous reactions. Patients experiencing severe hypersensitivity reactions had to be discontinued from the study. Premedication consisting of an oral corticosteroid, such as dexamethasone 16 mg per day in divided doses for 3 days starting 1 day prior to docetaxel administration, unless contraindicated, might be used. Similarly, prophylactic G-CSF might be used to mitigate the risk of hematological toxicities. Dose delays and reductions were permitted and prophylactic GCSF to maintain the dosing schedule. Docetaxel was administered every three weeks for four cycles.

Any overdose or incorrect administration of study drug had to be noted on the Study Drug Administration electronic Case Report Form (eCRF). Adverse events associated with an overdose or incorrect administration of study drug had to be recorded on the Adverse Event eCRF.

5.4.1.2 Adjuvant (Post-Operative) Treatment

Each patient received trastuzumab Q3W for 14 cycles using the same formulation (SC or IV) of the preoperative phase.

Trastuzumab IV:

Trastuzumab IV was administered at a dose of 6 mg/kg as an IV infusion on Day 1 of each cycle. If the interval between surgery and Cycle 1 exceeds 4 weeks a reloading dose of 8 mg/kg was required. Trastuzumab IV was administered every three weeks for 14 cycles.

Trastuzumab SC:

Trastuzumab SC was administered at a fixed dose of 600 mg, irrespective of the patient's weight. The trastuzumab SC dose was a fixed dose and did not need to be adjusted for body weight. No loading dose was required. Trastuzumab SC was administered every three weeks for 14 cycles.

5.5 Statistical considerations and analytical plan

5.5.1 Primary and Secondary Study Endpoints

5.5.1.1 Definition of Analysis Populations

Intent to Treat Population:

All patients receiving any amount of study medication were included in the intent-to-treat population. (Patients were assigned to treatment groups as randomized for analysis purposes). Any patient who was assigned a patient number, but did not receive any study medication, was not included.

On Treatment Analysis Population:

Patients who have received ≥ 6 cycles of study medication (in the neo-adjuvant setting Cycle 1-7), had received no other anti-cancer treatment (non-study medication or radiotherapy) and undergo surgery were included in the on-treatment analysis. This analysis only occured if this population differed by ≥ 15% of the intent-to-treat population.

Safety Population:

The safety population was the same group of patients as the intent-to-treat and includes patients who had received at least one dose of study medication and at least one safety assessment performed at baseline. Patients were assigned to treatment arms as treated.

5.5.1.2 Primary Endpoint

The primary endpoint was post-surgery pathologic TIL rate on residual disease after either trastuzumab IV or trastuzumab SC. The primary endpoint in each sperimental arm evaluated the proportion of patients showing biological significant improvement (effect size = 0.3) after neoadjuvant chemo-immunotherapy against the proportion recorded in the literature [53, 68] for subjects receiving only neoadjuvant chemotherapy (p0 = 0.1).

5.5.1.3 Secondary Endpoints

- To evaluate associations between biomarkers (TILs [at baseline and on residual disease], TLA, and FcγR polymorphisms) and between each biomarker with clinical outcome variables.
- To evaluate safety and tolerability of each treatment regimen, including pre-operative (neoadjuvant) and post-operative (adjuvant) treatment. Toxicities were graded according to the NCI CTCAE version 4.0 and reported as cumulative incidence.
- To evaluate HRQOL during study treatment based on FACT-B:
    - Comparison between mean FACT-B scores assessed at enrolment and mean FACT-B scores assessed before surgery.
    - Comparison between treatment arms of FACT-B scores assessed at predefined time points (see Table 9) and expressed as the area under the curve (AUC).

- To describe prognostic aspects of each treatment regimen based on:
    - Comparison of response rates between treatment arms:
        - complete pathological response rate
        - clinical response rate
        - breast conserving surgery rate
    - Comparison of median survival times between treatment arms:
        - 5-year disease-free survival
        - 5-year progression-free survival
        - Time to clinical response

Definitions of clinical outcome variables:

Post-surgery pathological complete response rate (pCR)*:* pCR is defined at the time of surgery and the rate is the proportion of the intent-to-treat population that achieves a pCR.

Clinical response rate: Clinical response is defined as complete response (CR), partial response (PR) stable disease (SD) and progressive disease (PD) and is identified as per local practice using RECIST criteria v1.1 as a guide[96].

Time to clinical response: Time to clinical response rate is defined as the time from the date of first dose received to the date of assessment of clinical response.

Breast conserving surgery rate: This is defined as the proportion of patients who achieved breast conserving surgery out of the intent-to-treat population without inflammatory breast cancer, as these patients will receive mastectomy irrespective of their response to neoadjuvant treatment.

Disease-free survival (DFS): This is defined as the time from the first date of no disease (i.e. date of surgery) to the first documentation of relapse disease or death. Any evidence of contralateral disease in-situ will not be identified as progressive disease. DFS

was described separately in patients who achieve a pCR from those who did not and overall for all patients that had surgery. Patients who were withdrawn from the study without documented progression and for whom where existed eCRF evidence that evaluations had been made, were censored at the date of the last assessment when the patient was known to be disease-free.

Progression-free survival (PFS): This is defined as the time from the date of randomization to the first documentation of progressive disease or death. Any evidence of contralateral disease in-situ was not identified as progressive disease. Patients who were withdrawn from the study without documented progression and for whom there exists eCRF evidence that evaluations had been made, were censored at the date of the last assessment when the patient was known to be free from progressive disease. Patients without post baseline assessments but known to be alive were censored at the time of randomization.

5.5.1.4 Safety

Safety of the treatment regimen, including pre-operative (neoadjuvant) and post-operative (adjuvant) treatment were evaluated as follows:

- Incidence of symptomatic cardiac events and asymptomatic LVEF events.
- LVEF measures over the course of the study.
- Incidence and severity of adverse events and serious adverse events.
- Laboratory test abnormalities.

Toxicity (both predefined and not predefined side effects was recorded, classified, graded and managed according to NCI Common Terminology Criteria for Adverse Events (CTCAE; version 4). Health-related quality of life (HRQOL) was measured by the Functional Assessment of Cancer Therapy-for patients with Breast Cancer (FACT-B) questionnaire.

All patients who had received at least one dose of treatment and at least one post-base-line safety assessment were included in the safety evaluation.

5.5.2 Statistical and Analytical Methods

This was a non-comparative, phase II multicenter, open-label, neoadjuvant, randomized study. The purpose of randomization is to reduce bias owed to patient selection into treatments groups.

The primary endpoint of this study was the TIL rate on residual disease after either trastuzumab IV or trastuzumab SC.

Statistical analyses for secondary endpoints are described in a subsequent Section.

5.5.2.1 Hypothesis Testing and Sample Size

Because this was a phase II study with 2 non-comparative arms, Simon's optimal 2-stage design was used for each of the 2 study groups. For each arm we assume:

- p1 = 0.4, expected rate of subjects with high TILs on residual disease (see Section 5.4.2.1 for definition of TILs), which, if verified, would imply a biologically significant improvement with respect to p0
- p0 = 0.1, lowest limit of the subject rate, which if verified would imply the absence of an improvement of interest. This value is the rate that would be expected if only the chemotherapeutic treatment was administered
- alpha = 0.05
- beta = 0.20

For each arm, the first stage ends after 4 patients have been evaluated, and if none of them exhibited a biologically significant improvement, the study was terminated. Otherwise, enrolment continued until a sample size of 15 individuals had been reached. If at least 4 (out of 15 pts) showed a biological significant improvement, then the study

treatment was considered successful.

As stated above, considerations for one study group mirror those of the other, therefore sample size in the 2 groups was 30 patients.

Only those subjects for whom, at the end of neoadjuvant treatment, histological exam exhibited a residual tumor pattern, which was estimated to be present in 50% of cases, were assessed for the primary analysis. Thus, a total of 60 patients were recruited (first stage: 16 patients), to ensure the desired sample size of at least 30 patients, necessary for the primary analysis.

Subjects were defined with high TILs vs. low TILs on residual disease after evaluation of histological specimens obtained by breast surgery at 27 weeks (max 40) after enrolment. The threshold for classifying subjects with high TILs, or not, was defined as equal to 15%, according to the median TIL rate observed in primary tumors of patients with HER2-positive breast cancer as described by Savas et al.[68]. However, this value (15%) had been observed in primary tumor diagnostic biopsies before any chemotherapy treatment. Otherwise, our study was focused on the evalution of TILs on post-neoadjuvant residual disease. It was therefore necessary to assess the pertinance of the literature value against a value observed in our study subsample. When the 16th study patient had surgery, it was possible to calculate the median TIL rate of our subsample. The pertinance with the literature value was tested trough the Wilcoxon signed-rank test for single sample. If null hypothesis was not rejected, we used the literature value (median TIL rate of 15%) as robust value for our study. The choice of the subsample of 16 patients derived from the number of patients required for the primary endpoint evaluation. In fact, using two-stage Simon's design, stage I requires 4 patients for each arm but, as already discussed, the likelihood to observe a patient with residual disease after study neoadjuvant treatment is 50%. Therefore, a number of approximately 16 patients was necessary to be reached to have 8 subjects of interest. For ethical and organizational purposes, we decided to consider this aspect (likelihood to observe a patient with residual disease) by using

probability calculus. We hypotized that in this case the observation of 8 patients of interest (interpreted as success) is binomial distributed with p = 0.5. Therefore, the likelihood to exactly observe 8 patients of interest out of 16 is 19.6%, while the likelihood to observe at least 8 patients of interest out of 16 is 40.2%. Based on these considerations, we estimated that, with a probability of 75%, we needed to enroll up to 20 patients before observing at least 8 patients of interest. These probabilistic considerations suggest that it may be reasonable to add a 5% more of the planned sample size (total: 63 patients). If 63 subjects are enrolled, the likelihood to observe at least 30 subjects of interest increases by 16% (from 44% to 60%).

5.5.2.2 Secondary Endpoint Analyses

- Associations between biomarkers (TILs at baseline, TLA, and FcγR polymorphisms) and between each biomarker with pCR were evaluated by the Chi-square test for categorical variables. The choice of using a parametric method is justified in this case by the total number of study subjects (approximately 60) and by the variability reduction of dichotomous variables.
- Associations of TILs on residual disease with TILs at baseline and TLA were evaluated by the Fisher exact test for categorical variables. The choice of using a non-parametric method is justified in this case by the small study sample size. Diagnostic tests such as sensitivity, specificity, PPV, and NPV were recorded by comparing TILs at baseline with TILs on residual disease. The degree of concordance of these values was evaluated.

The Chi-square test was used to assess whether the polymorphic variants of FcγRIIa, FcγRIIIa and FcγRIIb in the study population were in Hardy–Weinberg equilibrium. Associations of FcγR polymorphisms with TILs (at baseline and on residual disease) and TLA were evaluated by the p for trend test. The choice of using this methodology is due to the small study sample size, which limits the use of multiple comparisons.

Furthermore, the hypothesis for the presence of an almost linear trend in transition from one homozygous genotype to the other is extremely plausible.

- Comparisons of median survival times (DFS, PFS, time to clinical response) by TILs (either present at diagnosis or on residual disease), TLA and FcγR polymorphisms were evaluated using the Log Rank Test.
- For exploratory comparison of TIL rates between the trastuzumab SC arm and the trastuzumab IV arm, relative risk (RR) was calculated as the ratio of patients with high TIL rates on residual disease allocated to arm B to patients with high TIL rates on residual disease allocated to arm A.
- To evaluate HRQOL:
- Life tables were calculated according to the Kaplan-Meier method. Comparison of the treatment arms were carried out using log-rank test analysis.
    - the difference between mean FACT-B scores assessed at enrolment and mean FACT-B scores assessed before surgery was evaluated using the Student t test for dependent samples;
    - the difference between the two treatment regimens in terms of mean FACT-B scores assessed at enrolment and before surgery was evaluated using the Student t test for independent samples;
    - FACT-B scores will be assessed at predefined time points (see Table 9) and comparison between treatment arms was expressed as the area under the curve (AUC).
- Toxicities were graded according to the NCI CTCAE version 4.0 and reported as cumulative incidence.
- For interpretive purposes, we might use an unconditional logistic regression model to explore the cause-effect relationships in a multivariate fashion.

According to this, for temporal aspects, hazard ratios and 95% confidence intervals will be obtained using semiparametric Cox multivariate regression model.

5.5.2.3 Randomization

Randomization list was created by the Research and Innovation Unit of the University Hospital of Parma (via Antonio Gramsci 14, 43126 Parma, Italy), which had not any active role in patient treatment. Since the evaluation of the primary end point was centralized, the enrolment was competitive for the 21 recruiting centers, and there was no relevant etereogenity between centers in terms of geographical distribution and patient accrual, the most appropriate kind of randomization was:

- Simple: each patient had the same likelihood to receive one of two study treatments.
- Balanced: the size in the two arm had to be the same.

Randomization was electronically performed by an application developed in the e-CRF.

5.5.2.4 Missing Data

The management of missing data in this study was espected to be limited to the evaluation of secondary end-points and to be absent for primary end-point analysis. Implementation of automatic check and automatism in the eCRF guaranteed a high quality level of data due to the lowering of missing data and the robustness of data inserted.

However, since this study had established a quality of life evaluation trough the FACT-B questionnaire, in this case, missing data could occur more frequently. To manage this risk, in order to obtain a high level of compliance, the operator who delivered the FACT-B questionnaire at patient's enrolment and before surgery, completed a Data Reporting Form. This form reported information about questionnaire completeness, patient's compliance to the questionnaire, and patient's clinical/emotional condition while compiling

the questionnaire. The operator who delivered the FACT-B questionaire before surgery had not to be the same who had delivered it at study enrolment.

If the questionnaire was not complete, totally or in part, the operator had to document the reasons of non-compliance. Questionnaires were considered valid, and used for analysis only if presenting more than 80% of completed items (at least 30).

In presence of missing data, prorating by subscale was acceptable as long as more than 50% of the items were answered (e.g., a minimum of 4 of 7 items, 4 of 6 items, etc.) with the exception of the last section “other problems”, where a 80% completeness (a minimum of 8 of 10 items) was acceptable. The total score was then calculated as the sum of the unweighted subscale scores. The FACT scale was considered to be an acceptable indicator of patient quality of life as long as overall item response rate was greater than 80% (e.g., at least 30 of 37 FACT-B items completed).

6. Results

6.1 Study population

The present study refers to 63 patients.

6.2 Patients characteristics

The characteristics of the 63 patients enrolled in the 13 Oncology Units participating in the trial (Parma, Verona, Bolzano, Piacenza, Ferrara, Reggio Emilia, Sassuolo, Verona, Bologna (2), Torino, Rimini, Bergamo) are shown in table 6.

Baseline patients and tumor characteristics:

- The median age at tumor diagnosis time is approximately 50,62 years. In detail: 2 have between 25-29 years old, 5 have between 30-34 years old, 3 have between 35-39 years old, 11 have between 40-44 years old, 9 have between 45-49 years old, 7 have between 50-54 years old, 13 have between 55-59 years old, 4 have between 60-64 years old, 3 have between 65-69 years old, 4 have between 70-74 years old, 2 have between 75-79 years old.
- 13 patients (20%) had a tumor size > 5 cm (T3 according to the TNM classification);
- 44 patients (70%) had lymphovascular invasion;
- 34 patients (54%) had a grading tumor G3, poorly differentiated;
- 44 patients (70%) had an estrogen-receptor-positive cancer;
- 25 patients (40%) had a progesterone-receptor-positive cancer;
- 53 patients (84%) had a high proliferative activity tumor (KI67 > 15%).
- The number of randomized patients is 63: 32 treated with Trastuzumab SC (50,8 %) and 31 treated with Trastuzumab IV (49,2%).

- The operations performed are 63: 27 mastectomy (42,85%) and 35 (55,55%) quadrantectomy.

6.3 Treatment toxicity

The main treatment toxicities are shown in table 7.

- Hematological toxicities (Leukopenia or Neutropenia) > G3-G4 were found in 23 patients (36,5%).
- Non-haematological toxicities (Alopecia, Diarrhea, Mucositis, Neurologic Toxicity) were found in 13 patients (20,6%).
- Dose reduction and treatment delays occurred in 12 (19%) and 20 (32%) patients, respectively, mostly due to haematological toxicity (G3-G4 neutropenia).

6.4 Final evaluation of the Primary-Endpoint

The primary endpoint is post-surgery pathologic TILs rate on residual disease after either trastuzumab IV or trastuzumab SC.

Because this is a phase II study with 2 non-comparative arms, Simon's optimal 2-stage design has been used for each of the 2 study groups.

Since the likelihood to observe a patient with residual disease after study neoadjuvant treatment is 50%, 63 patients have been enrolled up for the stage 2 evaluation. Under binomial distribution assumption, 63 patients enrolled ensure that, with probability of 69%, at least 30 patients with residual disease will be observed (15 for each arm).

Subjects will be defined with high TILs vs. low TILs on residual disease after evaluation of histological specimens obtained by breast surgery. The threshold for classifying subjects with high TILs, or not, is defined as equal to 15%, according to the

median TIL rate observed in primary tumors of patients with HER2-positive breast cancer as described by the literature. However, this value (15%) has been observed in primary tumor diagnostic biopsies before any chemotherapy treatment. Otherwise, our study is focused on the evaluation of TILs on post-neoadjuvant residual disease. It is therefore necessary to calculate the median TIL rate of our subsample.

As can be seen in table 8 below, on the first 63 patients enrolled, the number of patients that can be evaluated was 11 and 13 respectively for arm A and B. Based on our statistical projection, for considering the study conclusive we should have had at least 4 patients for each treatment arm with high values of TILs on the residual disease. In arm A patients with high values of TILs were 3 out of 11, but this is due to the fact that pCR rate was greater than expected (64.5%). Consequently, the study cannot be considered conclusive, but in any case it is not negative because if we had 15 patients with residual disease, most likely patients with high values of TILs would have been at least 4. Differently, in arm B patients with high TILs values were 6 out of 13, so the study is considered conclusive despite the fact that pCR rate was greater than expected (59.3%).

In graph 1 it can be observed that the Baseline Stromal TILs median value in our sample is approximately 10% for arm A, and around 5% for arm B; this justifies the median value of 7.5% reported in table 8. Considering the median TILs rate of our subsample, 3 and 6 patients for arm A and B are defined with High TILs on residual disease, respectively. The mean difference in lymphocyte infiltration between pre- and post-treatment in percentage points shows that in arm B there is an increase of TILs between pre- and post-treatment of 46% compared to the more moderate increase of 27% in arm A. Neoadjuvant therapy with either SC or IV trastuzumab in combination with pertuzumab and chemotherapy had a significant effect on sTIL expression after surgery. In particular, the SC trastuzumab-based arm exerted the most relevant enrichment of sTILS in post-treatment residual tumors. Therefore, the arm B seems to obtain a better biological response.

The clinical outcome was determined not only by the amount of lymphocytic infiltration but also by the infiltrate phenotype.

Interestingly, as can be seen in graph 2 below, a significant inverse correlation was observed between PDL1 expression on pre-treatment sTILs and the T cell co-receptor CD3 expressed on post-treatment sTILs (Pearson's $\rho = -0.70$). This finding was particularly evident in the arm B group ($\rho = -0.85$) as it can be highlighted from the graph 3.

In addition, as it can be deduced from the graph 4, high values of PD-L1 expressing lymphocytes in the primary tumor at diagnostic biopsy represent a predictive factor in favour of pCR only in arm A. Indeed, all the 7 patients with high PD-L1 lymph values at diagnostic biopsy who were treated with IV trastuzumab obtained pCR, while only 3 out of 5 patients with high PD-L1 lymph values at diagnostic biopsy who were treated with SC trastuzumab got a pCR.

According to these results, it is possible to say that SC trastuzumab is likely to induce an immunophenotypic change on post-treatment tumor lymphocytic infiltrate causing the loss of predictive value of PD-L1 expression in arm B.

6.4.1 Subtyping of TILs at final analysis

The composition of the lymphoid infiltrate has been characterized using a series of immunostaining markers as follows:

- LCA (clones, 2B11 e PD7/26)
- CD20 (L26)
- CD3 (2GLV6)
- CD4 (SP35)
- CD8 (SP57)
- CD56 (MRQ-42)
- CD25 (4C9)

- PD-1 (NAT105)
- PD-L1/CD274 (SP142)

6.5 Secondary endpoint

With reference to table 8, we can indirectly derive the percentage of pathologic complete response in our 63 patients. This is defined as no residual invasive or in situ residual tumor in breast tissue or in the lymph nodes after surgery following primary systemic therapy. In arm A, 11 patients have been evaluable for residual disease, while the remaining 20 patients obtained pCR after treatment. We can therefore estimate a pCR rate of 64.5%.

In arm B, 13 patients had residual disease after surgery while the remaining 19 patients obtained pCR, assessing a pCR rate of 59.3%.

These data are thus consistent with what we knew from the literature, where the pCR rate is about 50%, even though in our study treatment arm A showed higher values of pCR rates.

Another interesting result that we obtained concerns the tumor-specific lymphocyte cell activity on blood samples collected during NT at three pre-defined timepoints.

As can be seen in graph 5, blood immune cells' concentration decreases during the 3 cycles of first chemotherapy phase. It is interesting to note that the immunosuppression induced by chemotherapy was more relevant than expected.

However, a stable concentration of B lymphocytes, a small increase in NK cells and a more significant increase in CD3-positive T lymphocytes in the blood samples collected after trastuzumab-based treatment were reported.

NT with either SC or IV trastuzumab in combination with pertuzumab and chemotherapy had a significant effect on T lymphocytes' concentration in the blood and, specifically, the most relevant enrichment of T lymphocytes population in blood samples

after surgery was reported in the SC trastuzumab-based arm (graph 6).

In addition, with reference to graph 7, the increase in the concentration of CD3-positive cells in blood samples after neoadjuvant immune-chemotherapy is not predictive for pCR, given that it occurred also in those patients who did not achieve pCR. These results are in line with what we knew from the literature, as there are no recognized predictive factors for pCR on blood stream for breast cancer patients.

Focusing on patients who did not achieve pCR, we can see from graph 8 that patients who received SC trastuzumab had a considerable increase in CD3-positive T cells on their blood samples, unlike patients who received IV trastuzumab.

Is therefore possible that, in those patients who did not reached pCR, a relevant enrichment in TILs on post-treatment residual disease is related to a greater rise of CD3-positive cells on peripheral blood, particularly in the SC trastuzumab-based treatment arm.

These findings suggest a role for the SC administration of anti-HER2 mAbs in determining favorable variations of host immune response parameters among patients with HER2-positive early BC who had residual disease after NT.

About evaluation safety and tolerability, the most common adverse events of grade 3 or higher were neutropenia (15 [48.4%] pts in arm A, and 11 [34.4%] in arm B), neurotoxicity (1 [3.2%], and 2 [6.2%], respectively), and diarrhea (1 [3.2%], and 1 [3.1%], respectively). There were no events of congestive heart failure.

7. Discussion

7.1 A comparison between ours and literature results

HER2 is a 185-kDa transmembrane tyrosine kinase protein giving higher aggressiveness to breast cancers. HER2-targeted therapy has transformed the outlook for both early and metastatic HER2-positive breast cancer, beginning with trastuzumab and continuing through the development of several new HER2-targeting agents. Since the initial studies of trastuzumab in metastatic breast cancer, its use has been extended to both the adjuvant and neoadjuvant settings in early breast cancer, leading to improved survival and increased rates of pCR in the breast and regional lymph nodes in the two disease settings respectively.

The Fc portion of anti-tumor monoclonal antibodies is a major component of their therapeutic activity, through binding to Fcγ receptors expressed by effector cells present in the tumor microenvironment. ADCC is a well-recognized immune effector mechanism in which antigen-specific antibodies direct immune effector cells (such as NK cells and monocytes/macrophages) of the innate immunity to the killing of the antigen-expressing cancer cells[62]. ADCC depends on the bifunctional structure of immunoglobulin G molecules and comprises an antigen-binding fragment (Fab) that engages the tumor cell and a crystalline fragment (Fc) that binds a FcγR on an effector cell such as NK, monocyte or macrophage[62]. The concomitant binding of Fab to tumor cell antigen and of Fc to an effector cell through its FcγR brings to the activation of immune cells with consequent destruction of the tumour cell[62].

Six phase III multicenter randomized controlled trials investigated the efficacy and safety of adjuvant trastuzumab IV in combination with or after standard adjuvant chemotherapy in the treatment of early breast cancer. All four pivotal randomized controlled trials (HERA, N9831, B31 and BCIRG-006) demonstrated significantly improved

DFS, and three (HERA, B31 and BCIRG-006) demonstrated significantly improved OS.

In the neoadjuvant setting clinical data indicated a great benefit from combining a drug targeting the HER2 receptor with chemotherapy, with an unexpected high rate of pCR. This result indicates that the addition of neoadjuvant and adjuvant trastuzumab to neoadjuvant anthracycline-taxane-based chemotherapy is an effective treatment for patients with HER2-positive locally advanced or inflammatory breast cancer to improve event-free survival, survival, and clinical and pathological tumor responses[43]. This approach is based on the higher pCR of 40% seen with the addition of trastuzumab, compared with a 17% pCR with chemotherapy alone[44]. Patients with a pCR after chemotherapy and trastuzumab showed a significantly better outcome compared with those who did not have a pCR.

Other anti-HER2 molecules (mAbs, antibody-drug conjugates, small molecule TKIs) have also demonstrated efficacy in patients with HER2-positive tumors. In fact the pCR can be increased to 75% with dual HER2-receptor blockade and chemo-therapy[45].

Pertuzumab was approved for the treatment of HER2-positive mBC in combination with trastuzumab and docetaxel in patients who have not received previous anti-HER2 therapy or chemotherapy for metastatic disease, it has been shown to significantly prolong both progression-free survival and overall survival.

Pre-clinical studies have suggested that both trastuzumab and pertuzumab works at different levels by blocking dimerization of HER2 with inhibition of intracellular signaling pathways, inducing apoptosis, or activating immune response by ADCC[70]. Innate and adaptive immune responses are components of an integrated system of anti-tumor host defense in which numerous cells and molecules function cooperative. Evasion of innate and adaptive immunity is thought to be critical for BC growth and progression. Increasing evidence suggests a significant contribution of both innate and adaptive immunity to clinical efficacy of anti-HER2 monoclonal antibodies (mAbs)[66]. It is, thus, possible that co-

administration of trastuzumab and pertuzumab may synergically enhance both innate and adaptive anti-HER2 immunity in HER2-positive breast cancer.

Moreover, the massive tumor cell death secondary to concomitant chemotherapy administration, and the resulting liberation of large amounts of tumor antigens being presented to APCs, may contribute to the creation a favorable immune microenvironment for anti-HER2 mAbs activity[64,66,69].

Trastuzumab for subcutaneous administration has been developed to address the limitations of IV administration, but its therapeutic indications are the same as for the intravenous formulation. Trastuzumab SC has a pharmacokinetic profile and efficacy non-inferior to standard IV administration, with a similar safety profile to trastuzumab IV, and, therefore, offers a valid treatment alternative[53].

Trastuzumab administered subcutaneously undergoes several steps through the peripheral lymphatic system and central lymph nodes and only then is poured into the blood stream. It may therefore experience an “early contact” with immune cells (B and T lymphocytes) in the lymph nodes (recognized sites of encounters between lymphocytes and antigens)[65].

Since the kinetics and distribution of trastuzumab SC is different from that of trastuzumab IV[63,65], it is possible that trastuzumab SC acts at different immunologic levels. It is thus possible that trastuzumab administrated by the IV route could affect in particular the “humoral” component of the pathological immune response[77,78]. On the contrary, trastuzumab given by the SC route could act on the “cellular” component thus affecting the response of memory CD4+ T cells or other cellular players such as dendritic cells[66,67]. In this case, the onset of immune effects of trastuzumab can be delayed but prolonged in time[75,78].

Therefore, by modifying the modality of administration of trastuzumab, it would be possible to interfere with different pathways of the immune system and to exert a beneficial/favorable immunomodulation in HER2-positive breast cancer. In contrast to

normal breast tissue, breast tumours and their adjacent stroma display higher levels of immune-cell infiltrates[81]. TILs are more commonly found in TNBC and HER2-positive breast cancers[82]. They are mononuclear immune cells that infiltrate neoplastic tissue and represent the attempt of the immune system to rise an anti-tumoral response. They are usually assessed by H&E- stained sections performed by trained pathologists and scored by semi-quantitative or qualitative system. The composition and functional status of the immune infiltrate could vary widely between patients, stages of disease, and tumor types. CD8+ cytotoxic T cells, T-helper 1 cells, and NK are generally associated with efficient anti-tumor immune response; immunosuppressive effects are seen with Th2 and FOXP3+ Treg cells; plasma cells can have both anti- or pro-tumoral activity depending on contextual factors.

NK, monocytes and neutrophils recognize and kill tumor cells in an antigen-independent manner (innate immunity). T and B lymphocytes react against BC specific antigens, such as HER2, recognizing and destroying tumor cells (adaptive immunity).

TILs could be intra-tumoral (itTILs), defined as lymphocytes in tumor nests having direct cell-to-cell contact, and stromal (sTILs), dispersed in the stroma between cancer cells. TILs can form tertiary lymphoid structures, aggregates which recapitulate the components and architecture of a lymph node, and correlate with better prognosis. A diffuse and solid tumor-growth pattern, consisting of dense tumor-cell nests with only limited intervening stroma, is rapidly evaluated as having a high level of TILs because the stromal compartment is small.

On the other hand, tumors with a more infiltrative and dissociative growth pattern and larger areas of stroma will generally be scored as having lower TILs[84]. TILs have been shown to provide prognostic and potentially predictive value, particularly in triple-negative and human epidermal growth factor receptor 2-overexpressing BC[63,64]. The presence of high levels of lymphocytic infiltration has been consistently associated with a more-favorable prognosis in patients with early stage triple-negative and HER2

positive breast cancer[63,64]. These infiltrates, that reflect favorable host antitumor immune responses, suggest that immune activation is important for improving survival outcomes[65].

In one of the largest studied cohorts of women with lymphnode-positive disease, the median percentage of stromal tissue infiltrated with TILs was 10% in oestrogen receptor ERpositive and HER2negative samples, 15% in HER2-positive samples, and 20% in ERnegative and HER2negative disease[82].

TILs have been evaluated in nearly 16,000 patients in prospective studies with clinical follow up data available. In literature several studies dissected the predictive role of TILs to anti-HER2 target therapies.

In the neoadjuvant trials, Neosphere[66] and NeoALTTO[67], TILs were not related with treatment response. In the NeoALTTO study, in which both pCR and event-free survival end points were assessed, TILs provided prognostic information independent of pCR rates[63]. In particular, those patients with high TILs had the most-favorable outcomes, even if they did not achieve a pCR. By contrast, those patients who did not achieve a pCR and had low levels of TILs had a poor EFS at 3 years (67%)[63].

In the same setting, Tryphaena [69], GeparSixto[96], GeparQuinto[97], GeparQuattro[98] and CherLob[99] trials showed patients with higher sTILs and itTILs were more likely to achieve a pCR. Different drugs were administered in the cited studies. All trials included anthracyclines in their chemotherapy backbone[100], except NeoSphere[66] and NeoALTTO[101]. All trials tested the efficacy of trastuzumab, lapatinib and their combination, except Neosphere and Tryphaena which evaluated the activity of pertuzumab[66, 61, 100].

The GeparDuo and GeparTrio clinical trials of neoadjuvant anthracycline and taxane chemotherapy enrolled a total of 1,058 patients with samples that could be assessed for TILs[49,64]. All histological subtypes were included, but trastuzumab was not used. The pCR rate was roughly 30% higher in tumours with TILs ≥60% compared with tumours with ≤60% in GeparDuo[101,79], and this finding was also observed in the GeparTrio trial[101,79].

Most of the immunotherapy agents benefits seem to correlate with an improvement

in OS but not in response rate or PFS[102]. Interestingly, in the NeoALTTO[101] trial the addition of lapatinib to trastuzumab increased pCR rate, but the same combination failed to improve DFS and OS in the adjuvant setting (ALTTO trial[103]), suggesting that, in this case, pCR is not a good surrogate of survival and partially explaining why TILs were not predictive. It is worth to note that TILs composition and activation have not been fully dissected in most of the previous studies, even if they may play a crucial role in predicting anti-HER2 response: in the NeoSphere trial, concomitant PD1 and PD-L1 low expressions were related to higher pCR rates, independently of TILs level[66].

According to the metanalysis of Solinas et al., we can conclude that, in the neo-adjuvant setting, TILs are predictive factors of response to trastuzumab and lapatinib, considering the peculiarity of NeoALTTO trial; few data are available to reach conclusion for pertuzumab[100]. NACT plus anti-HER2 treatment can result in the accumulation of TILs in the residual tumor[63,64], and depletion of immunosuppressive TREG cells, which is expected to portend a favourable outcome. However, NACT might also promote tumor infiltration by macrophages, which are associated with an increased chance of recurrent disease. The obtaining of a pCR, while strongly associated with a good prognosis, is not a validated surrogate for improvement in more-robust survival endpoints, such as event-free survival and overall survival. These results suggest the possibility that a group of HER2-positive patients with high TILs on residual disease could have an excellent outcome independently from achieving a pCR and that they could only require trastuzumab as single agent in combination with chemotherapy, rather than more expensive and toxic combination therapies.

In the adjuvant setting patients with TNBC display a robust linear relationship between an increase in TILs numbers over time and improved recurrence-free survival (RFS) end points, as noted in retrospective–prospective analyses performed on the Breast International Group (BIG) 2–98 trial, Finland Herceptin Trial (FinHER), Eastern Cooperative Oncology Group (ECOG) 2197 and ECOG 1199 trials, and the National

Epirubicin Adjuvant Trial (NEAT)/BR9601 trial[77,82,87,88].

A similar relationship between RFS and TILs population was seen for patients with HER2-positive tumors in the NeoALLTO trial and the HER2-positive patient cohorts reported by Ali et al[89]. Trastuzumab benefit seems to be greater in patients with higher TILs numbers, as seen in the FinHER data[88] and no significant prognostic value has been found for TILs in ERpositive HER2negative disease[79].

However, Perez at al.[104] reported that patients enrolled in N9831 trial with high sTILs did not take advantage from trastuzumab addiction to chemotherapy. On the other hand, an immune 14-genes signature was built from the mRNA samples of patients enrolled in N9831, and its expression was strongly associated with positive outcome after trastuzumab-based treatment, enhancing the importance of the anti-tumoral immune-melieu, independently from TILs percentage[68].

For metastatic disease, Cleopatra trial[102] was the first-line study testing the efficacy of docetaxel, pertuzumab and trastuzumab combination; it showed no correlation between PFS and TILs but, once again, an OS advantage for patients with high TILs rate (> 20%), irrespective of treatment arm, was observed.

The efficacy of trastuzumab-based treatment was associated with the enhanced in situ infiltration of interferon-γ producing CD8+ T cells and CD4+Th lymphocytes[92,93], and with decreased number of circulating Treg/CD4+ and reduced Treg/inflammatory Th17 ratios[92,93]. Patients with HER2-overexpressing cancers had a retained immune proficiency and higher CD8+ T cell responses against several tumor-associated antigens (TAAs) if compared to HER2-negative cases, whose general immune background, on the contrary, appeared compromised[79].

After NAC, HER2-positive patients showed a significant increase in the number of NK cells and regulatory T cells irrespective of the pathological response, whereas patients undergoing a pCR disclosed higher percentages of T helper cells. Notably, a significant increase in the number of activated NK cells was observed only in

HER2-positive patients achieving a pCR.

All these findings indicate that maintenance of functional T cell responses against selected antigens and improvement of NK cell proficiency during NAC are probably critical requirements for pCR induction, especially in HER2-positive BC patients.

7.2 Brief summary of results

In this non-comparative, phase II, open-label, randomized neoadjuvant trial, Stage 2 of Simon's Optimal Design has been overcome: out of the first 63 patients enrolled in our study, 11 (arm A) and 13 (arm B) had post-surgery pathologic TILs rate on residual disease (primary endpoint of the study) after either trastuzumab IV or trastuzumab SC. At least 3 patients for each arm are defined with high TILs on residual disease on the basis of the median value described in literature by Savas et al.

Considering the median TILs rate of our subsample of 7,5%, 3 patients for arm A and 6 patients for arm B are defined with High TILs on residual disease. What is interesting to note is that in arm B there is an increase of TILs between pre- and post- treatment of 46% compared to the more moderate increase of 27% in arm A. Therefore, arm B seems to obtain a better biological response.

Moreover, the subtyping of TILs obtained using immunostaining markers demonstrates a significant inverse correlation between PD-L1 expression on pre-treatment sTILs and T cell co-receptor CD3 expression on post-treatment sTILs, particularly in the arm B. These results suggest the possibility of a more relevant cellular-mediated immune response activation after treatment with subcutaneous formulation of trastuzumab. Furthermore, the decrease in PD-L1 expressing lymphocytes on residual disease could potentially have predictive value in term of response to immune-checkpoints inhibitors.

In addition, high values of PD-L1 Lymph on primary tumor at diagnostic biopsy represent a predictive factor for pCR only in arm A, while this predictive effect is lost in arm

B, suggesting a biological change in disease behavior due to a different immune response activation depending on trastuzumab formulation.

As regard to the secondary endpoints, peripheral blood samples collected during NT for tumor-specific lymphocyte activity analyses show that there is a significant increase in CD3-positive population after neoadjuvant chemo-immunotherapy, and this result is much more significant in arm B.

The increase in the concentration of CD3-positive cells in blood stream after neoadjuvant immuno-chemotherapy is not predictive for pCR, given that it occurred also in those who did not get pCR. Focusing especially on patients who did not achieve a pCR, we can see from graph 8 that patients who received SC trastuzumab had a substantial increase in T cells in their blood, unlike patients who received IV trastuzumab .

Is therefore possible that, in patients who did not reach a pCR, SC trastuzumab exerts a modulation of the immune response which determines an immunophenotypic change in TILs population on residual disease and a subsequent increase in T cell population on peripheral blood.

Our future perspective will be to observe whether the increase in TILs on residual disease and in CD3-positive cells on peripheral blood can provide a prognostic information independently of the pCR rate, conferring a good prognosis in an otherwise poor prognostic group.

It will be thus necessary to wait for the long-term follow up of our patients in order to assess the disease-free survival (DFS) and overall survival (OS) as secondary endpoints of the trial. The aim will be to confirm the good outcome of these patients, despite not achieving a pCR after neoadjuvant treatment, related to the subcutaneous administration of trastuzumab and its ability to modulate the immune system against HER2-positive cancer cells.

8. Conclusion

NT with either SC or IV trastuzumab in combination with pertuzumab and chemotherapy had a significant effect on sTIL expression at surgery. In particular, the SC trastuzumab-based arm exerted the most relevant enrichment of sTILS in post-treatment residual tumors and a greater rise of CD3 cells on peripheral good. These findings suggest a role for the SC administration of anti-HER2 mAbs in determining favorable variations of host immune response parameters among pts with HER2-positive early BC who had residual disease after NT.

9. Tables and graphs

Table 1 – Efficacy of Trastuzumab IV in the Adjuvant EBC Setting Study

Study	Median FU, mo	Interventions	No. of Patients*	DFS	OS
SEQUENTIAL TRASTUZUMAB					
HERA (Piccart-Gebhart et al. 2005; Gianni et al. 2011) N=5,090	48	CT±RT→OBS	1698	2-yr DFS: 81% 4-yr DFS: 72%	2-yr OS: 95% 4-yr OS: 88%
		CT±RT→T (x1 yr)	1703	2-yr DFS: 87% HR 0.64, p<0.0001 4-yr DFS: 79% HR 0.76, p<0.0001	2-yr OS: 97% HR 0.66, p=0.012 4-yr OS: 89% HR 0.85, p=0.11
		CT±RT→T (x2 yrs)	1701	NR	NR
NCCTG N9831 (Perez et al. 2009) N=3,505 (1,097 sequential)	66	AC→P	1,087	5-yr DFS: 72%	NR
		AC→P→T	1,097‡	5-yr DFS: 80% HR 0.70, p=0.0005	HR 0.86, p=0.281
		AC→P + T→T	949	5-yr DFS: 84% HR 0.75, p=0.019	NR
CONCURRENT TRASTUZUMAB					
NSABP B31 (Perez et al. 2011) N=2,101	47	AC→P	1,046	4-yr DFS: 72%	NR
		AC→P + T	1,055	4-yr DFS: 85%	NR
B31 + N9831** (Romond et al. 2005; Perez et al. 2007; Perez et al. 2011) N=3,351	47	AC→P	1,679	3-yr DFS: 75% 4-yr DFS: 74%	3-yr OS: 92% 4-yr OS: 86%
		AC→P + T	1,672	3-yr DFS: 87% 4-yr DFS: 86% HR 0.51, 95%CI: 0.44-0.59	3-yr OS: 94% 4-yr OS: 93% HR 0.59, 95%CI: 0.48-0.73
BCIRG-006 (Slamon et al. 2009, Slamon et al. 2011) N=3,222	65	AC→D	1,073	5-yr DFS: 75%	5-yr OS: 87%
		AC→D + T (x1yr)	1,074	5-yr DFS: 84% HR 0.64, p< 0.001	5-yr OS: 92% HR 0.63, p<0.001
		D+Carbo+T (x1yr)	1,075	5-yr DFS: 81% HR 0.75, p=0.04	5-yr OS: 91% HR 0.77, p=0.04

Abbreviations: AC: doxorubicin plus cyclophosphamide; Carbo: carboplatin; CEF: cyclophosphamide, epirubicin and fluorouracil; CI: confidence interval; CT: chemotherapy; D: docetaxel; DFS: disease-free survival; FU: follow-up; HR: hazard ratio; NSS: not statistically significant; OBS: observation; OS: overall survival; P: paclitaxel; RT: radiotherapy; T: trastuzumab

* Number of patients denotes patients included in the efficacy analyses.
** Joint Analysis of the NSABP B31 and NCCTG N9831 trials
‡ Excluded from the joint analysis by Romond et al. (Perez et al. 2007)

Table 2 – Overview of the Clinical Development Program of trastuzumab SC

Studies	**Status**	**Design**	**Primary Objective**
Trastuzumab SC (vial)			
Phase Ib Dose-finding Study (BP22023, CP2)	Completed	Dose-finding/dose confirmation study OL, PG, single dose, MC	Select the dose of trastuzumab SC which results in comparable exposure to that achieved from an IV dose of trastuzumab
Phase III Clinical Study (BO22227, HannaH)	Completed	PK, efficacy and safety study in the neoadjuvant/adjuvant setting OL, PG, randomised, multiple-dose, MC	Non-inferiority of pre-surgery trastuzumab Ctrough and Pcr
Phase I Device Qualification Study (BO25532, CP3)	Completed	PK bridging to injection device OL, PG randomised, single dose, DC	PK comparability of trastuzumab SC dosing via a SID or via hand-held needle and syringe used in previous clinical studies
Additional studies			
Phase II patient preference study (MO22982, PrefHER)	Completed	Patient preference and HCP satisfaction study randomised, MC, crossover study and PK study	To evaluate patient preference for trastuzumab SC administration using a SID/hand-held needle and syringe or trastuzumab IV
Phase III Safety Study [MO28048, SafeHer]	Ongoing	Safety study non-randomised two-cohort, MC, OL	To evaluate the safety of assisted- and self-administered trastuzumab SC as adjuvant therapy

HCP: Health Care Professional; MC: Multicentre; OL: Open-label; pCR: pathological complete response; PG: Parallel group; PK: pharmacokinetic; SID: single use injection device

Table 3 – NEOSPHERE (WO20697) and TRYPHAENA (BO22280): Overview of efficacy

NEOSPHERE (WO20697)				
Parameter	**Trastuzumab+ Docetaxel** N=107	**Perjeta+ Trastuzumab+ Docetaxel** N=107	**Perjeta+ Trastuzumab** N=107	**Perjeta+ Docetaxel** N=96
pCR rate in the breast (ypT0/is) n (%) [95% CI][1]	31 (29%) [20.6;38,5]	49 (45.8%) [36.1;55.7]	18 (16.8%) [10.3;25.3]	23 (24.0%) [15.8;33.7]
Difference in pCR rates[2] [95%CI][3]		+16.8% [3.5;30.1]	-12.2% [-23.8; - 0,5]	-21,8% [-35.1; - 8,5]
p-value (with Simes corr. for CMH test)[4]		*0.0141 [vs. Trastuzumab+ Docetaxel]*	0,0198 [vs. Trastuzumab+ Docetaxel]	0,0030 [vs Perjeta+ Trastuzumab+ Docetaxel]
pCR rate In the breast and lymph node (ypT0/is N0) n (%) [95% CI]	23 (21.5%) [14.1;30.5]	42 (39.3%) [30.3;49.2]	12 (11.2%) [5.9;18.8]	17 (17.7%) [10.7;26.8]
ypt0 N0 n (%) [95% CI]	13 (12.1%) [6.6;19.9]	35 (32.7&) [24.0;42.5]	6 (5.6%) [2.1; 11.8]	13 (13.2%) [7.4;22.0]
Clinical response	79 (79.8%)	89 (88.1%)	69 (67.6%)	65 (71.4%)

1. 95% CI for one sample binomial using Paerson-Clopper method.
2. Treatment perjeta + trastuzumab + docetaxel and perjeta + trastuzumab are compared to trastuzumab + docetaxel while perjeta + docetaxel is compared to perjeta + trastuzumab + docetaxel.
3. Approximate 95% CI for difference of two response rates using Hauck-Anderson method.
4. p-value from Cochran-Mantel-Haenszel test, with Simes multiplicity adjustment.
5. Clinical response represents patiens with a best overall response of CR or PR during the neoadjuvant period (in the primary breast lesion).

TRYPHAENA (BO22280)			
Parameter	**Perjeta+ Trastuzumab+ FEC → Perjeta+ Trastuzumab+ Docetaxel** N=73	**FEC→ Perjeta+ Tratsuzumab+ Docetaxel** N=75	**Perjeta+ TCH** N=77
pCR rate in the breast (ypT0/is) n (%) [95% CI][1]	45 (61.1%) [49.5;72.8]	43 (57.3%) [45.4;68.7]	51 (66.2%) [54.6;76.6]
Difference in pCR rates[2] [95%CI][3]	NA	NA	NA
p-value (with Simes corr. for CMH test)[4]	NA	NA	NA
pCR rate In the breast and lymph node (ypT0/is N0) n (%) [95% CI]	41 (56.2%) [44.1;67.8]	41 (54.7%) [42.7;66.2]	49 (63.6%) [51.9;74.3]
ypt0 N0 n (%) [95% CI]	37 (50.7%) [38.7;62.2]	34 (45.3%) [33.8;57.3]	40 (51.9) [40.3;63.5]
Clinical response	67 (91.8%)	71 (94.7%)	69 (89.6%)

FEC: 5-fluorouracil, epirubicin, cyclophosphamide; TCH: docetaxel, carboplatin and trastuzumab, CMH: Cochran-Mantel-Haenszel

1. 95% CI for one sample binomial using Paerson-Clopper method.
2. Treatment perjeta + trastuzumab + docetaxel and perjeta + trastuzumab are compared to trastuzumab + docetaxel while perjeta + docetaxel is compared to perjeta + trastuzumab + docetaxel.
3. Approximate 95% CI for difference of two response rates using Hauck-Anderson method.
4. p-value from Cochran-Mantel-Haenszel test, with Simes multiplicity adjustment.
5. Clinical response represents patiens with a best overall response of CR or PR during the neoadjuvant period (in the primary breast lesion).

Table 4 – Adjuvant Trials in which TILs have been Assessed

Trial analysed	Trial type	Treatment	TILs assessment	Population	N	Recurrence endpoints
BIG 2-98	Adjuvant Prospective RCT	Doxorubucin Cyclophosphamide CMF Docetaxel	Stromal on H&E	ER-/HER2- HER2+ TNBC	1.079 297 256	Not significant Not significant For each 10% increment of sTILs: DFS,HR=0,84 (95% CI 0.74-0.98, P=0.025)
FinHER	Adjuvant Prospective RCT	Docetaxel Vinorelbine FEC Trastuzumab	Stromal on H&E	ER-/HER2- HER2+ TNBC	591 209 134	Not significant Not significant For each 10% increment of sTILs: DDFS,HR=0,79 (95% CI 0,64-0,98, P=0,032)
E2197 and E1199	Adjuvant Prospective RCT	Doxorubicin Cyclophosphamide Docetaxel	Stromal on H%E	TBNC	481	For each 10% increment of sTILs: DFS,HR=0,84 (95% CI 0.74-0.95, P=0,005)
SEARCH, BCCA NBCS, NEAT	Prospective Observational RCT (NEAT)	Various,not standardised No trastuzumab	IHC for CD8 in stroma (sCD8) IHC for CD8 in tumour (iCD8)	ER- (including HER2+) ER-/HER2+ TNBC	8,775 3,591	Presence versus absence of iCD8: Breast cancer specific survival, HR=0,95 (95% CI 0.85-1.07, P=0.43) Presence versus absence of sCD8: Breast cancer-specific survival, HR=0,79 (95% CI 0,67-0,93, P=004)
NeoALTTO	Neoadjuvant Prospective RCT	Trastuzumab Lapatinib Paclitaxel FEC	Stromal on H&E	HER2+	387	3% decrease in rate of recurrence (event free survival) for every 1% increase in TILs P=0.002

Trials overall include a total of 15,800 patiens. BIG, Breast International Group; CMF, cyclophosphamide, methotrexate, 5-fluorouracil; DDFS, distant disease-free survival; DFS, disease-free survival; ER, oestrogen receptor; FEC, 5-fluorouracil, epirubicin, cyclophosphamide; H&E, haematoxylin and eosin; HR, hazard ratio; IHC, immunohistochemistry; PR, progesteron receptor; RCT, randomized controlled trials; sTIL, stromal TIL; TIL, tumour-infiltrating lymphocyte; TNBC, triple-negative breast cancer.

Table 5 – Neoadjuvant Trials that have Assessed TILs

Trial and treatments	***Subtype***	***n***	***TILs***	***Outcome***	***Multivariate analysis***
GeparDuo Doxorubicin Docetaxel Cyclophosphamide	*All*	*218*	sTILs and iTILs on H&E	>60% sTILs:pCR 41.7% <60% sTILs:pCR 9.3%	*OR 1.38 of pCR per 10% iTILs (95% CI 1.08-1.78,P=0,012)*
GeparDuo Doxorubicin Docetaxel Cyclophosphamide Vinorelbine Capecitabine	All	*840*	sTILs and iTILs on H&E	> 60% sTILs: pCR 40% <60% sTILs: pCR 13.9%	OR 1.21 of pCR per 10% iTILs (95% CI 1.08–1.35, P = 0.001)
GeparQuattro Epirubicin Cyclophosphamide Docetaxel Capecitabine Trastuzumab	HER2+	*156*	sTILs on H&E	> 50% sTILs: pCR 47.4% <50% sTILs: pCR 31.7%	OR 1.16 of pCR per 10% sTILs (95% CI 1.01–1.32, P = 0.038)
GeparQuinto Epirubicin Cyclophosphamide Taxane Everolimus	ER- and TNBC	*313*	sTILs and iTILs on H&E	> 60% sTILs: pCR 36.6% <60% sTILs: pCR 14.3% (P<0.001)	OR 1.2 of pCR per 10% sTILs (95% CI 1.0–1.3, P = 0.01)
GeparSixto Paclitaxel Liposomal Doxorubicin Carboplatin Bevacizumab Trastuzumab	HER2+ and TNBC	*580*	sTILs and iTILs on H&E	> 60% sTILs: pCR 59.9% <60% sTILs: pCR 33.8% (P<0.001) Significant test for interaction between increased TILs and response to carboplatin therapy	OR 1.2 of pCR per 10% sTILs (95% CI 1.11–1.29, P<0.001) OR 2.66 of pCR for > 60% versus <60% sTILs (95% CI 1.76–4.02, P<0.001)
EORTC 10994 and BIG 00-01 fec Docetaxel	ER-	*111*	gTILs	High gTILS: pCR 74.2% Low gTILs: pCR 31.3%	OR 6.42 of pCR for high versus low gTILs (95% CI 2.08–19.83, P = 0.001)
CHER-LOB50 Trastuzumab Paclitaxel FEC	HER2+	*105*	sTILs and iTILs on H&E	> 60% sTILs: pCR 59% <60% sTILs: pCR 27% (P<0.015)	Not reported

Trials overall include a total of 2,323 patiens. ER, oestrogen receptor; FEC, 5-fluorouracil, epirubicin, cyclophosphamide; gTIL, gene-expression surrogate TIL; H&E, haematoxylin and eosin; iTIL, intratumoural TIL; OR, odds ratio; pCR, pathological complete response; sTIL, stromal TIL; TIL, tumour-infiltrating lymphocyte

Table 6 – Patients characteristics

	Patients (63)	% (100)
Median age	50,62	
T3	13	20
N+	44	70
G3	34	54
ER POS	44	70
PR POS	25	40
KI 67>15%	53	84
Trastuzumab SC	32	50,8
Trastuzumab IV	31	49,2
Mastectomy	27	42,8
Quadrantectomy	35	55,5

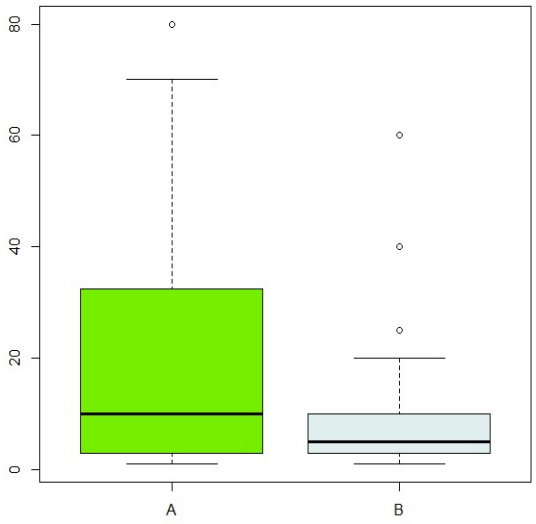
Table 7 – Treatment toxicity

Variable	Patients (63)	% (100)
Hematological toxicity > G3-G4	23	36,5
Non hematological toxicity > G3-G4	13	20,6
Dose reduced/ Treatment delayed	32	50

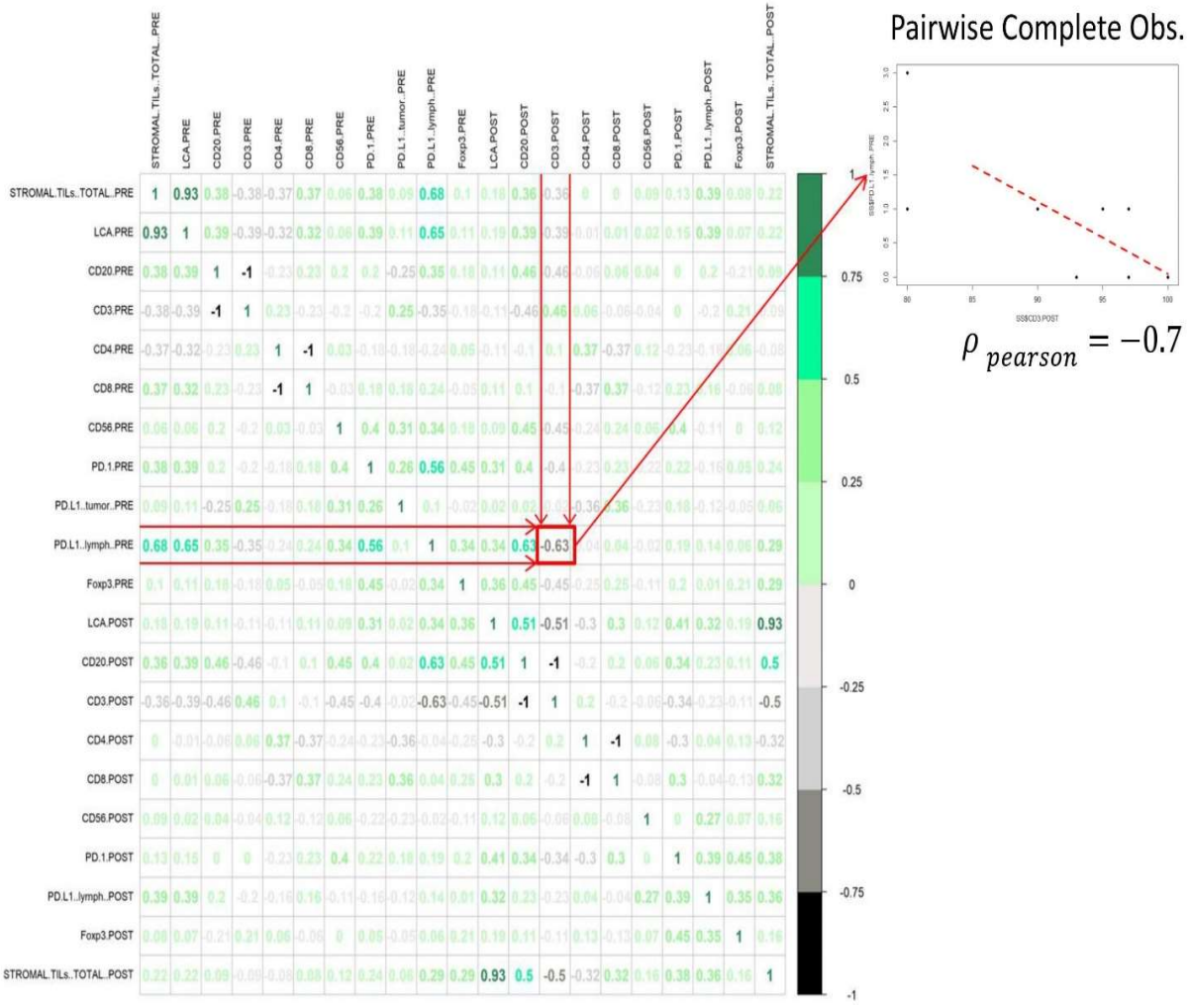
Table 8

1. *Median value described by Savas et al.* ***2.*** *Median value of our sample (63 pts)*

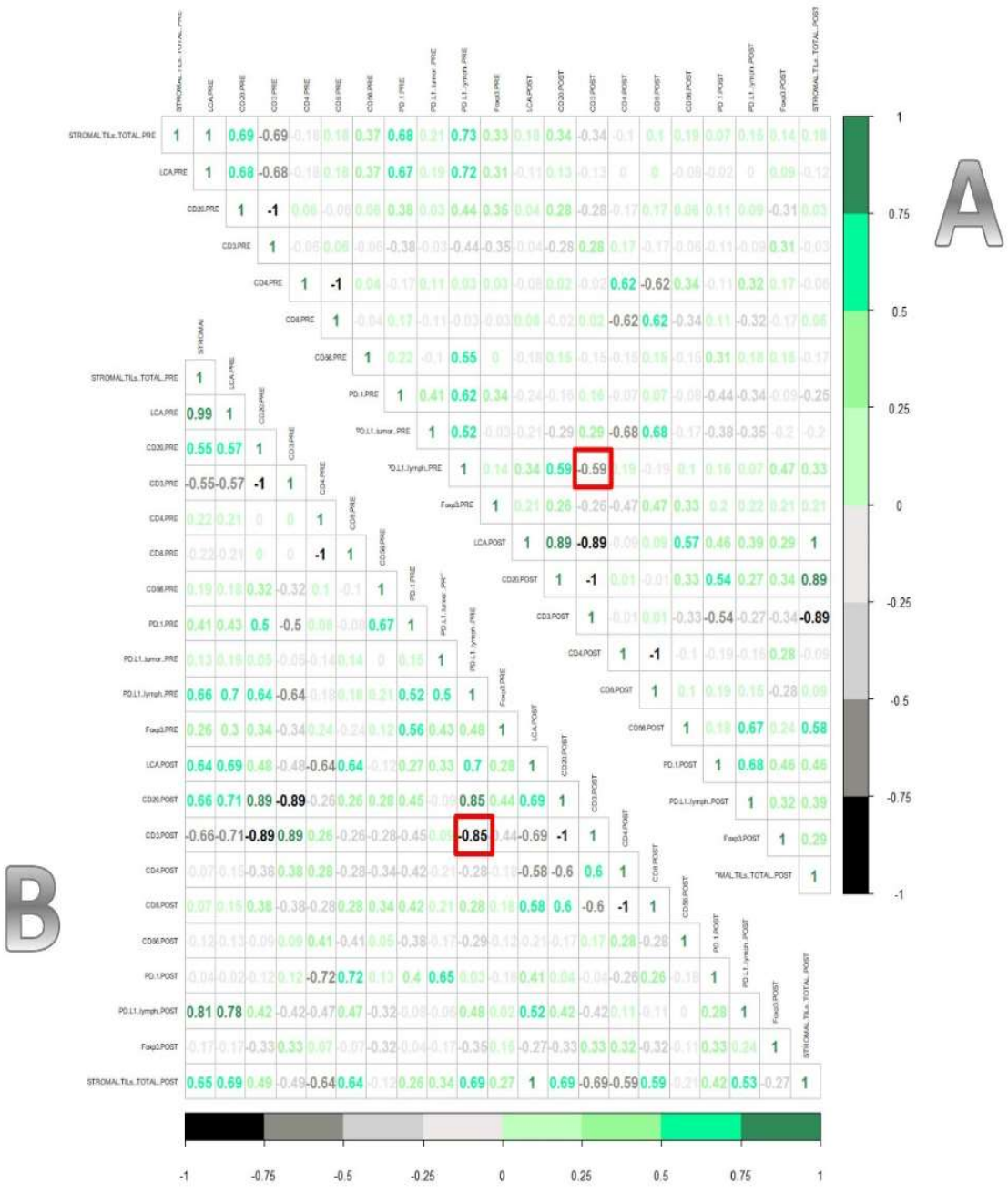
On 63 patients	Arm A	Arm B
# evaluable	11	13
# High TIL (>15%) (1)	3	3
# High TIL (7.5%) (2)	3	6



Graph.1 – Median value of our sample



In Graph 2 a no linear correlation between several patterns of distribution of immune cells, in pre and post-treatment phase, are shown. The increasing green color represent a strong positive correlation; on the contrary, the more intense grey color indicates an inverse correlation. Specifically, in the red boxes, an inverse correlation between PDL1 Lymph PRE and CD3 cells POST treatment is revealed. A significant effect on sTIL expression at surgery after neoadjuvant therapy with either SC or IV trastuzumab in combination with pertuzumab and chemotherapy is suggested by this data.



In Graph 3 the correlation between pre- and post-treatment lymphoid infiltration distinguished in the 2 treatment arms A and B is shown. The inverse correlation between PDL1 lymph PRE and CD3 POST becomes less significant in arm A (-0,59) while it becomes very significant in arm B (-0,85). A more relevant enrichment of sTILS in post-treatment residual tumors is exerted by SC trastuzumab-based arm.

PCR by interaction of ARM&PDL1LymphPRE

High values of PDL1Lymph

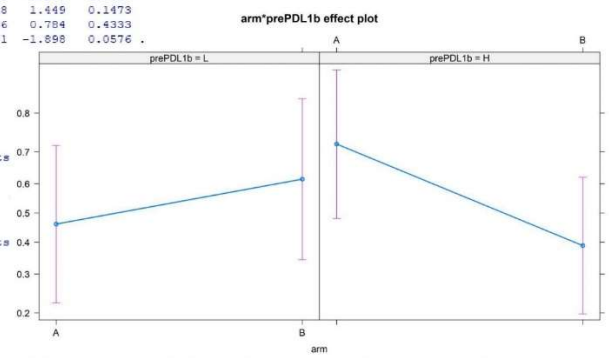
PCR	Treatment A	Treatment B
NO	0	2
YES	7	3

Low values of PDL1Lymph

PCR	Treatment A	Treatment B
NO	12	14
YES	12	12

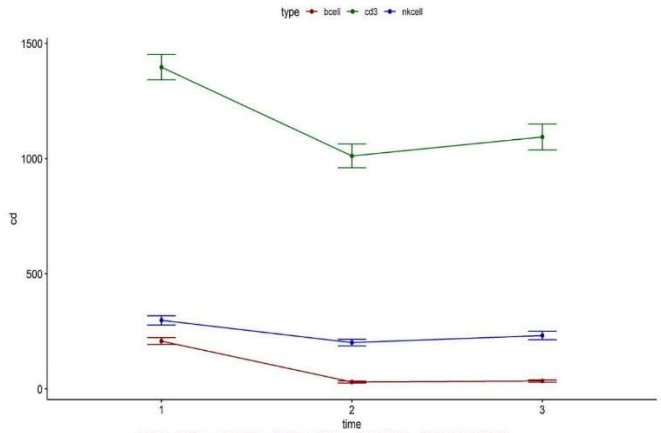
Logistic regression

```
Coefficients:
             Estimate Std. Error z value Pr(>|z|)
(Intercept)   -0.1542     0.5563  -0.277   0.7817
prePDL1bH      1.1097     0.7658   1.449   0.1473
armB           0.6242     0.7966   0.784   0.4333
prePDL1bH:armB -2.0317    1.0701  -1.898   0.0576 .

 prePDL1b*arm effect
        arm
prePDL1b         A         B
       L 0.4615385 0.6153846
       H 0.7222222 0.3888889

 Lower 95 Percent Confidence Limits
        arm
prePDL1b         A         B
       L 0.2236408 0.3435875
       H 0.4810368 0.1978771

 Upper 95 Percent Confidence Limits
        arm
prePDL1b         A         B
       L 0.7183461 0.8302436
       H 0.8794161 0.6214354
```



Logistic regression

In Graph 4 patients' division into two groups based on PDL1 Lymph values of the tumor samples from the primary tumor (diagnostic biopsy), prior to neoadjuvant chemo-immunotherapy, is reported. In each group PCR between treatment arms A and B is evaluated. High values of PDL1 Lymph at the diagnostic biopsy are a predictive biomarker of PCR only in arm A.

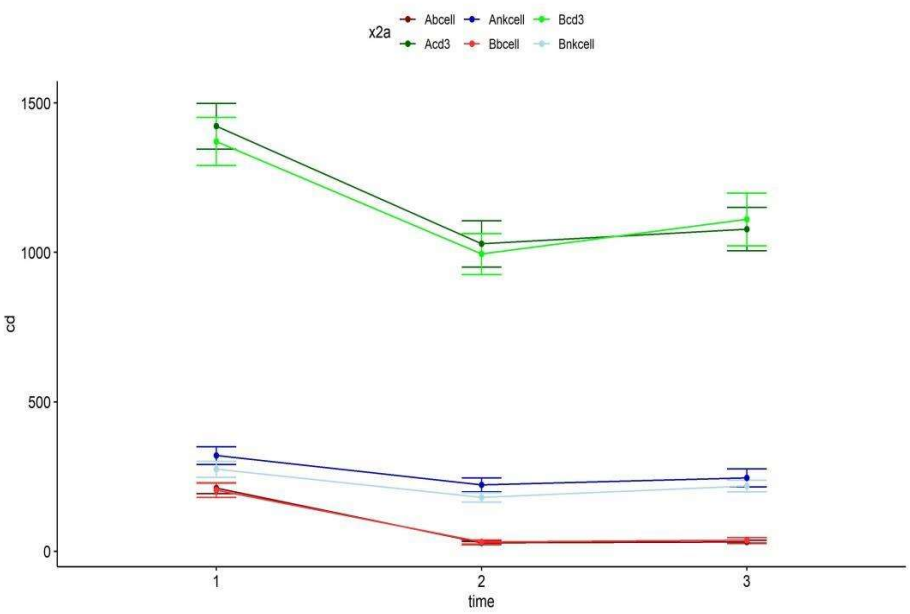



```
> res.aov3 <- aov(cd ~ type + time + time:type, data = my_data)
> summary(res.aov3)
             Df   Sum Sq  Mean Sq F value   Pr(>F)
type          2 128313933 64156966 906.420  < 2e-16 ***
time          2   5204139  2602069  36.762   1e-15 ***
type:time     4   1590916   397729   5.619 0.000194 ***
Residuals   558  39495581    70781
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

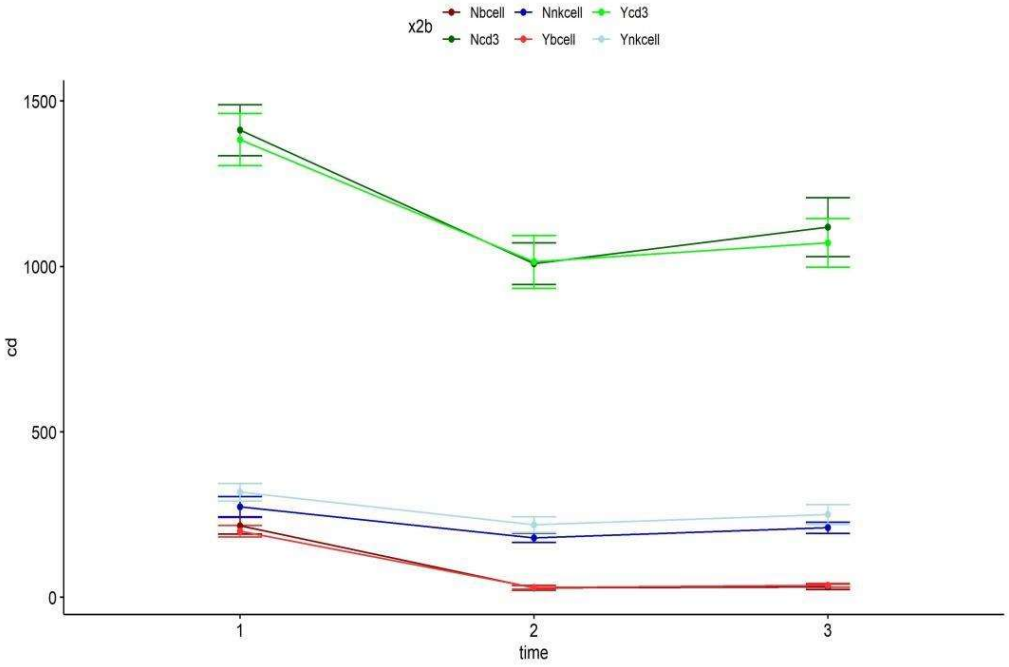
Secondary measure

ANOVA RM BF WI

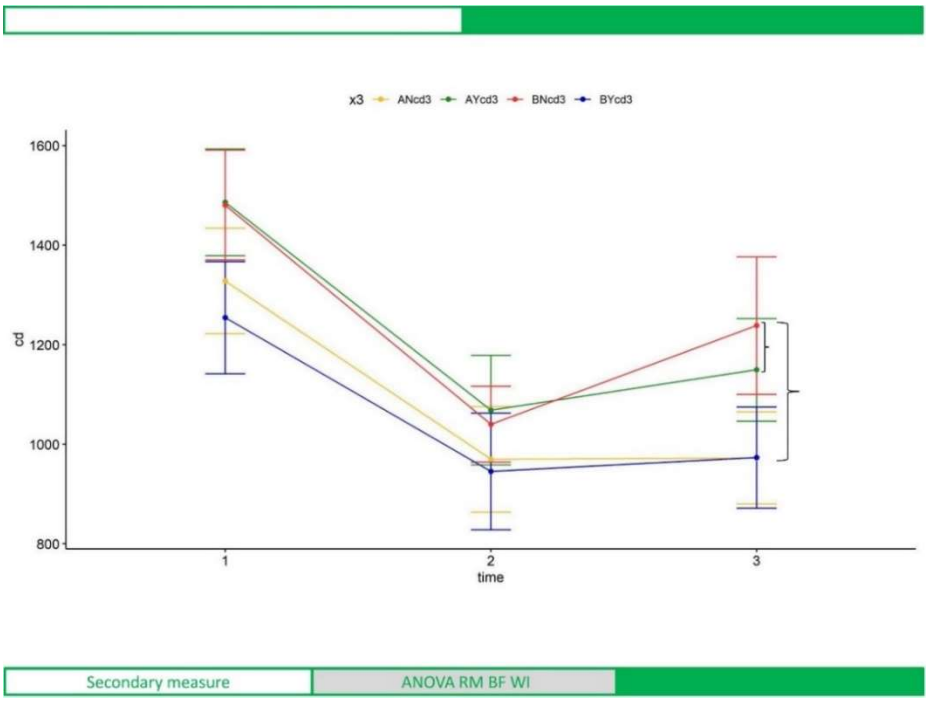
In Graph 5 blood concentrations of B lymphocytes (red line), T lymphocytes (green line) and NK cells (blue line) at 3 predefined time points is reported: 1) before initiation (within ≤ 2 weeks) of the run-in chemotherapy phase; 2) before initiation (within ≤ 1 week) of the trastuzumab-containing treatment; 3) after completion of trastuzumab-containing neoadjuvant therapy (up to 7 days prior to surgery). Immune cells' concentration decrease in blood during the 3 cycles of chemotherapy phase is shown. However a stable concentration of B lymphocytes, a small increase of NK cells and a more significant increase of T lymphocytes in the blood during the neoadjuvant therapy containing trastuzumab is reported. An activation of the immune system, specifically of T lymphocytes, after neoadjuvant therapy containing trastuzumab is suggested by this data.



In Graph 6 blood concentrations of B lymphocytes (red lines), T lymphocytes (green lines) and NK cells (blue lines) at 3 predefined time points (as in the previous graph) distinguished in the 2 treatment arms A and B is shown. A very significant increase in T lymphocytes after completion of trastuzumab-containing neoadjuvant therapy in the B arm rather than in the A arm is reported.



In Graph 7 blood concentrations of B lymphocytes (red lines), T lymphocytes (green lines) and NK cells (blue lines) at 3 predefined time points (as in the previous graph), according to the present of PCR or not, is reported. The increase in the concentration of cd3 in the blood (green lines), in time 2, is not predictive of pCR given that it occurred also in those who did not get pCR.



In Graph 8 concentration of blood T-cells at 3 predefined time points, as in the previous graph, divided in the 2 treatment arms A and B, according to the present of PCR or not, is reported. A significant increase in the concentration of cd3 cells in the blood has occurred in patients in the treatment arm B who did not have PCR after chemo-immunotherapy neoadjuvant as highlighted by the red line.

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