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## TITOLO TESI

## Efficacy of neoadjuvant immunotherapy and chemotherapy for Luminal B-like breast cancer: results of the phase II GIADA trial.

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# 1 ABSTRACT

**Background.** Among hormone receptor (HR)-positive tumors, Luminal B-like breast cancer (BC) harbors immunogenic features as high proliferation rate and high mutational load that can promote sensitivity to immune checkpoint inhibitors. Furthermore, cytotoxic drugs and hormonal treatments have been shown to modulate the immune system.

The role of immunotherapy in HR-positive, HER2-negative early BC is underexplored.

**Methods.** The prospective multicentric phase 2 GIADA trial enrolled premenopausal patients with Luminal B-like BC (HR-positive, HER2-negative, with Ki67>20% and/or histologic Grade 3) candidate to neoadjuvant chemotherapy from four Italian Institutions. Patients received: three 21-days cycles of intravenous epirubicin (90 mg/m2) and cyclophosphamide (600 mg/m2) followed by eight 14-days cycles of intravenous nivolumab (240 mg), intramuscular triptorelin (3.75 mg every 28 days) started concomitantly to chemotherapy, and oral exemestane (25 mg daily) started concomitantly to nivolumab. Tumor tissue samples were collected at baseline (t0), after chemotherapy before nivolumab (t1), and at surgery (t2). BC intrinsic subtypes were determined using PAM50 assay. Tumor infiltrating lymphocytes (TILs) were centrally assessed following TILs Working Group Recommendations. Primary endpoint was pathological complete response (pCR; ypT0/is, ypN0). At least 8 pCR were required to satisfy the statistical hypothesis.

**Results.** A pCR was achieved by seven out of 43 patients (16.3%; 95%CI 7.4-34.9). The rate of Residual Cancer Burden class 0-1 was 25.6% (n=11), and 70.6% patients (n=24 of 34 evaluable) obtained an objective response in the breast. pCR rate was significantly higher for patients with PAM50 Basal BC (50%, 4/8) as compared to other subtypes (Luminal A 9%, 1/11; Luminal B 8%, 2/24; p=0.017).

Immune-related biomarkers including TILs and gene expression signatures tracking immune processes were significantly associated with pCR. A combined score of Basal subtype and TILs had an AUC of 0.95 (95%CI 0.89-1.00) for pCR prediction.

A significant enrichment in TILs occurred from t0 to t1.

Most common Grade $\geq$3 treatment-related adverse events (AEs) during nivolumab were alanine aminotransferase (16.7%, n=7) and aspartate aminotransferase (9.5%, n=4) increase. Most common immune-related AEs were endocrinopathies, mostly hypothyroidism (14.3%, n=6), all of Grade 1-2.

**Conclusion.** Although the trial did not meet its primary endpoint, the results show that a subset of Luminal B-like BC patients may respond to sequential anthracyclines and anti-PD-1, especially in presence of a state of tumor inflammation and/or Basal subtype. Our data provide novel hints to trace the path of immunotherapy development in this context.

# 2 INTRODUCTION

## 2.1 Epidemiology of breast cancer

Breast cancer (BC) is the most common malignancy in women, accounting for more than 2 million new diagnosis per year, and the second leading cause of cancer death in women all over the world .[1] The overall mortality has decreased during the last three decades due to the effect of screening programs and improvements in treatments.[2]

The incidence of BC increases with age. This trend is at least partially due to crucial role of sex hormones in carcinogenesis. In fact, exposure to such hormones has a pro-tumor role in human BC, proven by the association with reproductive factors as a long fertile life (i.e. early menarche, late menopause) and reduced parity.

## 2.2 Biological heterogeneity and breast cancer subtypes

### 2.2.1 Breast cancer classification and clinical implications

Based on immunohistochemical evaluation of estrogen receptors (ER), progesterone receptors (PR) and Human Epidermal Growth Factor Receptor 2 (HER2) expression, BC is classified into three subgroups: hormone receptor (HR)-positive, HER2-positive, triple negative (TN), which is defined by the lack of expression of HR and HER2. This definition has important implications in clinical practice, as it guides treatments choice. Therefore, endocrine manipulation represents the mainstay of treatment for HR-positive

tumors, HER2 positive patients benefit from HER2-targeted treatments, while TNBC is actually treated with chemotherapy.

### 2.2.2 Gene-expression profiling and intrinsic molecular subtypes

During the last two decades, gene expression profiling has improved the understanding of BC biology. Four intrinsic molecular subtypes of BC (Luminal A, Luminal B, HER-2 enriched, Basal-like) and a normal breast-like group have been extensively characterized.[3,4]

These entities show several differences in terms of incidence, behavior, prognosis and treatment sensitivity, giving additional information from that provided by HR, PR and HER2 expression. Molecular intrinsic subtypes only partially overlap with tumor phenotype as defined by immunohistochemistry.

Several studies (for a total of 5994 independent samples) have compared the classification of tumors based on the PAM50 gene expression predictor with the IHC and ISH pathology-based surrogate definitions and a discordance rate of around 30% was observed.[5]

*Luminal A*

Luminal A is the most common BC subtype, and represents about 50-60% of all breast invasive tumors. It is characterized by the expression of genes activated by the ER transcription factor, typically expressed in the luminal epithelium lining the mammary ducts.[6] As compared to Luminal B tumors, Luminal A have lower expression of proliferation/cell cycle-related genes or proteins (e.g. MKI67 and AURKA) and higher

expression of luminal related proteins such as PR, but not ER, which is similarly expressed between the two luminal subtypes.[7] At the DNA level, Luminal A tumors show a lower number of mutations, lower number of chromosomal copy-number changes, less TP53 mutations (12% vs. 29%), and more PIK3CA (45% vs. 29%) mutations (13% versus 5%) as compared to Luminal B tumors.[8]

The immunohistochemistry profile is characterized by high expression of ER and PR and negativity for HER2. However, a subgroup of Luminal A tumors (6-8%) show HER2-amplification/overexpression.[5] Luminal A BCs are also frequently characterized by low rate of proliferation measured by Ki67, a low degree of nuclear polymorphism and a low histological grade.

Patients with Luminal A BC have good prognosis, with lower risk of relapse as compared to other subtypes. Bone are the most common site of recurrence.

*Luminal B*

Luminal B represents 10-20% of all BCs.[6] It is characterized by a more aggressive phenotype than luminal A tumors, with higher histological grade, higher proliferative index and a worse prognosis.[3] Up to 20% of Luminal B tumors present HER2-overexpression .[5] In clinical practice, the level of Ki67 with an arbitrary cutoff of 20% distinguishes luminal A and B tumors.[9]

The pattern of distant relapse also differs as compared to Luminal A tumors and, although the bone is still the most common site of recurrence, visceral involvement is more frequent.[8]

Although endocrine therapy represents the mainstay of treatment for Luminal B-like BC, unique molecular features of this subtype make it a good candidate for cytotoxic chemotherapy. Indeed, results from several neoadjuvant studies suggest that Luminal B

tumors are more chemosensitive than Luminal A, since they are associated with higher rates of pCR.[5,10] However, even with the most effective therapies, Luminal B BC patients have a poorer prognosis as compared with luminal A.[5]

*HER2-enriched*

HER2 is a trasmembrane tyrosin-kinase receptor involved in cell's proliferation and tumor growth. An over-expression/amplification of HER2 is present in about 15-20% of all BC.

HER2-enriched subtype is characterized by high expression of HER2 related genes and/or HER2 amplicon located in the 17q12 and often presents high rate of PIK3CA and p53 mutations.[11] These tumors also present intermediate expression of luminal-related genes and proteins (e.g. ESR1 and PR) and low expression of basal-related genes and proteins (e.g. Keratin 5 and FOXC1).

From a clinical point of view, HER2-enriched subtype is characterized by an aggressive course and a poor prognosis, with frequent visceral and CNS involvement.[3,11] However, the introduction of anti-HER2 targeted treatment dramatically improved outcomes in both the metastatic and early setting .[12,13]

*Basal like*

The Basal subtype accounts for almost 15% of all BC. This subtype is characterized by the high expression of proliferation-related genes 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 .[5] Basal like tumors are

characterized by the highest mutational load among BC and can present mutation in p53 and alteration in pRB pathway. A high lymphocyte infiltrate has been observed. In 70-80% of cases basal subtype and triple negative tumors overlap, although 2-17% present HER2 overexpression/amplification.[5]

Patients diagnosed with Basal like cancers have the poorest prognosis among all BC patients, with higher relapse rates than luminal ones within the first 3 years from diagnosis and frequent metastases to the lung or CNS.[3]

A claudin-low subtype, characterized by the low expression of genes involved in cell-cell adhesion, has also been described.

*Normal like*

When using the PAM50 subtype predictor, about 5%-10% of all BCs are categorized as normal-like. These are poorly characterized and have been grouped into the classification of intrinsic subtypes with normal breast samples.

There are few studies on this subtype and its clinical significance remains undetermined. There are even doubts about its actual existence as a BC subtype. In fact, some researchers believe they could be a technical artefact from high contamination with normal tissue. Indeed, in a large series of samples where the neoplastic cells were isolated by microdissection, no cases of normal breast-like subtype were found, supporting this hypothesis.[6]

## 2.3 Neoadjuvant treatment for Luminal B breast cancer

Pre-operative treatment, initially established for locally advanced, inoperable disease, is widely used in operable BC patients with the main aim of obtaining tumor downstaging in order to consent or facilitate a more conservative surgery and to improve esthetic outcome. Neoadjuvant therapy, as adjuvant treatment, has the potential to eliminate micro metastases, thus reducing the risk of disease recurrence and improving survival. Moreover, neoadjuvant therapy enables the rapid assessment of tumor sensitivity to chemotherapy, offering the unique opportunity of test, in vivo, biological activity of new drugs or combination .[14–17]

The maximum response to neoadjuvant chemotherapy is pathological complete response (pCR), defined as the absence of infiltrating cells in both the breast and axillary nodes (pTNM ypT0/is, ypN0). Achieving a pCR is a prognostic factor for long-term survival. Generally, more aggressive tumor characteristics (HER2 iperexpression/amplification, high proliferation rate, triple negative subtype) implies higher pCR rates.

A pooled analysis of twelve trials for a total of about 12000 patients confirmed that the complete eradication of invasive tumor in both breast and axilla is strongly associated with better event-free survival (EFS) (hazard ratio [HR] 0.48, 95% CI 0.43-0.54) and improved overall survival (OS) (HR 0.36, 95% CI 0.31-0.42), probably as a result of a prompt eradication of micromestatic disease. This association between pCR and long-term outcomes was stronger in patients with TNBC (EFS: HR 0.24, 95% CI 0.18-0.33; OS: HR 0.16, 95% CI 0.11-0.25) and in HR-negative/HER2-positive patients who received trastuzumab (EFS: HR 0.15, 95% CI 0.09-0.27; OS: HR 0.08, 95% CI 0.03, 0.22).[18]

On the other hand, patients with residual invasive disease after neoadjuvant treatment have poor prognosis and show a shorter disease-free survival (DFS) than those with pCR. Among Luminal BCs, Luminal B express the highest chemosensitivity and are more likely to achieve pCR as compared to Luminal A. Moreover, the role of pCR as a

surrogate endpoint for Luminal B but not for Luminal A tumors have been demonstrated.[19]

Although a number of chemotherapy combinations showed efficacy as preoperative treatment in Luminal B BC, the standard of care includes anthracycline- and taxane-based regimens.

## 2.4 Immunotherapy in breast cancer

The immune system plays an active role in cancer growth and response to treatment. The concept of immunoediting, first introduced about 50 years ago, contemplate 3 phases. In the first phase (elimination), the immune system is capable of recognize and eliminate malignant cells, thus controlling tumor growth; the second phase (equilibrium) takes place in case of incomplete elimination and is characterized by a coexistence of immune cells and quiescent cancer cells; in the third phase (escape), malignant cells become able to bypass the host's immune system, resulting in tumor growth.[20–22]

In this context, maintaining the status of intact and active immune system could grant tumor growth control.

PD-1 (programmed cell death protein 1) is a transmembrane protein member of the cluster of differentiation 28 (CD28) family of T cell co-stimulatory receptors normally present on the surface of T cells, B cells and Natural killer, while its ligand, PD-L1 (programmed cell death protein ligand 1) is predominantly expressed on dendritic cells. Once PD-L1 activates PD-1, CD28-mediated upregulation of co-inflammatory signals as interleukin (IL)-2, IL-3, IL-10, interferon (IFN) γ and T cells activation are hampered. As a result, migration, proliferation, secretion and activity of cytotoxic immune cells are suppressed,

thus mitigating an excessive immune response.[23] Moreover, this phenomenon results in the inactivation of anti-tumor response.[24–26]

It has been shown that PD-1 and PD-L1 are commonly up-regulated on tumor-infiltrating lymphocytes and on many different tumor cells, respectively, with the consequent establishment of a highly immunosuppressive tumor milieu.

The past decade has seen the raise of immunotherapy. Immune checkpoint inhibitors directed against the axis PD-1/PD-L1, with the aim of remove the block of the immune system in order to reinstate a proper anti-tumor response, have been proven to be effective in several solid tumors. Preclinical and clinical data confirmed the potential effectiveness of such therapy in breast cancer, in both the advanced and early setting. The Impassion130 study investigated the combination of atezolizumab plus nab-paclitaxel as first line treatment for mTNBC. After a median follow up of 12.9 months, the addition of atezolizumab resulted in a significantly prolonged PFS in both the intention to treat (ITT) and the PD-L1 population (PFS: 7.2 months vs 5.5 months [HR 0.80, 95% CI 0.69-0.92; p=0.002] and 7.5 months vs 5 months [HR 0.62, 95% CI 0.49-0.78; p<0.001]).[27] A clinically meaningful benefit in OS, although not statistically tested, was evident in the PD-L1+ population, with a median of 25.4 months for patients receiving atezolizumab as compared with 17.9 months for patients in the placebo arm (HR 0.67, 95% CI 0.53-0.86).[28] To date, atezolizumab (anti-PD-L1), given in combination with nab-paclitaxel, is the only immunotherapeutic drug approved by European Agencies as first line treatment for metastatic TNBC patients with positive PD-L1.

In the early setting, the addition of pembrolizumab to platinum-based standard chemotherapy demonstrated statistically significant improvement in pCR rates in a population of newly diagnosed TNBC patients (64.8% vs 51.2%, p<0.001). This benefit was independent from PD-L1 status. Furthermore, a trend for a superior EFS in the pembrolizumab arm was observed (91.3% versus 85.3%; HR: 0.63; 95% CI 0.43-0.93).[29]

Based on these results, FDA (but not EMA) approved pembrolizumab as neoadjuvant treatment for early stage TNBC in combination with chemotherapy.

Due to the fact that HR-positive/HER2-negative BC is usually considered as a cold tumor, the role of immunotherapy in this subtype is underrated.[30–34] However, several data suggest that immunity may play an important role also in Luminal subtype. In fact, high stromal tumor infiltrating lymphocytes (TILs) can be present in about 10% of HR+ BC.[33,35] In a large pooled analysis by Denkert et al, high TILs levels were associated with significantly higher rates of pCR, although, counterintuitively, this translated into a worse overall survival in HR-positive/HER2-negative population.[33]

Luminal B BCs are defined by high proliferation rate and can present a high mutational load and express potentially targetable biomarkers as immune checkpoint. The rate of PD-L1 expression among HR-positive breast tumors ranges between 9 and 41% for respectively Luminal A and Luminal B subtypes, reflecting the extreme heterogeneity of the disease; furthermore, PD-L1 expression has been associated with higher pCR rates in HR-positive BC.[36,37]

In a phase 1b trial, the monoclonal anti-PD-1 antibody pembrolizumab showed clinical activity in a cohort of pretreated HR-positive/HER2-negative metastatic BC patients, with a clinical benefit rate of 20% a median duration of response of 12 months.[38] The I-SPY2 study, a phase 2 platform trial with an adaptive design, showed a probable benefit of neoadjuvant pembrolizumab added to chemotherapy across all BC subtypes, with an estimated rate of pCR of 30% vs 13% in HR-positive HER2-negative patients; furthermore, the rate of patients with residual cancer burden (RCB) II-III was 54% vs 75% for respectively the experimental arm and the control arm.[39] In a cohort of 14 endocrine resistant HR-positive HER2-negative mBC patients enrolled in a phase 2 study, the combination of pembrolizumab plus chemotherapy lead to a prolonged clinical benefit

(partial response + stable disease >6 months) in 28%, with one patient remaining on treatment without progression for longer than 1 year.[40]

The combination of pembrolizumab plus chemotherapy in high-risk HR-positive BC is under investigation in the phase 3 Keynote-756 trial.[41]

In Luminal B tumors, the modulation of immune milieu may involve complex loops, including estrogens, soluble inflammatory mediators such as IL6, TGF β and TNF α, and immune cells, further influenced by the effect of both endocrine and cytotoxic treatments. In particular, anthracyclines are capable to induce cell apoptosis, with the release of antigens that can elicit specific immune response.[42] This process, known as immunogenic cell death, entails different events such as calreticulin exposure, caspase activation, reactive oxygen species (ROS) production, oxidative modification of DAMPs (danger associate molecular patterns such as HMGB1), that trigger immune system activation.[43] In this context, the standard chemotherapy combination of epirubicin + cyclophosphamide (EC) represents an optimal candidate to be tested as an induction treatment prior to immunotherapy.

Some of the most recent trials exploring the combination of chemotherapy with immune checkpoint inhibitors including Luminal B early BC patients are shown in **Table 1**.

**Table 1.** Clinical trials of immunotherapy plus chemotherapy in HR+ early breast cancer.

| Study | Phase | Setting | Intervention | Primary Endpoints |
|---|---|---|---|---|
| NCT01042379[39] | 2 | Neoadjuvant | Pembro four-arm /placebo + paclitaxel followed by AC | pCR |
| NCT03515798[44] | 2 | Neoadjuvant | Pembro+(F)EC → paclitaxel vs (F)EC → paclitaxel | pCR, DLT |
| NCT02957968[45] | 2 | Neoadjuvant | Decitabine+pembro → dose-dense AC → paclitaxel | TIL |
| NCT03815890[46] | 2 | Neoadjuvant | Cohort 1: nivo; Cohort 2: nivo+doxo | |
| NCT03356860[47] | 1/2 | Neoadjuvant | Paclitaxel → EC +/- durva | Toxicity, pCR |
| NCT03875573[48] | 2 | Neoadjuvant | Control arm: paclitaxel → ddAC and RT. Experimental arm 1: durva + control arm. | RCB |

| | | | | |
|---|---|---|---|---|
| | | | Experimental arm 2: olectumab + control arm | |
| NCT02999477[49] | 2 | Neoadjuvant | pembro + nab-paclitaxel | Change in PD-L1 expression at 2 weeks |
| NCT03725059[41] | 2 | Neoadjuvant/Adjuvant | CT +/- pembro | pCR, EFS |

Abbreviations: pembro, pembrolizumab; pCR, pathogical complete response; AC, anthracycline-cyclophosphamide; (F)EC, (fluorouracil)-epirubicin-cyclophosphamide; DLT, dose-limiting toxicity; TIL, tumor infiltrating lymphocytes; nivo, nivolumab; doxo, doxorubicin; durva, durvalumab; dd, dose dense; RT, radiotherapy; RCB, residual cancer burden; PD-L1, programmed death ligand 1; CT, chemotherapy.

Preclinical models suggest that aromatase inhibitors (AI) can downregulate the differentiation of regulatory T cells, thus preventing the establishment of an immunosuppressive milieu. Furthermore, AI proved to be capable of modifying tumor immune infiltration, in favor of a cytotoxic polarization, by reducing FOXP3+ T cells and increasing CD8+/FOXP3+ T cell ratio in HR-positive BC.[35,50]

For these reasons, AI seem to be a suitable companion for immune checkpoint inhibitors.[51]

**Table 2** summarizes trials with the combination of immunotherapy and hormonal therapy for eBC.

**Table 2.** Clinical trials of immunotherapy plus hormonal therapy in early breast cancer.

| Study | Phase | Setting | Intervention | Primary Endpoints |
|---|---|---|---|---|
| NCT03874325[52] | 2 | Neoadjuvant | Durva+AI | PEPI score |
| NCT02997995[53] | 2 | Neoadjuvant | Treme+exe → Durva+exe | pCR |
| NCT02971748[54] | 2 | Adjuvant | Pembro+HT | DFS |
| NCT03573648[55] | 2 | Neoadjuvant | Ave + tam +/- palbo | cCR |

Abbreviations: durva, durvalumab; AI, aromatase inhibitors; PEPI, Preoperative endocrine prognostic index; treme, tremelimumab; exe, exemestane; pCR, pathological complete response; pembro, pembrolizumab; HT, hormonal therapy; DFS, disease-free survival; ave, avelumab; tam, tamoxifen; palbo, palbociclib; cCR, clinical complete response.

## 2.5 Nivolumab mechanism of action and safety

Nivolumab is a fully human IgG4 kappa monoclonal antibody (mAb) that inhibits PD-1, by binding it on the surface of activated immune cells, thus preventing the activation of its downstream signaling cascade.[56,57]

In vitro, nivolumab binds to PD-1 with high affinity, preventing it from binding its cognate ligands-PD-L1 and PD-L2, and ultimately promoting a reproducible enhancement of both proliferation and IFNγ release by lymphocytes.

In vivo blockade of PD-1 by a nivolumab murine analog proved to enhance the antitumor immune response, resulting in tumor rejection in several immunocompetent mouse tumor models (MC38, SA1/N, PAN02).

Nivolumab has been studied, either alone or in combination with chemotherapy, in different tumor types. In Europe, nivolumab monotherapy is indicated for melanoma as adjuvant therapy and for treatment of advanced or metastatic disease, previously treated metastatic non-small cell lung cancer (NSCLC), previously treated advanced renal cell carcinoma (RCC), advanced or metastatic urothelial carcinoma after a previous platinum-based therapy, locally advanced/recurrent esophageal cancer, and for the treatment of previously treated recurrent or metastatic squamous cell carcinoma of the head and neck (SCCHN). In addition, the combination of nivolumab and Ipilimumab has recently been approved for first line treatment of malignant mesothelioma, for advanced NSCLC in association with platinum-based chemotherapy, for previously treated MSI-high metastatic colorectal cancer.

In the adaptive phase 2 TONIC trial, nivolumab in combination with different induction strategies (i.e. chemotherapy or radiotherapy) in a population TNBC patients, showed an ORR of 20% and 35% in overall and doxorubicin-treated population, respectively.[58] Different studies investigating the role of nivolumab in BC patients are shown in **Table 3**.

**Table 3.** Studies with nivolumab in breast cancer.

| Study | Phase | Population | Intervention | Primary Endpoints |
|---|---|---|---|---|
| NCT02499367[58] | 2 | mTNBC (≤3 previous therapy) | Induction → nivolumab versus No induction → nivolumab* | PFS |
| NCT04109066[59] | 3 | High-risk early HR+/HER2- BC | Neoadjuvant phase: nivolumab/placebo + weekly paclitaxel → AC; Adjuvant phase: nivolumab/placebo + HT | pCR, EFS |
| NCT03414684[60] | 2 | mTNBC (≤1 previous therapy) | Carboplatin +/- nivolumab | PFS |
| NCT03789110[61] | 2 | HER2- mBC (≤3 previous CT/≥1 previous endocrine therapy if HR+) | Ipilimumab + nivolumab single arm | ORR |
| NCT03316586[62] | 2 | mTNBC (≤3 previous CT/≥1 previous endocrine therapy if HR+) | Cabozantinib + nivolumab single arm | ORR |
| NCT03546686[63] | 2 | TNBC with residual disease after taxane-based NACT | Ipilimumab + nivolumab and biopsy/cryoablation→surgery and adjuvant nivolumab | EFS |
| NCT03430479[64] | 1/2 | HR+ mBC | Nivolumab + radiotherapy + endocrine therapy | DLT |

Abbreviations: mTNBC, metastatic triple negative breast cancer; PFS, progression-free survival; HR, hormone receptors; HER2, human epidermal growth factor 2; AC, adriamicyn-cycophosphamide; pCR, pathogical complete response; EFS, event-free survival; CT, chemotherapy; ORR, overall response rate.
* Induction: radiation; doxorubicin; cyclophosphamide; cisplatin

Overall, the safety profile of immunotherapy in combination with chemotherapy for early BC has been investigated in several studies.

In phase I trials, the rate of treatment-related adverse events (trAEs) of any grade ranged from 75% to 89%, with fatigue being the most common.

The phase 1b Keynote-173 study randomized 60 TNBC to six different regimens of pembrolizumab plus chemotherapy. Potential immune-related adverse events (irAEs) were reported in 18 patients (30%) across all cohorts, including grade 1-2 hypothyroidism and hyperthyroidism (n=10, 17%); two patients (3%) reported grade 3 colitis, three grade

3 (5%) skin reaction, and one (2%) experienced grade 3 hepatitis. Eleven patients discontinued pembrolizumab due to an irAE.[65]

Among the 69 patients enrolled in the I-SPY2 trial, the most common immune-related adverse events (irAEs) were pruritus (n=22, 32%) and thyroid disorders (n=11, 16%). Five patients (7%) experienced grade 3-4 adrenal insufficiency. No grade 5 irAE was observed.[39]

The combination of neoadjuvant chemotherapy (nab-paclitaxel, followed by epirubicin/cyclophosphamide) with the anti-PD-L1 durvalumab in TNBC has been assessed in the phase 2 GeparNuevo study; a total of 174 participants were enrolled and 92 were randomized to immunotherapy. The most common irAEs of any grade was thyroid disfunction (n=46, 50%). However, the addition of durvalumab did not lead to a higher frequency of chemotherapy dose reduction or discontinuation.[66]

In the phase 3 Keynote-522 trial exploring the addition of pembrolizumab to standard of care chemotherapy as neoadjuvant treatment for TNBC, the incidence of trAEs of any grade was similar across the two arms. With regards to potential irAEs, hypothyroidism and hyperthyroidism of any grade were observed in respectively 107 (14%) and 36 (5%) patients; five patients (0.6%) experienced a grade $\geq$3 hypo/hyperthyroidism. A grade $\geq$3 was evident in 30 patients (3.8%). Ten patients (1.3%) had a grade $\geq$3 adrenal insufficiency.[29]

One of the initial BC studies assessing the combination of checkpoint inhibitors plus AI was a Phase 1 study of tremelimumab, an anti-CTLA-4 antibody, with exemestane, in 26 women with heavily pretreated ER+ metastatic BC.[51]

At the time this thesis was draft, no data on the combination of Nivolumab and endocrine treatment for early BC patients was available.

### 2.6 Rationale for the study

As previously discussed, HR-positive disease is a heterogeneous entity, comprising tumors with low proliferation rate (Luminal A) and tumors that harbor high expression of proliferation-related genes and a high mutational load (Luminal B). Although Luminal B BC is associated with higher pCR rates compared to Luminal A (10-15 vs 5-7%), especially in young patients, pCR rates are still suboptimal.[18,19,67,68]

A critical research question regarding the treatment of early stage, high risk HR-positive tumors is the identification of effective strategies to improve pCR rates, as this could impact long-term survival. Due to its unique characteristics among endocrine sensitive tumors, Luminal B is a good candidate for immune-modulating strategies. Both chemotherapy and endocrine therapy, which are standard options for Luminal B BC patients, may be integrated in immunotherapy strategies with the aim to maximize the immune system engagement.

On these premises, we designed a phase 2 study in order to assess the efficacy of an innovative neoadjuvant regimen including the anti- PD-1 nivolumab and to exploit the immunomodulatory features of chemotherapy and endocrine therapy, with the aim of improving pCR rates among premenopausal Luminal B breast cancer patients.

## 3 METHODS

### 3.1 Trial overview

The GIADA trial was designed as an independent, investigator-driven, single arm, multicentric phase 2 neoadjuvant study, conducted at four Italian Institutions and coordinated by the University of Padova.

## 3.2 Patients' population

The target population was composed of Luminal B-like premenopausal patients with indication to undergo neoadjuvant chemotherapy as for clinical practice.

To be enrolled, patients had to meet the following inclusion criteria:

- female, age ≥18 years, premenopausal, ECOG Performance Status 0-1;
- primary diagnosis of invasive breast cancer, stage II-IIIA, HR positive (ER ≥ 10% and/or PgR ≥10% by IHC) and HER2 negative (IHC 0/1+ and/or FISH/CISH negative) according to local assessment;
- histologic grade 3 and/or Ki67 >20% according to local assessment;
- eligible for neoadjuvant chemotherapy according to multidisciplinary evaluation;
- normal organ and marrow function;
- availability of tumor tissue suitable for biological and molecular examination before starting primary treatment.

Key exclusion criteria included stage IIIB-IV BC, contraindication to anthracyclines and other common exclusion criteria in immunotherapy trials, such as uncontrolled autoimmune disease or conditions requiring systemic corticosteroids or immunosuppressant therapy.

The study was conducted according with Good Clinical Practice Guidelines and the World Medical Association Declaration of Helsinki.

All patients provided written informed consent.

## 3.3 Study procedures

Eligible patients with Luminal B-like BC and indication to neoadjuvant chemotherapy were identified after multidisciplinary discussion in the context of the Breast Unit.

After informed consent signature, eligibility was confirmed by screening procedures, which included hematology and blood chemistry, cardiologic examination and radiological evaluations to exclude stage IV disease. Tumor assessment at the baseline included tumor evaluation by mammogram and ultrasound.

The type of surgery indicated in the absence of neoadjuvant treatment was collected at baseline.

Enrolled patients received three 21-days cycles of intravenous epirubicin 90 mg/m2 and cyclophosphamide 600 mg/m2, followed by eight 14-days cycles of intravenous nivolumab 240 mg. Oral exemestane 25 mg daily was started concomitant to nivolumab, and intramuscular triptorelin 3.75 mg every 28 days was started concomitant to chemotherapy. Endocrine therapy was maintained until surgery.

Patients underwent surgery two to five weeks from the last nivolumab dose, after repeating breast tumor evaluation by mammogram and ultrasound. Adjuvant therapy was at physician's discretion and included both endocrine therapy and, in some cases, chemotherapy depending on pathological response.

Formalin-fixed paraffin-embedded (FFPE) tumor tissue specimens were collected at baseline (core biopsy, t0), after chemotherapy within seven days prior to the first nivolumab dose (core biopsy, t1) and at surgery (surgical sample, t2). Fresh-frozen samples from diagnostic core biopsies were also collected and stored at -80°C.

Study design is represented in **Figure 1**.

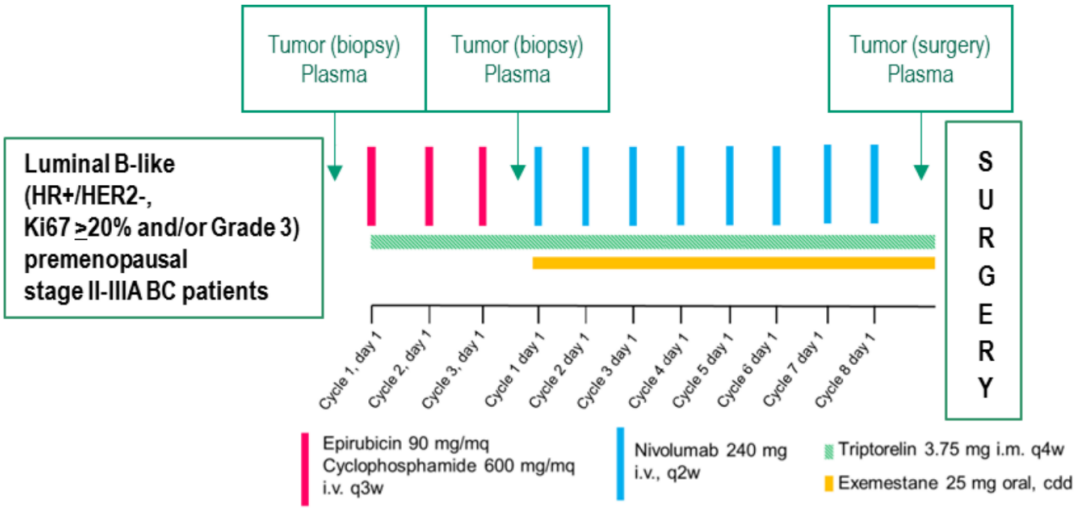


**Figure 1.** GIADA study design

## 3.4 Tumor tissue analysis

### 3.4.1 RNA extraction from baseline biopsies

Fresh frozen baseline tumor biopsies were reviewed by a pathologist for tumor tissue quality and quantity. All the biopsies contained at least 40% of tumor cells.

Frozen tumor tissues were disrupted in liquid nitrogen using a mortar and pestle. Ground tissues were resuspended in lysis buffer, RTL buffer (Qiagen) plus β-mercaptoethanol, and homogenized by passing the lysate at least 5 times through a 20-gauge needle. Total RNA was extracted using RNeasy Plus mini kit (Qiagen) following the manufacturer's instructions.

If fresh frozen tumor biopsy was not available, Formalin-Fixed Paraffin-Embedded (FFPE) baseline tumor biopsies were evaluated by a pathologist for tumor tissue quality and quantity. From each FFPE baseline tumor biopsy, five 10 µm sections were cut and RNA was extracted using RNeasy FFPE Kit (Qiagen) following manufacturer' instructions.

RNA concentration and quality were assessed with Nanodrop 1000 Spectrophotometer (Thermo Scientific NanoDrop Products), Qubit™ RNA HS Assay Kit on Qubit fluorometer 1.0 (Invitrogen, Life Technologies) and TapeStation 4200 (Agilent Technologies, Germany) to ensure they met the specifications for purity (260/280 ratio between 1.7 and 2.3) and concentration (≥10 ng/μL).

### 3.4.2 Gene expression analysis

Gene expression analysis was carried out with the NanoString Breast Cancer 360 assay (BC360TM) on a NanoString nCounter SPRINT Profiler (NanoString Technologies Inc., Seattle, WA, USA).

The Breast Cancer 360TM assay covers genes from different independent signatures, including the PAM50 signature. The panel includes 758 target probe pairs, 18 housekeeping genes used for normalization, 6 exogenous positive control RNA targets that range linearly from 128 fM to 0.125 fM, and 8 exogenous negative control sequences. The samples were processed according to manufacturer's instructions and kits provided by NanoString Technologies. Briefly, the starting material was 100ng RNA for fresh frozen tissue and 200 or 300ng for FFPE samples depending on the percentage of fragments with dimensions greater than 200bp (DV200 value). RNA concentration and quality were assessed with Nanodrop 1000 Spectrophotometer (Thermo Scientific NanoDrop Products) and Qubit™ RNA HS Assay Kit on Qubit fluorometer 1.0 (Invitrogen, Life Technologies) to ensure they met the specifications for purity (260/280 ratio between 1.7 and 2.3) and concentration (≥10 ng/μL). RIN and DV200 were evaluated with the High Sensitivity RNA kit on TapeStation 4200 (Agilent Technologies, Germany). Sample RNA was hybridized with panel probes for 17 hrs at 65°C and then

complexes were processed on the nCounter Analysis System. Cartridges were scanned at 555 FOVs.

Zero counts on the raw scale are converted to ones prior to normalization.

Gene expression data for genes non-included in the PAM-50 or TIS signatures were normalized using a ratio of the expression value to the geometric mean of all housekeeping genes on the panel. Genes in the TIS signature are normalized using a ratio of the expression value to the geometric mean of the housekeeper genes used only for the TIS signature, while genes in the PAM50 signature are normalized using a ratio of the expression value to the geometric mean of the housekeeper genes used only for the PAM50 signature.

Genes not in the PAM50 signature were additionally normalized using a ratio of the housekeeper-normalized data and a panel standard run on the same cartridge or on the same codeset lot as the observed data.

Data was then Log(2) transformed. Forty-eight gene signatures covering several aspects of breast cancer biology and predefined by manufacturer for the Breast Cancer 360 Panel® (Nanostring Technologies; Seattle, Washington, USA) were calculated, including intrinsic molecular subtyping was determined using the previously reported PAM50 subtype predictor (Parker J, JCO 2009). Gene signatures were adapted with constants to make scores comparable across research use only (RUO) and IUO assays.

### 3.4.3 TILs evaluation

Histopathologic scoring of TILs was performed on hematoxylin and eosin-stained sections from FFPE tumor samples following according to the International Tumor Infiltrating Lymphocytes Working Group recommendations established by Salgado et al.[69,70] This approach recommends reporting the percentage of stromal TILs, that is lymphocytes located into the stroma, between the tumor cells, and do not directly interact

with carcinoma cells. The percentage of stromal TILS results from the area of stromal tissue area occupied by mononuclear inflammatory cells over total intratumoral and stromal area.

TILs were evaluated on diagnostic core biopsy and on residual tumor after neoadjuvant chemotherapy.

#### 3.4.4 Residual cancer burden

Residual cancer burden (RCB) was estimated from residual disease in both the breast and the lymph nodes after neoadjuvant chemotherapy. The following variables were retrieved: largest bidimensional measurements of residual primary tumor bed, diameter of the primary tumor bed in the resection specimen, the proportion of the primary tumor bed that contains invasive carcinoma and/or in situ carcinoma, the number of positive axillary lymph nodes, and the diameter of the largest metastasis in an axillary lymph node. RCB has been calculated through the online calculator tool and declinated into four categories: RCB-0 (complete pathologic response = pCR), RCB-I (minimal residual disease), RCB-II (moderate residual disease) and RCB-III (extensive residual disease).

### 3.5 Sample size and statistical analysis

Sample size was estimated using a Simon's two-stage design. Assuming a 10% pCR rate with standard chemotherapy, a pCR of 25% with the study treatment was considered of interest. Setting $\alpha=0.05$ and $\beta=0.20$, a total of 43 patients were required. The first stage (at least three pCR out of 18 patients) was accomplished in November 2018. In the second stage additional 25 patients were enrolled. To fulfill the statistical hypothesis, at least eight pCR out of 43 patients were required.

Efficacy endpoints were evaluated on the intention-to-treat (ITT) population including all enrolled patients. Clinical objective response is reported for patients who underwent breast ultrasound both at baseline and immediately before surgery. Percentages and their 95% confidence intervals (95% CI) were calculated according to the Wilson method with continuity correction.

The incidence of treatment-related adverse events (trAEs, relation with study treatment determined by the physician) was calculated by treatment phase (i.e. anthracycline phase and nivolumab phase).

The association of molecular subtypes and TILs with pCR was studied using univariate logistic regression or the $\chi 2$ test. The association of gene signatures with pCR was studied using a linear model without a blocking factor with p-values adjusted using the Benjamini and Yekutieli False Discovery Rate (FDR) adjustment (models fitted using the limma package in R).

Bivariate correlation between gene signatures were assessed by Pearson's coefficient.

The combined score of Basal subtype and TILs was calculated from the estimated coefficient of each variable in a bivariate logistic model for pCR: TILs (%) x 0.15 + Basal (0=no, 1=yes) x 2.37. The performance of the score was estimated by determining the area under the ROC curve (AUC).

The level of significance was $p<0.05$. Data were analyzed with IBM® SPSS® Statistics (version 27), R software (version 4.0.3), and SAS (version 9.4).[71]

### 3.6 Outcomes

The primary endpoint was the rate of patients achieving a pCR by local pathology evaluation, defined as the absence of invasive cancer cells in breast and axilla (ypT0/is, ypN0).

Key secondary endpoints were: clinical objective responses in the breast, breast conservative surgery rate, conversion to conservative surgery from mastectomy, definition of molecular intrinsic subtypes by PAM50.

Clinical objective responses in the breast were defined as partial or complete responses according to the Modified Response Evaluation Criteria in Solid Tumors (version 1.1) criteria, based on ultrasound examination performed at baseline and immediately before surgery.

The rate of breast conservative surgery was calculated as the percentage of conservative procedures over total surgeries. The conversion from mastectomy was calculated as the percentage of patients initially candidate to mastectomy who underwent breast conserving surgery.

# 4 RESULTS

## 4.1 Patients' characteristics

From October 2017 to October 2019 43 patients were enrolled, received at least one dose of study treatment, and underwent surgery.

Patients' characteristics as the baseline are shown in **Table 4**.

**Table 4.** Patients' characteristics

| **Characteristic** | | **N tot= 43** |
|---|---|---|
| | | **N (%)** |
| **Age, years** | **median (range)** | 45 (31-54) |
| **Clinical stage** | **IIa**<br>**IIb**<br>**IIIa** | 21 (48.9%)<br>14 (32.6%)<br>8 (18.6%) |
| **Tumour size** | **T1**<br>**T2**<br>**T3** | 4 (9.3%)<br>32 (74.4%)<br>7 (16.3%) |
| **Lymph node status** | **N0**<br>**N1** | 21 (48.8%)<br>18 (41.9%) |

| | | |
|---|---|---|
| | **N2** | 4 (9.3%) |
| **Histological type** | **Ductal**<br>**Lobular** | 41 (95.3%)<br>2 (4.7%) |
| **Histologic tumour grade** | **Grade 1**<br>**Grade 2**<br>**Grade 3** | 1 (2.3%)<br>18 (41.9%)<br>24 (55.8%) |
| **ER expression** | **median %, (Q1:Q3)**<br>**≥10%** | 90 (75:95)<br>43 (100%) |
| **PR expression** | **median % (Q1:Q3)**<br>**≥10%** | 82.5 (40:90)<br>38 (88.4%) |
| **Ki67, %** | **median (Q1:Q3)** | 30 (25:41) |
| **HER2 status** | **IHC 0/1+**<br>**IHC 2+ and FISH neg**<br>**FISH neg** | 29 (67.4%)<br>6 (14.0%)<br>8 (18.6%) |
| **Phenotype** | **HR+/HER2-, Ki67≥20%, any Grade**<br>**HR+/HER2-, Ki67<20%, Grade 3** | 41 (95%)<br>2 (5%) |

Abbreviations: N, number; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor 2; IHC, immunohistochemistry; FISH, in situ fluorescence hybridization.

Median age at diagnosis of BC was 45 years (range 31-54). Most patients had ductal histology (95.3%) and high histologic grade (grade 3, 55.8%) tumors. The majority of tumors were T2 (74.4%). Nodal involvement was present in 51.2% of patients at diagnosis (nodal status N1 in 41.9% and N2 in 9.3%). Median Ki67 was 30% (range 25:41). Ninety-five % of the patients had a luminal B phenotype with Ki67 higher than 20%.

## 4.2 Outcomes

Seven out of 43 patients achieved a pCR (16.3%, 95%CI 7.4%-34.9%, not meeting the pre-specified hypothesis) and 11 achieved a RCB class 0-1 (25.6%, 95%CI 14.0%-41.8%).

Among the 34 patients with available ultrasound examination at the baseline, 24 obtained a clinical objective response (complete plus partial response 70.6%, 95% CI 52.3%-85.5%), seven had a stable disease (20.6% 95% CI 9.3%-38.8%), while 3 patients showed progressive disease (8.8% 95% CI 2.3%-25%).

A breast conservative surgery was performed in 13 patients (30.2%, 95% CI 17.7%-46.7%). The type of surgery in the absence of neoadjuvant treatment was indicated at baseline for 38 patients. Of the 28 patients initially candidate to mastectomy, seven underwent breast conservative surgery (conversion rate 25.0%, 95%CI 11.4%-45.8%). Primary and secondary efficacy endpoints are detailed in **Table 5**.

**Table 5.** Primary and secondary efficacy endpoints

| **Efficacy endpoint** | **N/tot (%)** | **95% CI** |
|---|---|---|
| pCR (ypT0/is, ypN0) | 7/43 (16.3%) | 7.4%-34.9% |
| RCB class 0-1 | 11/43 (25.6%) | 14.0-41.8% |
| Objective response by ultrasound (breast)* | | |
| Complete | 6/34 (17.6%) | 7.4%-35.5% |
| Partial | 18/34 (52.9%) | 35.4%-70.7% |
| Complete or Partial response | 24/34 (70.6%) | 52.3%-85.5% |
| Stable disease | 7/34 (20.6%) | 9.3%-38.8% |
| Progression disease | 3/34 (8.8%) | 2.3%-25.0% |
| Breast conserving surgery** | 13/43 (30.2%) | 17.7%-46.7% |
| Conversion from mastectomy to breast conserving surgery | 7/28 (25.0%) | 11.4%-45.8% |

Abbreviations: N, number; tot, total; CI, confidence interval; pCR, pathological complete response; RCB, Residual Cancer Burden.
*Calculated over a total of 34 patients who underwent breast ultrasound both at baseline and immediately before surgery. The remaining 9 patients underwent either breast magnetic resonance imaging or contrast-enhanced mammography as per local policy and were not assessable for objective response according to protocol criteria.
** Reasons to perform mastectomy in 30 patients were: tumor size/breast volume ratio (n=17), aesthetic reasons (n=3), prophylactic procedure (n=3), multicentric tumor (n=6), large calcification area (n=1).

### 4.3 Gene expression and TILs analysis

Gene expression data were available for the total population. According to PAM50 gene expression predictor, subtype distribution was Luminal B for 56% of patients (n=24), Luminal A for 25% (n=11), Basal for 19% (n=8).

The distribution of pCR according to intrinsic subtypes is shown in **Figure 2**. pCR rate was significantly higher for Basal (4 out of 8, 50%) as compared to other subtypes (pCR 9% and 8% for respectively Luminal A and B, p=0.017).

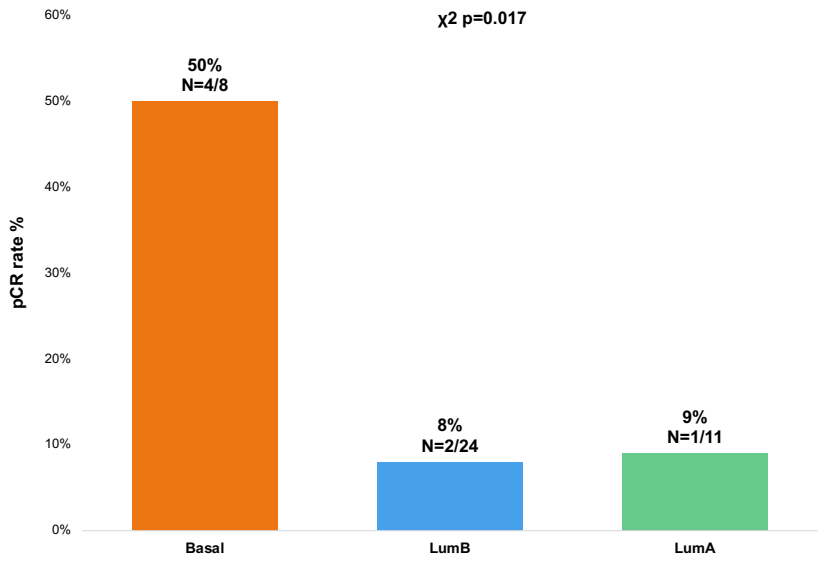


**Figure 2.** pCR rate according to intrinsic subtype by PAM50

**Figure 3** represents the heatmap of gene expression. The following inflammatory response and immune gene signatures were significantly overexpressed in pCR as compared to non-pCR patients (adjusted $p<0.05$): CD8 T-cells, cytotoxic cells, cytotoxicity, IFN gamma, inflammatory chemokines, macrophages, PD-L1, PD-L2, IDO-1, TIGIT (T cell immunoreceptor with immunoglobulin and ITIM domain) and tumor inflammation signature.

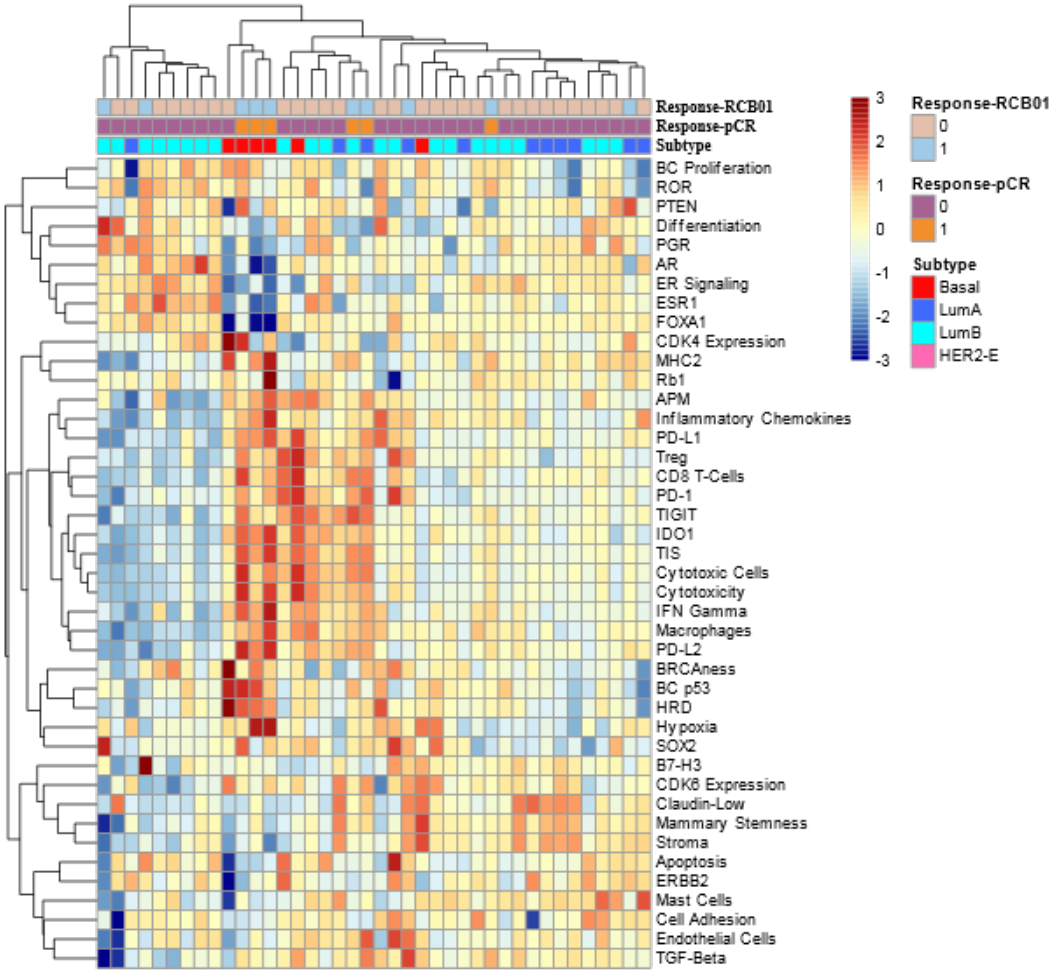


**Figure 3.** Heatmap of gene expression data for 40 patients using unsupervised hierarchical clustering to show relatedness among signature scores for each sample. The signatures are displayed in rows and listed to the right of the heatmap. Each column is a unique sample.

There was a weak to moderate positive significant correlation of Basal subtype signature with each of the above-mentioned immune signatures (Pearson's coefficients ranging from 0.319 to 0.606, **Figure 4**), suggesting a partial biological overlap only.

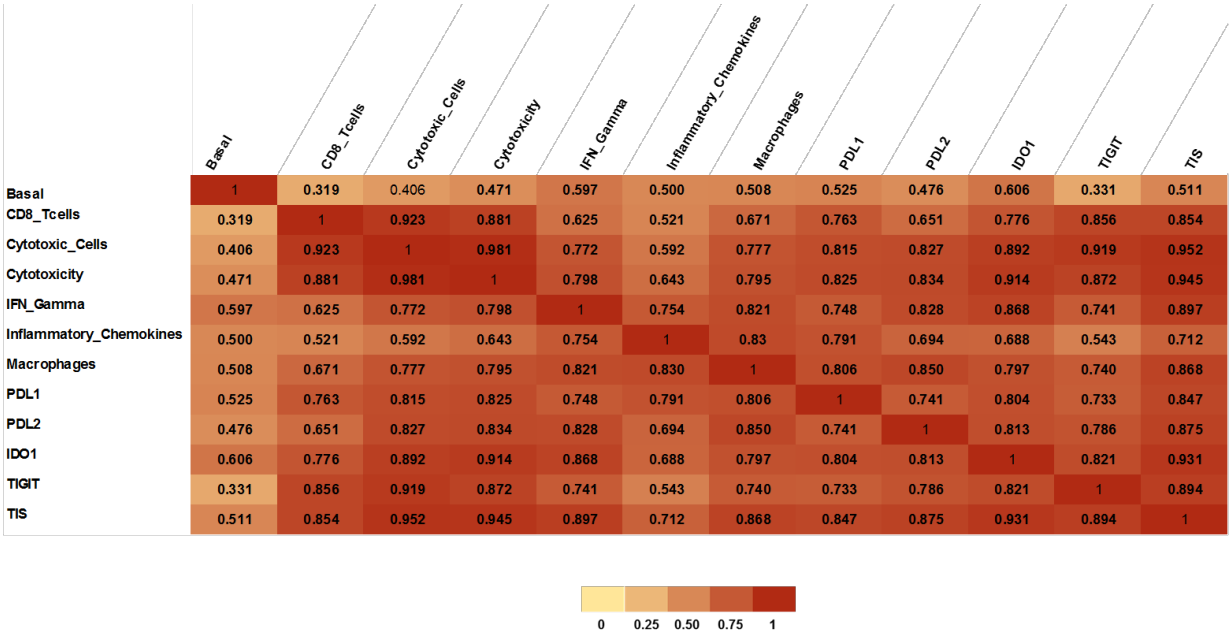


**Figure 4**. Correlation matrix showing the correlation between Basal PAM50 centroid signature and immune-related gene signatures that resulted significantly associated with pCR. Perason's correlation coefficients are shown.

TILs were evaluated from the diagnostic core biopsy (t0) and from the surgical sample (t2). The median level of TILs in baseline samples from patients achieving a pCR (15%; Q1:Q3, 4%:30%) was significantly higher as compared to non-pCR patients (2%; Q1:Q3, 1%:3%, p<0.001, **Figure 5**). TILs significantly increased after chemotherapy (p=0.029).

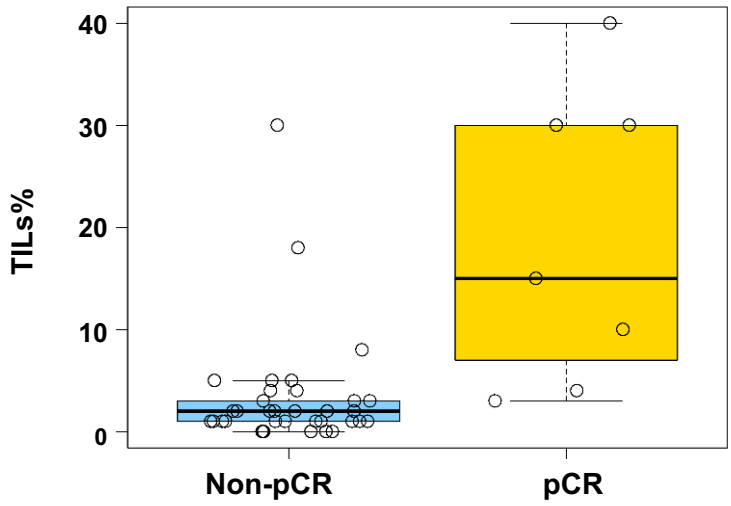


**Figure 5**. Boxplot showing TILs levels in pCR vs non-pCR patients

We performed a bivariate logistic regression analysis considering TILs level and Basal subtype. Both factors were independently associated with pCR (odds ratio 1.16 95%CI 1.04-1.31, p=0.010 for each 1% TILs increment and odds ratio 10.71 95%CI 1.01-113.07, p=0.049 for Basal vs non-Basal subtype). The derived integrated score had an AUC of 0.95 (95%CI 0.89-1.00) for pCR prediction (**Figure 6**). According to the optimal cut-off derived by Receiving operator curve (ROC) analysis (1.74), pCR rate was 58.3% (7/12) vs 0% (0/31) for high vs low score (p<0.001).

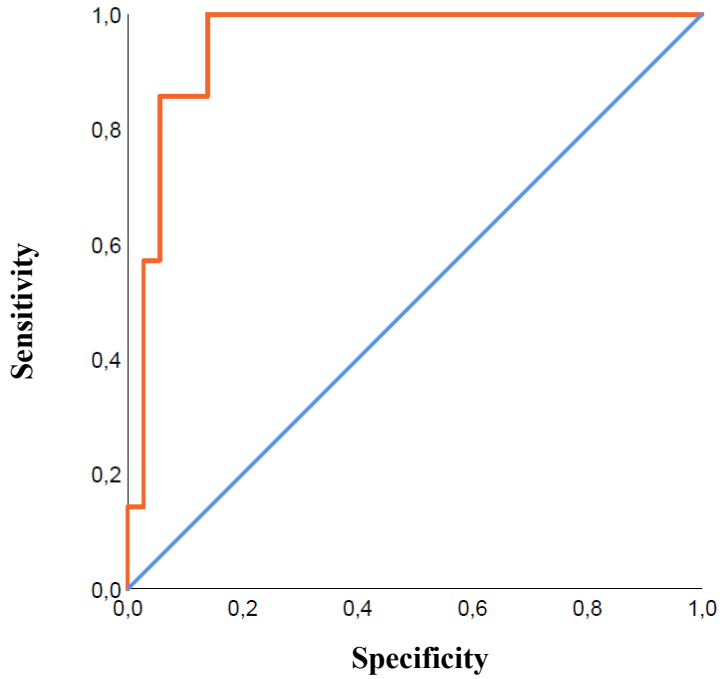


**Figure 6**. ROC for combined Basal subtype and TILs to predict pCR.

## 4.4 Safety

**Table 6** summarizes trAEs according to treatment phase. As expected, nausea was the most common event during anthracycline treatment, followed by neutropenia (n=24 [55.8%] and n=16 [37.2%] patients, respectively). Four patients (9.3%) experienced

grade 4 neutropenia. Among the 42 patients who received at least one nivolumab dose, increases in GGT (n=7 [16.7%]), ALT (n=7 [16.7%]), and AST (n=4 [9.5%]) accounted for the most common grade 3 trAEs. Most frequent potentially immune-related adverse events during nivolumab phase were endocrinopathies (all of grade 1-2), including hyperthyroidism (n=5 [11.9%]), hypothyroidism (n=6 [14.3%]), adrenal insufficiency (n=1 [2.4%]) and ACTH decrease (n=2 [4.8%]). Immune-related skin toxicities occurred in five patients, including one grade 3 erythema nodosum. We also observed a case of grade 3 immune-related pancreatitis. Serious adverse events during nivolumab were ALT and/or AST and/or GGT increase (n=3), and immune-related pancreatitis (n=1). Serious adverse events during chemotherapy included febrile neutropenia (n=2). Overall, 81.4% of patients received at least six courses of nivolumab. Nine patients permanently discontinued nivolumab for safety reasons, and three patients discontinued nivolumab for other reasons (including two with local progression). One patient permanently discontinued study treatment after the three courses of chemotherapy due to febrile neutropenia and did not receive nivolumab.

**Table 6**. Summary of trAEs

| **trAEs occurring in >5% of patients (any Grade)** | **Chemotherapy phase, n=43** | | | |
|---|---|---|---|---|
| | **Any Grade, n (%)** | **G1-2, n (%)** | **G3, n (%)** | **G4, n (%)** |
| Nausea | 24 (55.8%) | 24 (55.8%) | 0 | 0 |
| Neutropenia | 16 (37.2%) | 6 (14.0%) | 6 (14.0%) | 4 (9.3%) |
| Fatigue | 15 (34.9%) | 15 (34.9%) | 0 | 0 |
| Anemia | 7 (16.3%) | 7 (16.3%) | 0 | 0 |
| ALT increased | 7 (16.3%) | 5 (11.6%) | 2 (4.7%) | 0 |
| White blood cells decreased | 6 (14.0%) | 4 (9.3%) | 2 (4.7%) | 0 |
| GGT increased | 5 (11.6%) | 5 (11.6%) | 0 | 0 |
| AST increased | 3 (7.0%) | 2 (4.7%) | 1 (2.3%) | 0 |
| Vomiting | 3 (7.0%) | 3 (7.0%) | 0 | 0 |
| **trAEs occurring in >5% of patients (any Grade)** | **Nivolumab phase, n=42** | | | |
| | **Any Grade, n (%)** | **G1-2, n (%)** | **G3, n (%)** | **G4, n (%)** |
| ALT increased | 14 (33.3%) | 7 (16.7%) | 7 (16.7%) | 0 |
| AST increased | 13 (31.0%) | 9 (21.4%) | 4 (9.5%) | 0 |
| Arthralgia | 9 (21.4%) | 9 (21.4%) | 0 | 0 |
| GGT increased | 8 (19.0%) | 1 (2.4%) | 7 (16.7%) | 0 |
| Fatigue | 5 (11.9%) | 5 (11.95) | 0 | 0 |
| Anemia | 4 (9.5%) | 4 (9.5%) | 0 | 0 |

| | | | | |
|---|---|---|---|---|
| Nausea | 4 (9.5%) | 4 (9.5%) | 0 | 0 |
| Lymphocyte count decreased | 3 (7.1%) | 2 (4.8%) | 1 (2.4%) | 0 |
| **Potentially irAEs (any incidence, any Grade)** | **Nivolumab phase, n=42** | | | |
| | **Any Grade, n (%)** | **G1-2, n (%)** | **G3, n (%)** | **G4, n (%)** |
| Hypothyroidism* | 6 (14.3%) | 6 (14.3%) | 0 | 0 |
| Hyperthyroidism* | 5 (11.9%) | 5 (11.9%) | 0 | 0 |
| Skin** | 5 (11.9%) | 4 (9.5%) | 1 (2.4%) | 0 |
| ACTH decreased | 2 (4.8%) | 2 (4.8%) | 0 | 0 |
| Infusion related reaction | 2 (4.8%) | 2 (4.8%) | 0 | 0 |
| Adrenal insufficiency | 1 (2.4%) | 1 (2.4%) | 0 | 0 |
| Pancreatitis | 1 (2.4%) | 0 | 1 (2.4%) | 0 |

Abbreviations: trAEs, treatment-related adverse events; n, number; ALT, alanine aminotransferase; GGT, γ-glutamil transferase; AST, aspartate aminotransferase; ACTH, adrenocorticotropic hormone.
*Two patients had both hyperthyroidism and hypothyroidism.
**Including: pruritus, maculo-papular rash, acneiform rash, erythema nodosus

# 5 DISCUSSION AND CONCLUSIONS

We reported the results of the first trial evaluating a multimodal treatment with immunotherapy, chemotherapy and hormonal therapy, dedicated to a population of HR-positive/HER2-negative pre-menopausal patients including gene expression profiling.

Seven out of 43 enrolled patients achieved a pCR, with a total rate of 16.3%, not meeting the primary endpoint. With regards to RCB, 25.6% of patients were categorized as class 0 or I. Furthermore, objective response rate in breast was reported in 68.6% of evaluable patients.

The I-SPY2 trial previously evaluated immunotherapy in a population of HR-positive HER2-negative early BC at high risk of recurrence according to Mammaprint.[39] The addition of pembrolizumab to chemotherapy resulted in an estimated 17% improvement in pCR (total rate 30%) and a shift in RCB distribution to a lower disease burden, with 46% of patients in the experimental arm classified as RCB class 0-I. Although a comparison between the studies seems inappropriate, due to the different design, chemotherapy backbone and population, the I-SPY2 indicates that immunotherapy for

patients with high-risk Luminal disease is worth exploring, supporting our background hypothesis.

Although the primary endpoint was not met, the GIADA trial results somehow overcome the reported rate of pCR ranging from 5 to 10% after anthracycline- and taxane-base chemotherapy in Luminal B early BC.

Two recent studies evaluated response to neoadjuvant treatment according to gene expression. The randomized phase 2 CORALLEEN trial compared a combination of aromatase inhibitor letrozole and cycline-dependant kinase (CDK) 4/6 inhibitor ribociclib versus standard anthracycline and taxane for patients with HR-positive/HER2-negative BC classified as Luminal B by PAM50 assay. The pCR rate in the chemotherapy arm was 5.8% (95% CI 1.4-16.6) and the rate of patients achieving a RCB class I or 0 was 11.8 (95% CI 4.5-27.8).[72]

In the phase 2 NeoPAL study, a population of HR-positive/HER2-negative patients Prosigna®-defined Luminal B, or Luminal A and node-positive, stage II-III BC, were randomized to receive letrozole and CDK4/6 inhibitor palbociclib or chemotherapy with anthracycline and taxane as neoadjuvant treatment. In the Luminal B subgroup (defined by PAM50) the rates of patients who achieved a pCR and a RCB class 0-I with chemotherapy were 4.4% and 8%, respectively.[73]

Both the trials included mostly patients with stage II disease, although the enrollment was restricted to post-menopausal patients, as opposed to our population. Indeed, our choice of pre-menopausal women probably determined a higher risk group, with potential better response to chemotherapy. Furthermore, the host and tumor immune environment may turn to a less immunogenic state with increasing age. To the other hand, estrogens may play an immunesuppressive role, thus decreasing the anti-tumor immune response.[35,74]

According to PAM50, our population was enriched with Luminal B (56%) and Basal subtype (19%) as compared to other cohorts of HR-positive, HER2-negative BC patients,

probably as a result of the trial inclusion criteria (premenopausal patients, high grade and/or high Ki67). We observed a pCR in 50% of Basal patients. Although this subtype is known to have the highest rate of pCR (about 35% in HR-positive/HER2-negative) after neoadjuvant anthracycline- and taxane-based chemotherapy, our results after three courses of anthracycline seems unlikely solely due to the intrinsic chemosensitivity.[75] As a matter of fact, gene expression data revealed the contribution of immune-related processes in the modulation of pCR. Even if, as expected, immune signatures were positively correlated with Basal subtype, the strength of the correlation was weak to moderate, suggesting that the biologic information is not completely superimposable.

To better understand the role of the immune system, we evaluated TILs on tumor samples. Higher TILs at the baseline were predictive of a better response to chemotherapy. TILs have been previously associated with response to neoadjuvant chemotherapy in HR-positive, HER2-negative BC patients, with pCR rates of about 15% in case of TILs>10%.[33] With all the limitations of cross-studies comparisons and potential biases related to patients' selection, in our trial we observed a 71% pCR rate (5/7) for patients with TILs>10%. Moreover, the finding that TILs increased after anthracycline emphasizes the role of chemotherapy as a primer to entangle the immune system, consistently with previous evidences.[58] Our result adds another piece of information to the role of TILs in BC, by suggesting that this biomarker may serve in future trials of immunotherapy for Luminal B BC as a stratification or selection factor.

Other authors have reported intrinsic subtype and TILs as predictor of response to chemotherapy.[76–80] We condensed the independent association of TILs and Basal subtype with pCR in a score that showed a high sensitivity and specificity for pCR prediction. Although this score should be further validated, our findings suggest that both biomarkers should be incorporated into the design of trials of chemo-immunotherapy for HR-positive, HER2-negative BC.

With regard to chemotherapy safety, no unexpected events were reported, and the totality of the patients received the planned three courses of epirubicin and cyclophosphamide.

Among the 42 patients that received at least one dose of nivolumab, the most frequent grade 3 AEs were ASL and ALT increase (17% and 10% respectively). Overall, our rates of hepatic toxicity were considerably higher than those reported by other experiences of immunotherapy for early BC, ranging from 2% to 5%.[29,66,81]

The most common irAEs, described in 26% of patients, were thyroid dysfunction. Hyperthyroidism reported in 11.9% and hypothyroidism in 14.3% of patients. These rates were consistent with other evidences in early BC, although slightly higher.[29,39,66,81]

The reasons for nivolumab safety profile of the GIADA trial are unclear. The chemotherapy backbone, sequence and timing and the addition of endocrine therapy could have had a role. Few evidences suggested the role of hormones in modulating toxicity to immunotherapy. In a retrospective analysis of melanoma patients treated with PD-1, sex was associated with the development of irAEs in multivariate analysis.[82] Intriguingly, pre-menopausal women were more prone to develop irAEs as compared to post-menopausal women.[82] Indeed, estradiol is associated with upregulation of CD4+ T cells, dendritic cells and autoreactive B cells, and these effects could at least partially explain our observations.[83–85] Nevertheless, a causal effect because of the small sample size could not been excluded.

This study has limitations: it is a non-randomized trial with a limited sample size, tissue samples at each timepoint were not evaluable for all included patients. Furthermore, it is impossible to fully disentangle the contribution of chemotherapy, immunotherapy and endocrine therapy. Relevant strengths include: the study design, being one of the first studies to test immunotherapy as part of the neoadjuvant treatment of HR-positive, HER2-negative BC and the inclusion of a selected Luminal B-like population further dissected by molecular subtype.

In conclusion, although the study did not meet its primary endpoint, we observed an interesting rate of clinical response rate, pCR and RCB class 0-1. Coupling clinical data with gene expression profile can individualize patients that are more likely to respond to neoadjuvant treatment. Overall, the combination of chemotherapy, immunotherapy and endocrine therapy resulted in a notable rate of trAEs, even though about 77% of patients completed the study treatment and more than 80% received six of the planned eight cycles of nivolumab. The choice of an optimistic target was taken to counterbalance the risk of exposing patients to potential side effects in a curative setting. Our results are hypothesis-generating and provide a new hint on the role of immunotherapy in early luminal B BC. A more extensive characterization of the immune microenvironment could help disentangle the complexity of response to immunotherapy in these patients. It is also of paramount importance for clinicians to acquire expertise in the management of irAEs, especially in the early setting.

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