Targeting triple negative BREAst cancer metabolism with a combination of chemoimmunotherapy and a FASTing-like approach in the preoperative setting: the BREAKFAST 2 trial

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PROTOCOL SIGNATURE PAGE

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A Study of Fondazione IRCCS Istituto Nazionale dei Tumori

I confirm that the Sponsor will comply with all obligations as detailed in the study protocol and in all applicable regulations and guidelines. I ensure that the Investigators will be informed of all relevant information that become available during the conduct of this protocol.

PRINCIPAL INVESTIGATOR SIGNATURE

	Signature	Date
Prof. Filippo G. de Braud	_____	_____

CO-PRINCIPAL INVESTIGATOR SIGNATURE

	Signature	Date
Dott. Claudio Vernieri	_____	_____

DECLARATION OF THE INVESTIGATOR

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I have read and understood the contents of the clinical protocol for the BREAKFAST 2 Trial dated October 1st, 2022 and agree to perform the study according to the study protocol and all applicable regulations and guidelines. I will ensure that the Investigators are informed of all relevant information becoming available during the conduction of this study.

Principal investigator name:

Institution:

Signature:

Date:

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1 ABBREVIATIONS AND DEFINITIONS OF TERMS

AEs	Adverse Event
BIA	Bioelectric Impedance Analysis
BMI	Body Mass Index
CESM	Contrast-enhanced mammography
CRF	Case Report Form
CT	Computed Tomography
CTCAE	Common Terminology For Adverse Events
DMFS	Distant Metastasis-Free Survival
DFS	Disease Free Survival
EC	Ethics Committee
ECOG	Eastern Cooperative Oncology Group
EFS	Event Free Survival
EORTC	European Organization For Research And Treatment Of Cancer
EOT	End Of Treatment
ER	Estrogen Receptor
FFPE	Formalin-Fixed Paraffin-Embedded
FLA	Fasting-Like Approach
IEC	Independent Ethics Committee
IEO	European Institute Of Oncology
IGF-1	Insulin-Like Growth Factor 1
IHC	Immunohistochemistry
INT	Fondazione IRCCS Istituto Nazionale Dei Tumori
ISH	In Situ Hybridization
KM	Kaplan-Meier
LVEF	Left Ventricular Ejection Fraction
mTORC	Mechanistic Target Of Rapamycin Complex 1
CE-MRI MUGA	Contrast-Enhanced Magnetic Resonance Imaging Multi-Gated Scintigraphic Scan
NGS	Next Generation Sequencing
OS	Overall Survival
OTC	Over-The-Counter

PBMCs	Peripheral Blood Mononuclear Cells
pCR	Pathologic Complete Response
PgR	Progesteron Receptor
PP2A	Protein Phosphatase 2 A
PS	Performance Status
SAE	Serious Adverse Events
SCFAs	Short-Chain Fatty Acids
SNLs	Sentinel Lymphonodes
TILs	Tumor Infiltrating Lymphocytes
WCRF	World Cancer Research Fund
WHO	World Health Organization

2 BACKGROUND

3 Standard preoperative therapy and unmet clinical needs in triple-negative breast cancer (TNBC) treatment

Breast cancer (BC) is the most commonly diagnosed neoplasm, with an estimated 2,3 million new cases diagnosed in 2020 worldwide. In Europe, BC accounts for 27.8.% of female cancers and 1 out of 12 women is estimated to develop breast cancer before the age of 75. Moreover, BC represents the leading cause of cancer-related deaths in women in the majority of European countries[1]. BC prevalence is rising, mainly as a result of increased incidence and population ageing but also of early detection and improvements in treatment efficacy.

The majority (~90%) of patients with BC are diagnosed with localized disease;[2] however, about 20-30% of resected BCs eventually recur with metastatic disease, which is almost invariably incurable.

Approximately 15%-20% of all BCs are classified as Triple-Negative Breast Cancer (TNBC), as defined by absent or minimal (<1%) expression of oestrogen receptor (ER) and progesterone receptor (PgR) at immunohistochemistry (IHC), as well as by absence of HER2 overexpression at IHC and *HER2* gene amplification. TNBC is characterized by an aggressive behavior, high metastatization potential and fast acquisition of resistance to chemotherapy. These features portend the worst prognosis among BC subgroups[3,4]. This is in part due to the lack of ER/PgR expression, which makes endocrine manipulations ineffective, but mostly to its intrinsic biological characteristics, resulting in fast acquisition of resistance to chemotherapy and tendency to spread to vital organs (such as liver, lungs and brain) in addition to lymph nodes and bones[5].

Due to high proliferation rate and common alterations in DNA repair enzymes, TNBC is exquisitely sensitive to cytotoxic chemotherapy, in particular to anthracyclines, cyclophosphamide and taxanes. The administration of systemic chemotherapy before surgery (neoadjuvant or preoperative chemotherapy) is the recommended approach for TNBC patients with clinically/radiologically node-positive (N+) disease and/or primary tumors larger than 1 cm in their maximum diameter[6].

In the preoperative setting, TNBC presents higher response rates when compared to hormone-sensitive neoplasms[7].

The use of preoperative chemotherapy is associated with several potential advantages. Firstly, it is associated with higher rates of breast-conserving surgery[8]. Secondly, it gives the opportunity to rapidly test tumor sensitivity to standard chemotherapy (plus/minus experimental treatments). Moreover, it provides prognostic indications; indeed, achieving pCR with preoperative chemotherapy is associated with significantly better long-term outcomes in patients with TNBC, including relapse free survival (RFS), distant-metastasis free survival (DMFS) and overall survival (OS)[9]. Finally, it allows to test the predictive role of biological (patient- or tumor-related) biomarkers of sensitivity to the proposed treatment in the pre-chemotherapy tissue, and to study how these biomarkers are modulated during the treatment in patients who fail to achieve pCR.

The standard preoperative treatment for TNBC has consisted for years in the sequential administration of anthracyclines- and taxanes-based chemotherapy regimen, which is associated with an expected pCR rate in the range of 31%-48%[10-12].
Several phase III trials recently tested the addition of carboplatin to standard anthracycline-taxane preoperative chemotherapy in patients with TNBC.
By promoting DNA interstrand cross-linking, carboplatin induces DNA breaks that result in enhanced cytotoxicity in tumors with impaired DNA damage response, including the subgroup of patients with germline or somatic inactivating mutations of *BRCA1/2*[13]. Clinical evidence comes from the work of Tutt et al. where carboplatin monotherapy demonstrated to double the response rate compared to docetaxel monotherapy in patients with advanced TNBC holding germline BRCA1/2 mutation[14].
In the early-stage disease treatment setting, several trials have been carried out to assess the efficacy of adding platinum compounds to standard anthracycline and taxane-based preoperative chemotherapy in TNBC. A meta-analysis[10] of 9 different randomized controlled trials (RCT) showed a consistent increase of pCR rate from 37% to 52.1% with the addition of carboplatin. Of note, in this context, the presence of inactivating *BRCA 1/2* mutations did not predict higher pCR rates with the addition of platinum compounds to standard preoperative chemotherapy[10]. A recent update of this meta-analysis showed that platinum-based neoadjuvant chemotherapy, as compared with platinum-free regimens, is associated with significantly longer EFS (HR 0.70, 95% CI 0.56-0.89) and with a non-significant 18% reduction in the risk of death (HR 0.82, 95% CI 0.64-1.04)[15]. Based on the reported improvement of TNBC patient EFS with the addition of carboplatin to anthracycline-taxane-based neoadjuvant chemotherapy, the chemotherapy backbone of neoadjuvant treatment in patients with stage II-III TNBC (node positive and/or primary tumors of maximum diameter higher than 2 cm) should contain an anthracycline, a taxane and carboplatin. In particular, 12 weekly administration of carboplatin-paclitaxel doublet, followed by for triweekly/biweekly injections of anthracyclines (doxorubicin/epirubicin)-cyclophosphamide have emerged as the most effective chemotherapy backbone in the neoadjuvant treatment setting. Since the efficacy of neoadjuvant carboplatin is not associated with the presence/absence of *BRCA1/2* alterations, this chemotherapy regimen has become the standard-of-care backbone chemotherapy in several European and US Institutions in recent years[6].
Among BC subtypes, TNBC is the most immunogenic one, as it is characterized by a relatively high mutational burden and a tumor microenvironment characterized by immune cell infiltration; in addition, tumor infiltrating lymphocytes represent a strong prognostic factor in early-stage TNBC[16]. For these reasons, several clinical trials investigated the antitumor activity of adding immune checkpoints inhibitors (ICIs) in combination with neoadjuvant chemotherapy in patients with advanced TNBC. Among these studies, the Impassion 130 and the Keynote 355 trials demonstrated a clinically meaningful benefit from the addition of immunotherapy to standard first line treatment in advanced TNBC expressing high PDL1 levels[17,18].
Recently, several clinical trials showed that the addition of ICIs inhibitors to standard cytotoxic chemotherapy is effective also in the neoadjuvant setting, leading to a significant increase in pathological complete response (pCR) rates[19-21]. Among these trials, both the phase II GeparNuevo[22], which investigated Atezolizumab in

combination with Nab-paclitaxel followed by Epirubicin plus Cyclophosphamide, and the phase III KEYNOTE 522 trial, in which Pembrolizumab was combined with anthracycline, taxane and carboplatin-based chemotherapy, showed an improvement in terms of pCR rates and long-term efficacy outcomes, such as DFS and EFS, respectively, in patients receiving ICIs. Importantly, the clinically meaningful EFS improvement demonstrated by the KEYNOTE-522 (HR 0.63; P = 0.0003), set the new standard for stage II-III early-stage TNBC.
However, despite these improvements, a non-negligible proportion of TNBC patients treated in the early-stage setting will eventually recur, especially among those not achieving pCR at surgery. For instance, in the KEYNOTE-522 trial, the 3-year EFS rate in pembrolizumab-treated patients with residual disease at surgery is only 67.4%, highlighting the urgent need to develop new effective therapeutic strategies to improve treatment efficacy for TNBC patients.
In addition, selecting patients more likely to benefit from the addition of immunotherapy to neoadjuvant chemotherapy remains a clinical challenge. Indeed, differently from the metastatic setting, PD-L1 positivity do not seem to have a predictive value in TNBC patients treated with neoadjuvant ICIs, as patients with PD-L1+ TNBC obtain a higher pCR rate than those with PD-L1– cancers independently from the treatment received.

3.1 Rationale for targeting TNBC metabolism in combination with neoadjuvant chemoimmunotherapy

When compared to other breast cancer subtypes, TNBC is characterized by higher histologic grade, higher proliferation rate and clinical aggressiveness. These biological and clinical characteristics correspond, from a metabolic point of view, to highly glycolytic tumors; indeed, enhanced glycolysis represents the major source of anabolic precursors for the biosynthesis of amino acids and nucleotides, which are required by rapidly growing and proliferating cancer cells to synthesize or repair macromolecules (proteins, DNA). For instance, serine biosynthesis from the glycolysis intermediate 3-phosphoglycerate is frequently upregulated in human TNBC, while inhibition of serine synthesis results in inhibition of cancer cell growth and proliferation[23].
However, TNBC are heterogeneous neoplasms from a metabolic point of view. For instance, Basal-Like 2 (BL2) tumors, as defined according to the classification of Lehmann[24], are characterized by upregulation of genes involved in glycolysis. On the other hand, mesenchymal-like TNBC mostly rely on lipid and cholesterol metabolism. More recently, a multi-omics analysis of TNBC tumor samples identified three metabolic subtypes of TNBCs, characterized by distinct metabolic gene expression, metabolite abundance, genomic drivers, and sensitivity to various metabolic inhibitors[25].
Based on this heterogeneity, different metabolic interventions could produce differential anticancer activity depending on the specific metabolic tumor dependencies.

3.2 The Fasting-Like Approach (FLA) as a strategy to target cancer metabolism and to improve the tolerability of cytotoxic chemotherapy

Effects of nutrient starvation in TNBC

In *in vitro* experiments, reducing the concentration of glucose and serum - the major sources of growth factors and amino acids- in cell growth media strongly sensitizes models of TNBC to different chemotherapeutical agents, including doxorubicin, cyclophosphamide and platinum compounds[26,27]. This effect mainly results from the fact that rapidly growing and proliferating cancer cells are exquisitely sensitive to metabolic deprivation, especially when they are exposed to DNA-damaging agents. Indeed, under such conditions, they are unable to activate repair processes that require energy units (in the form of ATP) and anabolites.

Rationale for fasting during cancer treatment

Cyclic fasting is the simplest and most effective way to recapitulate *in vivo* the metabolic effects of *in vitro* starvation of glucose, amino acids and growth factors.
In particular, water-only fasting reduces the blood concentration of metabolites (e.g., glucose) and growth factors (e.g., insulin and insulin-like growth factor or IGF-1) that foster tumor growth and proliferation, and it also increases the blood concentration of metabolites that inhibit energy production in cancer cells (e.g., ketone bodies) [28].
Preclinical *in vitro* and *in vivo* evidence suggests that starvation/fasting is more toxic to highly replicating cancer cells than to normal tissues[29]. This phenomenon, known as differential stress response (DSR), is the consequence of the intrinsically different proliferative and metabolic characteristics of most cancer cells when compared to their normal counterpart. When deprived of glucose, growth factors (e.g., insulin, IGF-1) and other metabolites (e.g., glutamine), most normal cells typically enter a quiescent proliferative state that is characterized by reduced energy expenditure and inhibition of anabolic functions (such as DNA, protein and lipid synthesis), and by a parallel activation of catabolic processes, such as autophagy, which result in the replenishment of essential metabolites. This response to nutrient starvation allows normal cells to fulfill essential biological functions, such as DNA-repair and *de novo* synthesis of damaged proteins, lipids and macromolecular structures (e.g., ribosomes, mitochondria). These protective activities become especially important when cells need to repair chemotherapy-induced damage to macromolecules and other cellular structures.
On the contrary, constitutive activation of oncogenic pathways in cancer cells makes them less capable of halting growth and proliferation during nutrient starvation; indeed, most cancer cells go on growing and proliferating irrespective of nutrient availability in the extracellular environment. As a consequence of this uncoupling between cell proliferation and the concentration of extracellular metabolites, cancer cells are exposed to rapid energy/metabolite depletion that leads to energetic crisis and the impossibility to synthesize new DNA, protein and lipid molecules to sustain their growth.

The *in vitro*-observed DSR between normal and cancer cells predicts that fasting during ChT administration could reduce ChT-induced damage to normal tissues, while improving its *in vivo* anticancer activity.

In vivo evidence for combining fasting with cytotoxic chemotherapy

Consistent with *in vitro* experiments, cycles of short-term fasting (STF), which recapitulate *in vitro* starvation by reducing the concentration of blood glucose, insulin and IGF-1, significantly reduced the *in vivo* growth of several tumor models, including orthotopic models of TNBC (including syngeneic ones). Moreover, cyclic fasting also synergized with different cytotoxic agents, including anthracyclines and platinum compounds, against murine tumors, while protecting healthy tissues from ChT-induced damage[23]. These results are consistent with the DSR hypothesis and indicate that fasting has the potential to improve the therapeutic index of some chemotherapeutic agents by potentiating their anticancer activity and protecting normal tissues from chemotherapy-induced AEs. The fact that the fasting-chemotherapy combination not only slowed down tumor growth, but also prolonged animal survival and rescued the lethality of high-dose chemotherapy, further supports the concept of a DSR, and indicates the potential safety and clinical usefulness of this combination.
So far, only a few, small studies have been performed in cancer patients to test the tolerability of STF. The first published case series suggested that water-only fasting for 1-5 consecutive days during ChT is quite well tolerated by patients with different tumor types, and could also reduce hematological and gastrointestinal toxicities associated with cytotoxic treatments, consistent with preclinical experiments[30]. Then, a small randomized study in women with breast cancer showed that 48 hour-water only fasting in combination with (neo)adjuvant ChT is associated with a reduction of ChT-induced hematological toxicities, including thrombocytopenia and anemia[31]. Another randomized trial showed that 72 hour fasting in combination with platinum-based ChT is more effective than 24- or 48-hour fasting in modifying systemic metabolism and in reducing DNA damage to peripheral blood neutrophils and lymphocytes; these data are indicative of a protective effect of fasting during ChT, and also support a positive correlation between fasting duration and normal tissue preservation[32]. Although the population of patients enrolled in this trial was heterogeneous in terms of tumor types, stage and treatment received, the fasting-chemotherapy combination was associated with a high rate of tumor responses, which was superior to what expected on the basis of published studies. While this could simply be the result of selecting patients with better prognostic features (e.g., patients who were not malnourished), it is important to note that fasting did not seem to be detrimental in the population of patients considered. Finally, one randomized trial performed in patients with breast or ovarian carcinoma recently showed that 48-hour fasting may improve the quality of life of patients receiving cytotoxic ChT.[33]

Several concerns have been raised about the use of complete (water-only) fasting in cancer patients, because of the risk of inducing progressive weight loss or sarcopenia. This could be especially relevant for those tumors that frequently induce anorexia (such as tumors of the head and neck tract or the esophagus) or cachexia (such as pancreatic or gastric cancers), especially in the advanced stage. Conversely, cachexia is not

commonly observed in patients with limited-stage TNBC, and it is unlikely that cycles of fasting, alternated with refeeding periods, could directly induce cachexia. Indeed, intentional fasting induces systemic metabolic changes that significantly differ from metabolic changes occurring during cachexia. During intentional short-term fasting, the appetite is conserved, loss of lean mass and sarcopenia do not occur, and fat reserves are used to mobilize energy sources in the form of triglycerides and fatty acids. Conversely, cachexia is characterized by an intense systemic inflammatory response, which leads to loss of appetite and increased degradation of proteins in muscle cells, and finally results in loss of lean tissues and sarcopenia.

The Fasting-Like Approach (FLA) as a safe alternative to complete fasting

Since complete fasting is unlikely to be feasible for the vast majority of cancer patients, studies conducted in recent years have been focused on less drastic dietary interventions that are able to produce metabolic changes comparable to those induced by complete fasting. In particular, fasting-like approaches (FLAs), i.e., plant-based, severely calorie-restricted, low-carbohydrate, low-protein diets, have recently emerged as an equally effective, and potentially better tolerated alternative to complete fasting.
Studies performed in murine TNBC models have demonstrated that cycles of three-days FLA enhance the anticancer activity of anthracycline- or platinum-based chemotherapy, while at the same time promoting intratumor infiltration by cytotoxic $CD8^+$ lymphocytes[26] and reducing tumor infiltration by immunosuppressive regulatory T cells (Tregs). Even more importantly, the depletion of CD8+ T cells in these experiments resulted in impaired efficacy of FLA, thus demonstrating that an active immune system is essential to mediate the antitumor effects of cyclic FLA in syngeneic mouse TNBC models. Moreover, we have recently shown that cycles of FLA deplete TNBC stem cells, and cancer stem depletion makes TNBCs exquisitely responsive to the inhibition of the PI3K/AKT/mTORC1 axis[34].
Based on these data, as well as on results of other preclinical works[35,36], the FLA can be considered a valuable alternative to water-only fasting in preclinical tumor models, including models of TNBC.

Safety, feasibility and metabolic effects of the FLA in cancer patients

Cyclic FLA has been recently tested in relatively large clinical trials in cancer patients. The first of these studies, namely the DIRECT trial, was a multicentric, phase II-III, randomized clinical trial that investigated the antitumor activity of a severely calorie-restricted (~1200 Kcal on day 1; ~200 Kcal on days 2-4), 4-day, plant-based, low amino-acid diet combined with neoadjuvant chemotherapy in 131 breast cancer patients. Unfortunately, the study was prematurely interrupted because of poor patient compliance with the experimental diet; nevertheless, promising antitumor activity was observed in the subset of patients who were compliant with the dietary intervention[37].
The results of this trial highlighted the importance of a proper management of patients undergoing severe calorie restriction to monitor their compliance with experimental dietary regimen[38].
The second trial was a phase Ib study, in which a less severely calorie-restricted dietary intervention regimen (~1100 Kcal on day 1; ~700 Kcal on days 2-5) was combined with standard antitumor therapies in a heterogeneous population of 90 cancer patients.

In this study, the experimental diet was well tolerated, metabolically active, and associated with promising antitumor activity when combined with standard anticancer therapies in a subset of BC patients[35,39].

The third, and most recently published trial, has been conducted by our group at Fondazione IRCCS Istituto Nazionale dei Tumori between January 2017 and January 2020[40]. The study, directed by Dr. Claudio Vernieri and Prof. Filippo de Braud, investigated the safety, feasibility and biological effects of a severely calorie-restricted FLA regimen in combination with their standard anticancer therapies in a heterogeneous cohort of 101 patients with different tumor types. Specifically, the FLA scheme used in this study consisted in a 5-day, vegetal-based, low-calorie (600 Kcal on day 1, 300 Kcal on days 2 to 5), low-carbohydrate, low-protein diet, with no restriction in the intake of non-caloric beverages.

Among 101 patients enrolled in the study, 100 patients were able to complete at least one cycle of FLA. The trial met its primary endpoint, with an incidence of severe FLA-related AEs of 12.9%, i.e., lower than the hypothesized 20%. The most common FLA-related AE was fatigue, which occurred in 90.2% of patients and was G3/G4 in 4% of patients. Other severe (G3/G4) FLA-related AEs consisted of hypoglycemia (5%), nausea (1%), dizziness (1%) and increased AST levels (1%). Serious adverse events (SAEs) occurred in 4 patients (4%), and in 2 cases they were FLA-related (syncope, severe fatigue). Patient compliance to the prescribed FLA regimen was globally excellent, with a total number of 23 minor deviations (22.8% of all patients) and 6 major deviations (5.9%) to the prescribed FLA regimen. Overall, 72 (71.3%) patients were fully compliant with the FLA (absence of major and minor deviations during all FLA cycles), while 95 (94.1%) patients were fully compliant, or they reported only minor deviations. Considering individual FLA cycles in all patients, the global rate of compliance was 91.8% (404 out of 440 cycles), with minor and major deviations being reported in 29 (6.6%) and 7 (1.6%) cycles, respectively.

During the first FLA cycle, in 99 evaluable median plasma glucose levels were reduced by 18.6% (range: [-63.1%; + 67.8%]), serum insulin by 50.7% (range: [-91.3%; + 697%]) and IGF-1 by 30.3% (range: [-72.3%; +139.8%]), while there was an increase of average urinary ketones (from 0.18 mg/dl to 59.9 mg/dl). Similar metabolic changes occurred in 9 healthy volunteers following the same 5-day FLA regimen, but not in a control population of 13 breast cancer patients receiving ChT alone. Of note, we observed qualitatively and quantitatively similar modifications of key metabolic parameters during eight subsequent FLA cycles, thus excluding the occurrence of metabolic adaptation to the FLA. Similar metabolic changes occurred in patients with different tumor types, and regardless of concomitant treatment (ChT *vs* other treatment types) or tumor stage (limited-stage *vs* advanced).

In addition to promising biological and immunomodulatory effects[40], cyclic FLA resulted in complete and long-lasting tumor remissions in some patients with highly aggressive, deadly and treatment-resistant neoplasms, such as pancreatic cancers, lung cancers and triple-negative breast cancer[41]. These “excellent tumor responses” were observed when cyclic FLA was combined with chemotherapy or immunotherapy.

Based on these promising results, on the rationale for using the FLA as an investigational dietary intervention to boost the antitumor efficacy of chemotherapy in TNBC, and also based on results of preclinical studies showing that calorie restriction

synergizes with the antidiabetic compound metformin to cause metabolic crisis and to induce apoptosis in cancer cells[42], in June 2020 we initiated a phase II, randomized clinical trial (BREAKFAST; NCT04248998) in which we aimed at investigating the antitumor activity of combining neoadjuvant anthracycline-taxane chemotherapy with cyclic FLA, plus/minus metformin, in patients with stage I-III TNBC. Between June 2020 and February 2022, the study has enrolled 30 patients, and it was amended to be transformed into a multicentric study to accelerate patient enrollment. However, after changes in the standard-of-care therapy of stage II-III TNBC patients, and in particular after the addition of carboplatin and pembrolizumab to neoadjuvant anthracycline-taxane regimens, we decided to prematurely interrupt the BREAKFAST study, and to design a new trial to combine standard chemoimmunotherapy with cyclic FLA (please see below).

Rationale for combining calorie restriction with immunotherapy

Preclinical studies showed that restricting the dietary intake of carbohydrates or proteins affects the frequency and activation status of myeloid and lymphoid cells in peripheral organs and in tumor microenvironment. For instance, in 4T1 and Py8119 TNBC cell mouse transplants, glycolysis inhibition prevented G-CSF and GM-CSF production by cancer cells, thus reducing myeloid-derived suppressor cells (MDSCs) mobilization and their recruitment to the tumor site[43]. In addition, short-term fasting can downmodulate monocytes in peripheral blood and spleen of healthy mice by inhibiting systemic CCL2 production[44]. Coherently, in a syngeneic murine 4T1 TNBC model, the combination of FLA and chemotherapy (doxorubicin) determined the accumulation of cytotoxic CD3+/CD8+ T cells in the tumor bed, which were shown to be crucial to mediate the synergic effect between FLA and chemotherapy.[26] As observed in a preclinical proof-of-concept study, short-term starvation could synergize with PD-1 blockade in murine models of lung cancer[45] by promoting a decrease of Treg cells and an increase of effector T cells in the tumor microenvironment. Of note, the observed enhancement of tumor sensitivity to PD-1 blockade occurring by short-term starvation was mediated by an antitumor CD8 T cell response and was strictly dependent upon the inhibition of IGF-1–IGF-1R signaling in tumor cells.
These desirable immunomodulatory effects have been recently confirmed and largely expanded in cancer patients enrolled in the recently published NCT03340935 trial[40].
Indeed, at the end of the first FLA cycle, flow cytometry analysis of peripheral blood mononuclear cells (PBMCs) revealed a significant decrease of total monocytes (CD14+) and of two highly immunosuppressive monocyte subsets, i.e., those lacking HLA-DR expression (CD14+HLA-DRneg), acknowledged as monocytic myeloid-derived suppressor cells (M-MDSCs) and CD14+ cells expressing PD-L1 (CD14+PD-L1+). The FLA also reduced low-density CD15+ granulocytes, which include polymorphonuclear MDSCs (PMN-MDSCs). Interestingly, the observed modifications in myeloid sub-populations were similar in patients undergoing the FLA in combination with ChT or with other standard antitumor therapies, but not in a control population of BC patients treated with ChT alone, which even resulted in a boost of CD14+PD-L1+ cells. In 8 healthy volunteers the FLA reduced CD14+, CD14+HLA-DRneg, CD14+PD-L1+ and CD15+ cells similarly to what observed in cancer patients. FLA-

induced reduction of myeloid cell subsets was paralleled by an increase of activated CD8+ T cells (co-expressing PD1 and CD69) and cytolytic CD3negCD16+CD56dim NK cells, while CD3+ T cells expressing the high affinity IL-2 receptor (CD3+CD25+ cells), which can be associated with Treg activity, were reduced. As in the case of myeloid cells, changes in lymphocytic populations occurred independently of concomitant antitumor therapies, and they were similarly observed in 8 healthy volunteers in a similar way as observed in patients. During the second FLA cycle, myeloid and lymphoid PBMC populations were modulated in a similar way as during the first FLA cycle, thus excluding the adaptation to FLA-induced systemic immunological effects. Inflammatory cytokines such as CCL2, G-CSF and IL-6, which are involved in myeloid cell mobilization from the bone marrow, were significantly reduced after the FLA. Finally, and even more importantly, an unplanned, interim analysis of the ongoing DigesT trial (NCT03454282) confirmed most of these systemic immunological modifications at the tumor level in 22 BC patients, where the FLA resulted in an increase of tumor-infiltrating T cells, activated T cells, lymphocytes with a memory phenotype, NKT and activated dendritic cells, paralleled by a decrease of M2 macrophages. Together, these data indicate that the FLA, alone or in combination with standard antitumor therapies, down-regulates immunosuppressive myeloid cell subsets, while at the same time increasing effector cells with an activated phenotype.

3.3 Study rationale and purpose

TNBC is the most aggressive subtype of breast cancer. TNBC patients who achieve pCR during neoadjuvant chemo-immunotherapy have significantly lower rates of disease recurrence or death. Preclinical studies indicate that combining nutrient starvation, in the form of cycles of FLA, with anthracycline- or platinum-based chemotherapy remarkably increases the therapeutic index of chemotherapy against murine and human models of breast cancer, including models of TNBC. In particular, the chemotherapy-fasting/FLA combination increases the anticancer activity of chemotherapy, while reducing treatment-related adverse events (AEs). Moreover, the FLA has demonstrated potent and desirable immunomodulatory effects both in *in vivo* studies and in patients with cancer, and the activation of antitumor immunity is a crucial mediator of the anticancer effects of the FLA, either alone or in combination with chemotherapy. Therefore, there is a strong biological rationale to combine cyclic FLA with ICIs in cancer therapy.
Based on these data, we hypothesize that combining the FLA with standard-of-care, preoperative, anthracycline-taxane-carboplatin chemotherapy plus Pembrolizumab can increase the rate of pCR in a population of patients with stage II-III TNBC.

4 STUDY DESIGN

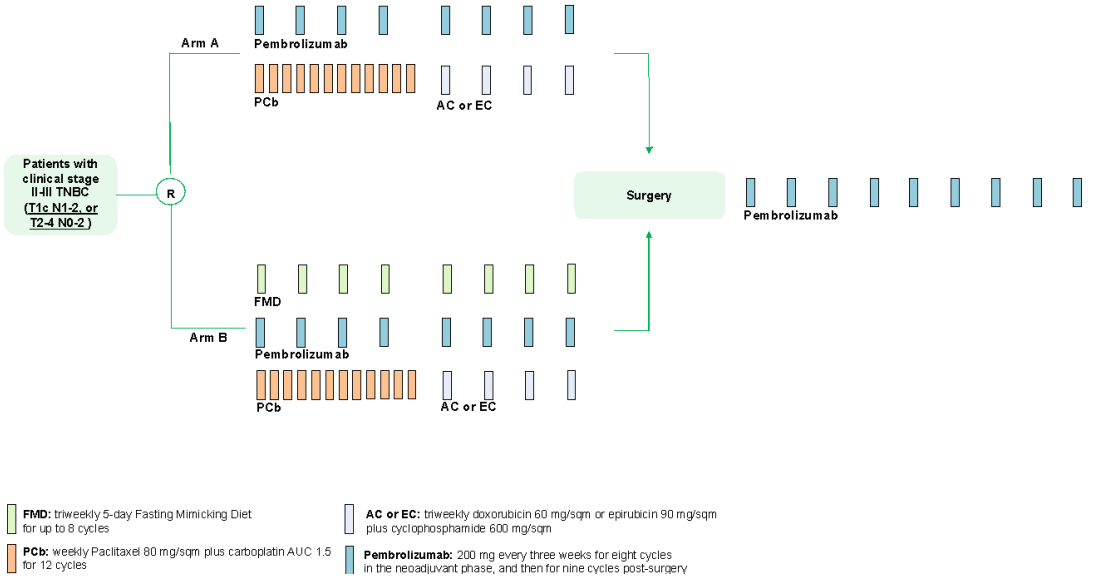
4.1 Description of study design

This is an Italian, multicenter, open-label, two-arm, comparative, randomized phase II study. This study is designed to investigate if the addition if the experimental metabolic intervention consisting in cycles of FLA, as administered every three weeks up to a maximum of 8 consecutive cycles, is able to increase the anticancer activity of standard preoperative chemo-immunotherapy consisting of antracycline-taxane-carboplatin-based chemotherapy plus pembrolizumab in patients with treatment naïve, localized (tumor stage T1c AND nodal stage N1-2, or tumor stage T2-4 AND nodal stage N0-2) invasive Triple Negative Breast Cancer (HER2 negative, ER <1%, PgR <1%). Bilateral and/or multifocal primary tumor is allowed, as well as inflammatory breast cancer, and the tumor with the most advanced T stage should be used to assess the eligibility. If multi-focal/multi-centric disease, TNBC needs to be confirmed for each focus. The primary study endpoint is pathologic complete response (pCR).
Patients will be randomly allocated to one of the following treatment arms:

- Arm A (control arm): 12 consecutive cycles of weekly paclitaxel plus carboplatin (PCb) combined with 4 triweekly cycles of Pembrolizumab, followed by 4 consecutive cycles of triweekly anthracycline (doxorubicin or epirubicin)-cyclophosphamide (AC or EC) chemotherapy combined with 4 triweekly cycles of Pembrolizumab. This combination treatment will be further referred to as “standard treatment”.
- Arm B (experimental arm): standard treatment in combination with up to a maximum of 8 consecutive triweekly cycles of 5-day FLA.

Enrolled patients will be randomized in a 1:1 ratio and stratified according to a) disease stage: stage II (T1N1, T2N0, T2N1, T3N0) vs. stage III (T3N1; any T4; any N2); b) patient body mass index (BMI ≥25 kg/m2 vs <25 kg/m2).
After completion of the experimental preoperative protocol, patients will undergo surgery between 14 and 28 days after the last chemotherapy administration.
After surgery, patients will receive 9 additional triweekly pembrolizumab administration at the same dosage, and regardless of the pathologic tumor response (pCR yes vs. no). After surgery, patients may receive local radiotherapy, depending on the pathological stage and according to local and international guidelines.

Figure 1: Study design.



5 OBJECTIVES AND ENDPOINTS

5.1 Objectives

Primary objective

To investigate if the experimental treatment, consisting of the administration of preoperative chemo-immunotherapy (PCb plus Pembrolizumab, followed by AC or EC plus Pembrolizumab) in combination with cyclic triweekly FLA, is able to increase the rate of pCR when compared to standard preoperative chemo-immunotherapy (PCb plus Pembrolizumab, followed by AC or EC plus Pembrolizumab).

Secondary objectives

- To assess the tolerability of the experimental treatment when compared to the control treatment, and in particular to evaluate the incidence of severe (grade 3/4) adverse events (AEs)
- To evaluate the safety of the experimental treatment, and in particular to evaluate treatment-related AEs
- To study patient compliance, as defined as the ability to adhere to the prescribed FLA regimen in combination with chemoimmunotherapy pharmacological treatment based on the analysis of daily food diaries filled during each of 5 days while on FLA
- To estimate clinical tumor responses, as assessed through clinical examination, breast US and, when available, breast MRI, according to RECIST 1.1 criteria after the first part of the neoadjuvant treatment (PCb plus Pembrolizumab

plus/minus FLA) and before surgery, in the experimental treatment arm when compared to the control arm
- To estimate patient disease-free survival (DFS) in the experimental treatment arm when compared to the control arm
- To estimate patient event-free survival (EFS) in the experimental treatment arm when compared to the control arm
- To evaluate distant metastasis-free survival (DMFS) in the experimental treatment arm when compared to the control arm
- To evaluate Overall Survival (OS) in the experimental treatment arm when compared to the control arm

Exploratory objectives

- To study the short-term (intra-cycle) and long-term (across subsequent cycles) effects of the experimental treatment on systemic metabolism by evaluating several blood metabolites, including plasma glucose, insulin, insulin-like growth factor 1 (IGF-1) levels, serum amino acids and whole blood and plasma lipid profile.
- To study the short-term (intra-cycle) and long-term (across subsequent cycles) effects of the experimental treatment on blood immune populations.
- To correlate experimental treatment-induced modifications of systemic metabolism and immunity with treatment activity, in terms of pCR, DFS, EFS, DMFS and OS.
- To correlate tumor gene expression profiles with the efficacy of the experimental treatment, defined on the basis of rates of pCR.
- To correlate the tumor mutational status with the efficacy of the experimental treatment, defined as the rate of pCR.
- To evaluate the differential activity of FLA in subgroups with different pathologic residual cancer burden (RCB).
- To explore the use of point-of-care ultrasound in the assessment of patients sarcopenia during anticancer treatments.
- To evaluate patient-reported tolerability of the experimental treatment, and in particular treatment-related AEs (PROs), and compare it with physician-reported tolerability.

6 ENDPOINTS

6.1 Primary endpoints

- Pathologic complete response (pCR), as defined as the absence of residual tumor cells in both breast tissue and axillary lymph nodes (ypT0/ypTis ypN0).

6.2 Secondary endpoints

Efficacy endpoints

- Disease free survival (DFS), as defined as the time from surgery to tumor recurrence, either local or distant, or death
- Event-free survival (EFS), as defined as the time from the date of randomization to the first documentation of progressive disease or death
- Distant metastasis free survival (DMFS), as defined as the time from surgery to the occurrence of distant metastases or death
- Overall Survival (OS), as defined as the time from randomization to the date of death. Patients alive at the time of data cut-off and analysis will be censored at their last contact date

Compliance endpoints

- Dose-intensity, that is the dose of effective drug administrated per unit of time (e.g., mg/m2/week)
- Percentage of patients with drug dose and/or time modifications
- Percentage of patients with experimental dietary regimen modifications
- Percentage of premature withdrawals

Safety endpoints

- Incidence, nature, severity and seriousness of AEs, according of NCI-CTCAE, version 5.0
- Maximum toxicity grade experienced by each patient for each specific toxicity
- Percentage of patients experiencing grade 3-4 toxicity for each specific toxicity
- Patients with at least a SAE

Exploratory endpoints

- Metabolic biomarkers (e.g., plasma glucose, insulin, insulin-like growth factor 1 (IGF-1) levels, serum amino acids and whole blood and plasma lipid profile, fecal microbiota) assessed at baseline and at each chemotherapy cycle
- Role of key DNA repair, metabolic, autophagy and immunologic pathways in the efficacy of the experimental treatments, defined as the rate of pCR.

7 STUDY POPULATION

7.1 Patient population

This study will enroll patients with treatment-naïve, stage II-III (tumor stage T1c AND nodal stage N1-2, or tumor stage T2-4 AND nodal stage N0-2), invasive Triple Negative invasive Breast Cancer (HER2 negative, ER <1%, PgR <1%) who are candidate to receive neoadjuvant carboplatin-paclitaxel-anthracycline-pembrolizumab chemoimmunotherapy. Bilateral and/or multifocal primary tumor is allowed, as well as inflammatory breast cancer, and the tumor with the most advanced T stage should be used to assess the eligibility. If multi-focal/multi-centric disease, TNBC needs to be confirmed for each focus.

The investigators involved in the study must ensure that only patients who meet all the following inclusion and none of the exclusion criteria will be offered the experimental treatment.
All young women with childbearing potential will be proposed to undergo fertility preservation methods before study treatment initiation and to accept the use of adequate contraceptive measures up to six months after the last treatment administration. In case of pregnancy desire, they will also be offered the use of GnRH analog in combination with the experimental study treatment to reduce the risk of premature ovarian failure and to reduce damage on fertility[46].
Patients enrolled in this study are not allowed to participate in additional parallel investigational drug or device studies.

7.2 Inclusion criteria

Patients eligible for inclusion in this study must meet **all** of the following criteria:

1. Female sex
2. Age ≥ 18 and ≤ 75 years.
3. Evidence of a personally signed and dated informed consent document (ICD) indicating that the patient has been informed of all pertinent aspects of the study before enrollment
4. Willingness and ability to comply with the prescribed FLA regimen, the scheduled visits, treatment plans, laboratory tests and other procedures.
5. Histologically confirmed diagnosis of invasive TNBC candidate to neoadjuvant chemo-immunotherapy and subsequent curative surgery. On the basis of International Guidelines, TNBC is defined by absent or minimal (<1%) expression of oestrogen and progesterone receptors at IHC, and absence of HER2 protein over-expression and *HER2* gene amplification, as defined as an IHC score of 0, 1+, or an IHC score of 2+ with *in situ* hybridization (ISH) analysis excluding *HER2* gene amplification. The expression of hormone receptors (ER and PgR) and HER2 will be evaluated through immunohistochemistry (IHC), according to International Guidelines[47,48]
6. Availability of a formalin-fixed, paraffin-embedded (FFPE) block containing tumor tissue, or at least 7 unstained tumor slides.
7. Patients with tumor stage T1c AND nodal stage N1-2, or tumor stage T2-4 AND nodal stage N0-2 according to TNM.
8. Presence of an Eastern Cooperative Oncology Group (ECOG) performance status 0 or 1.
9. Presence of adequate bone marrow and organ function as defined by the following laboratory values:
   a. ANC ≥ 1.5 x 10^3/l
   b. platelets ≥ 100 x 10^3/l
   c. hemoglobin ≥ 9.0 g/dl
   d. calcium (corrected for serum albumin) within normal limits or ≤ grade 1 according to NCI-CTCAE version 5.0 if not clinically significant
   e. potassium within the normal limits, or corrected with supplements
   f. creatinine < 1.5 ULN

g. blood uric acid < 10 mg/dl
h. ALT and AST ≤ 2 x ULN
i. total bilirubin < 1.5 ULN except for patients with Gilbert syndrome who may only be included if the total bilirubin is < 3.0 x ULN or direct bilirubin < 1.5 x ULN
j. Fasting glucose ≤ 250 mg/dl.

10. Female patients of childbearing potential must agree to sexual abstinence or to use two highly effective methods of contraception throughout the study and for at least six months after the end of the FLA. Abstinence is only acceptable if it is in line with the preferred and usual lifestyle of the patient. Examples of contraceptive methods with a failure rate of < 1% per year include tubal ligation, male sterilization, hormonal implants, established, proper use of combined oral or injected hormonal contraceptives, and certain intrauterine devices. Alternatively, two methods (e.g., two barrier methods such as a condom and a cervical cap) may be combined to achieve a failure rate of < 1% per year. Barrier methods must always be supplemented with the use of a spermicide. A patient is of childbearing potential if, in the opinion of the Investigator, she is biologically capable of having children and is sexually active.
11. Female patients are not of childbearing potential if they meet at least one of the following criteria:
    a. Have undergone a documented hysterectomy and/or bilateral oophorectomy
    b. Have medically confirmed ovarian failure
    c. Achieved post-menopausal status, defined as: ≥ 12 months of non-therapy-induced amenorrhea or surgically sterile (absence of ovaries); in women <45 years of age FSH level in the postmenopausal range may be used to confirm a post-menopausal state in women not using hormonal contraception or hormonal replacement therapy.

7.3 Exclusion criteria

Patients eligible for this study must not meet **any** of the following criteria:

1. Prior systemic treatment for breast cancer or other malignancies within 5 years of treatment enrollment, except for adequately treated basal cell or squamous skin cancer or in situ cervical cancer. Other malignancies diagnosed more than 5 years before the diagnosis of breast cancer must have been radically treated without evidence of relapse at the moment of patient enrollment in the trial.
2. Prior treatment with anthracyclines
3. Prior therapy with an anti-PD-1, anti-PD-L1, or anti-PD-L2 agent or with an agent directed to another co-inhibitory T-cell receptor (e.g., CTLA-4, OX-40, CD137)
4. Body mass index (BMI) < 19 kg/m^2.
5. History of alcohol abuse.
6. Non-intentional weight loss ≥ 5% in the previous 3 months, unless the patient has a BMI > 22 kg/m^2 and weight loss has been lower than 10% at the time of

enrollment in the study; or non-intentional weight loss of ≥ 10% in the previous 3 months, unless the patient has a BMI > 25 kg/m^2 and weight loss has been lower than 15% at the time of the enrollment in the study. In both cases, weight must have been stable for at least one month before study enrollment.

7. Active pregnancy or breast feeding.
8. Known active B or C hepatitis or human immunodeficiency virus (HIV) infection, or occasional finding of active hepatitis B/C infection during screening tests before chemotherapy initiation, as defined as positive polymerase chain reaction (PCR) testing for HBV-DNA and HCV-RNA and qualitative PCR for HIV-RNA, or requiring active treatment at study enrollment.
9. Serious infections in the previous 4 weeks before the FLA initiation, including, but not limited to, potential hospitalizations for complications of infections, bacteriemia or serious pneumonitis.
10. Active autoimmune diseases requiring systemic treatments (e.g., systemic steroids or immune suppressants). Replacement therapy (e.g., thyroxine, insulin, or physiologic corticosteroid replacement therapy for adrenal or pituitary insufficiency) is not considered a form of systemic treatment.
11. Active chronic therapy with systemic steroids at a dose ≥ 10 mg per day of prednisone or equivalent at study enrollment.
12. Diagnosis of type 1 or 2 diabetes mellitus requiring pharmacologic therapy (including, but not limited to, insulin or insulin secretagogues), with the exception of metformin. A diagnosis of type 2 diabetes mellitus not requiring pharmacological treatments, or only requiring treatment with metformin, based on the judgment of a diabetologist, is compatible with patient enrollment in the trial.
13. Anamnesis of clinically significant heart disease including:
    a. angina pectoris, coronary bypass, symptomatic pericarditis, myocardial infarction in the previous 12 months from the beginning of experimental therapy;
    b. congestive heart failure (NYHA III-IV).
14. Anamnesis of clinically meaningful cardiac arrhythmias, such as ventricular tachycardia, chronic atrial fibrillation, complete bundle branch block, high grade atrio-ventricular block like bi-fascicular block, type II Mobitz and third grade atrio-ventricular block, nodal arrhythmias, supra-ventricular arrhythmia.
15. Left ventricular ejection fraction lower than 50% at the cardiac scan with radionuclides or at echocardiography.
16. Previous episodes of symptomatic hypotension leading to loss of consciousness.
17. History of eating disorders (anorexia, bulimia).
18. Baseline plasma fasting glucose ≤ 60 mg/dL.
19. Medical or psychiatric comorbidities rendering the patient not candidate to the clinical trial, according to the investigator's judgement.
20. Other cardiac, liver, lung or renal comorbidities, not specified in the previous inclusion or exclusion criteria, but potentially exposing the patient to a high risk of lactic acidosis.
21. Known history of active TB (Bacillus Tuberculosis).

8 EXPERIMENTAL INTERVENTION

8.1 Study intervention

All patients enrolled in this study will receive preoperative chemo-immunotherapy, consisting in 12 consecutive cycles of weekly paclitaxel (at a dosage of 80 mg/m^2) plus carboplatin (area under the curve -AUC- of 1.5 mg/mL per min), plus triweekly Pembrolizumab (200 mg), followed by 4 triweekly cycles of doxorubicin (at a dosage of 60 mg/m^2) or epirubicin (at a dosage of 90 mg/m^2) plus cyclophosphamide (at a dosage of 600 mg/m^2 plus Pembrolizumab (200 mg). At enrollment, patients will be randomized in a 1:1 ratio to receive (arm B) or not (arm A) additional treatment consisting of triweekly 5-day FLA, up to a maximum of 8 consecutive cycles. At randomization, patients will be stratified according to tumor stage (II vs. III) and patient BMI (≥25 kg/m2 vs <25 kg/m2).

8.2 Chemo-immunotherapy

Patients will receive a sequential carboplatin-taxane-pembrolizumab and anthracycline-cyclophosphamide-pembrolizumab neoadjuvant chemo-immunotherapy program, consisting of two parts:

1) Paclitaxel 80 mg/m^2 plus Carboplatin AUC 1.5, weekly for 12 cycles (PCb) plus Pembrolizumab 200 mg flat dose every three weeks (part 1: approximately three months of therapy).
2) Doxorubicin 60 mg/m^2 or Epirubicin 90 mg/m^2 plus Cyclophosphamide 600 mg/m^2 (AC or EC) plus Pembrolizumab 200 mg flat dose on day 1 every 21 days for 4 cycles (part 2: approximately another three months of therapy).

All chemotherapy drugs will be administered i.v.. Patients may receive oral netupitant plus palonosetron (Akynzeo) plus 8 mg of i.v. dexamethasone the day of anthracycline-containing chemotherapy administration to prevent the occurrence of nausea and vomiting. Prophylaxis for potential paclitaxel-induced allergic reactions may include an oral antihistamine on the night before chemotherapy (e.g., cetirizine 10 mg), as well as an oral antihistamine plus 8 mg of i.v. dexamethasone on the day of treatment administration. However, premedication schemes used at each participating center can be chosen at the discretion of the treating physician of that specific site, with the exception of dexamethasone, which should be administered at 8 mg dosage only on day 1 of each treatment administration, and eventually escalated only in the case of adverse events (e.g., severe nausea, severe vomiting, allergic reactions).

NOTE: To avoid interference with the metabolic effects induced by the FLA (including a reduction of blood glucose and insulin levels), carboplatin must be dissolved in saline (0.9% NaCl solution), and not in 5% glucose-containing solution.

8.2.1 Pembrolizumab prescribing

Pembrolizumab has been approved by international regulatory agencies (i.e. FDA, EMA) as part of neoadjuvant treatment for early-stage TNBC (tumor stage T1c, nodal stage N1-2, or tumor stage T2-4, nodal stage N0-2). In Italy this treatment is actually prescribed through a compassionate use program. Centers will administer Pembrolizumab within the study through this compassionate program, after obtaining the specific additional informed consent provided by the pharmaceutical company. In case of formal registration and reimbursement from Italian regulatory agency (AIFA) during the course of the study, Pembrolizumab will be administered through National Health System.

8.3 FLA scheme (arm B)

Patients randomized to Arm B (experimental arm) will receive up to a maximum of eight cycles of FLA in combination with chemo-immunotherapy.
Each FLA cycle will consist of 5 consecutive days of a specific FLA scheme, which will be repeated with a three-week interval. The FLA will consist of a plant-based, low-calorie (about 600 Kcal on day 1; about 300 Kcal on day 2 to 5), low-protein, low-carbohydrate diet. This is the same FLA scheme whose safety, feasibility, metabolic and immunological effects have been evaluated in the recently conducted NCT03340935 trial at our Institution[35,40,41], and which has been combined with preoperative chemotherapy in the recently interrupted BREAKFAST trial (NCT04248998).
All patients will be prescribed the same FLA regimen. No modifications nor personalization of the prescribed FLA regimen are allowed.
The first FLA cycle will start two days prior to the day of first chemo-immunotherapy cycle administration and will continue for two more days after chemotherapy. In the absence of significant contraindications or severe adverse events, subsequent FLA cycles will recur with three-week intervals and will maintain the same timing with respect to chemo-immunotherapy administration. In case that the FLA is delayed for any reason, chemo-immunotherapy will be administered according to the schedules program; in case of FLA re-start, the FLA must be necessarily administered concomitantly with cycles 1st, 4th, 7th or 10th of weekly PCb, and concomitantly with triweekly AC/EC, and it must be started two days before chemotherapy administration. In particular, the FLA cannot be combined with cycles 2nd, 3rd, 5th, 6th, 8th, 9th, 11th or 12th of PCb, and it cannot be initiated in days other than -2 of each triweekly AC/EC cycle.
The full FLA scheme, with the approximated amount of calories contained in each food/beverage, is reported in ***Table* 1**. Mean caloric intake from individual FLA components were obtained from the Food Composition Database for Epidemiological Studies in Italy ("Food Composition Database for Epidemiological Studies in Italy" *by Gnagnarella P, Salvini S, Parpinel M. Version 1.2015 Website* http://www.bda-ieo.it/).

Table 1

The FLA diet scheme		
Day	**Meal**	**Food/beverage**
Day 1	Breakfast	One tea or non-caloric tisanes at the choice of the patient (without sugar or other sweeteners). In addition, possibility to drink one coffee (without sugar or other sweeteners).
	Lunch	A dish of mixed vegetables (up to a total of 300 grams), including spinach, cabbage, savoy cabbage, cauliflower, broccoli, salad or zucchini, consumed as raw, boiled or steamed (72 KCal), together with a tablespoon (12 grams) of extra virgin olive oil (108 KCal). 100 grams of whole wheat bread (224 KCal).
	Dinner	100 grams of salad (lettuce, valerian salad, rocket salad, endive salad, chard, red and green radicchio, chicories, friarielli; 30 KCal) with a teaspoon (6 grams) of extra virgin olive oil (54 KCal); 250 grams of orange, apple or pear (100 KCal).
Days 2-3-4	Breakfast	One tea or non-caloric tisanes at the choice of the patient, without sugar or other sweeteners. In addition, possibility to drink one coffee (without sugar or other sweeteners).
	Lunch	Around 200 grams of salad (lettuce, valerian salad, rocket salad, endive salad, chard, red and green radicchio, chicories, friarielli; 40 KCal) with a teaspoon of extra virgin olive oil (54 KCal). As an alternative, an equivalent amount of the following vegetables consumed as raw, boiled or steamed: spinach, cabbage, savoy cabbage, cauliflower, broccoli, zucchini, onions, artichokes.
	Dinner	25 grams of shelled walnuts or 30 grams of almonds (180 KCal).
Day 5	Breakfast	One tea or non-caloric tisanes at the choice of the patient (without sugar or other sweeteners). In addition, possibility to drink one coffee (without sugar or other sweeteners).
	Lunch	A dish of mixed vegetables (up to a total of 300 grams), including spinach, cabbage, savoy cabbage, cauliflower, broccoli, salad or zucchini, consumed as raw, boiled or steamed (75 KCal), together with a teaspoon (6 grams) of extra virgin olive oil (54 KCal). 200 grams of orange, apple or pear (100 KCal).

	Dinner	100 grams of salad (lettuce, valerian salad, rocket salad, endive salad, chard, red and green radicchio, chicories, friarielli; 20 KCal) with a teaspoon of extra virgin olive oil (54 KCal).

Any food/beverage, including spices, not explicitly contained in this list must be avoided.
Water intake will not be limited. Consuming around 2 liters of non-caloric beverages in the autumn-winter-spring seasons, and 3 liters during summer will be strongly recommended. A maximum of 2 tea cups, or 2 coffee cups, or unlimited herbal teas per day, as long as they are calorie-free, will be allowed.
A maximum of half lemon is allowed during each of the five FLA days.
The FLA will be repeated every 3 weeks, up to a maximum of 8 consecutive cycles, unless side effects requiring its temporary or permanent discontinuation do occur.

8.4 Dietary recommendation for patients randomized to arm A and maintenance diet during the refeeding periods for patients randomized to arm B

All patients enrolled in this study will be recommended to follow a diet based on established dietary guidelines for cancer prevention and cancer survivors regardless of the randomization arm (World Cancer Research Fund (WCRF) 2018, American Cancer Society 2012, European Code Against Cancer 2014).
Dietary recommendations will include:

- eating plenty of whole grains, legumes, non-starchy vegetables and fruits;
- choosing whole-grain breads, pasta and cereals (such as barley and oats) instead of the same products made from refined grains, and brown rice instead of white rice;
- limiting high-calorie foods (high in sugar or fat) and avoiding sugary drinks;
- avoiding processed meat and limiting red meat and foods high in salt;
- choosing fish, poultry, or beans instead of red meat (beef, pork and lamb);
- limiting alcohol intake.

Arm B patients are invited to follow these recommendations as a maintenance diet between FLA cycles, while Arm A patients will be recommended to adhere to these guidelines for the whole treatment period. In the experimental arm, this maintenance diet is aimed at avoiding rebound increase in blood glucose and growth factor (e.g., insulin, IGF-1) concentration after the FLA completion, while at the same time stimulating regain of the weight lost during the FLA. Weight regain after the FLA is important to prevent progressive weight loss and loss of lean tissue. Preventing loss of lean mass is especially important to ensure the maintenance of patient health status, and to prevent the progressive degradation of muscle proteins, which may result in increased blood amino acid concentration and in tumor overfeeding. Therefore, no quantitative restrictions in food intake (i.e., total amounts and calorie content) will be imposed during the maintenance diet. Moreover, since 8 consecutive FLA could induce significant weight loss, patients will be monitored by an expert team of oncologists and

nutritionists (Dr. C. Vernieri, dr. G. Fucà, dr. F. Ligorio, dr. L. Zanenga) throughout the study protocol in order to modify dietary recommendations according to weight kinetics, body composition and treatment-related adverse events (i.e., diarrhea, nausea, vomiting).

8.5 Trial Blinding/Masking and treatment allocation

This is an open-label trial. Patients will be randomized to arm A or arm B in a 1:1 ratio.

8.6 Stratification

At randomization, patients will be stratified according to the two following factors: a) clinical disease stage according to TNM classification: stage II (T1N1, T2N0, T2N1, T3N0) vs. stage III (T3N1; any T4; any N2); b) patient BMI (≥25 kg/m2 vs <25 kg/m2).

8.7 Concomitant Medications (Allowed & Prohibited)

Medications specifically prohibited in the exclusion criteria are not allowed during the trial. If there is a clinical indication for any of the prohibited medications, discontinuation from trial treatment may be required.

Acceptable Concomitant Medications

All treatments deemed necessary for a patient's welfare may be administered at the discretion of the investigator in keeping with the community standards of medical care. Granulocyte colony stimulating factors may be administered if deemed necessary by treating oncologists and according to National/International Guidelines. All concomitant medications will be recorded on the case report form (CRF) including all prescription, over-the-counter (OTC), and i.v. medications and fluids. Documentation of drug dosage, frequency, route, and date may also be included on the CRF in case of changes occurring during the trial period.

Prohibited Concomitant Medications

Patients are prohibited from receiving the following therapies during the Screening and Treatment Phase of this trial:

- Antineoplastic systemic treatments, except for the chemoimmunotherapy regimen explicitly specified in the study protocol.
- Investigational treatments, including chemical, physical and dietary interventions, other than study treatment.
- Chronic immunosuppressive drugs.
- Hyperosmotic Laxatives (e.g., lactulose, sorbitol, polyethylene glycol), unless absolutely required for the treatment of acute constipation occurring during the study.
- Herbal preparations/medications. These herbal medications include, but are not limited to: St. John's wort, Kava, ephedra (ma huang), gingko biloba, Aloe Vera and its derivatives; artemisinin and its derivatives; dehydroepiandrosterone

(DHEA), yohimbe, ginseng and any type of dietary supplements whose chemical composition is unknown. Patients should stop using these herbal medications at least 10 days prior to treatment initiation.
- Vitamin or food supplements, with the exception of Vitamin D. If the patient chronically takes dietary, salt- or food-containing supplements at the moment of study enrollment, she should discontinue them at least 10 days prior to treatment initiation. Continuation of chronic supplement intake during the FLA is only permitted if the supplements do not contain calories or high-dose vitamins and are limited to salts or low dosages of vitamins (below 1/10 of the Daily Recommended Dose). Any supplement with unknown chemical composition must be discontinued.
- Therapeutic doses of warfarin sodium (Coumadin®). Therapeutic anticoagulation may be accomplished using low-molecular weight heparin.
- Inhibitors of pancreatic alpha-amylase and lipase (e.g., Orlistat).
- Anorectic drugs (e.g., dexamphetamine, phenmetrazine and benzphetamine).

Rescue Medications & Supportive Care

Patients should receive appropriate supportive care measures according to the investigator's judgment as per clinical practice.

9 VISIT SCHEDULE AND ASSESSMENTS

The Study Flow Chart (Table 2) lists the study procedures and indicates with an "X" the time points for visits and other evaluations to be performed. All screening assessments have to be completed no more than 28 days before initiation of the experimental treatment, with the exception of radiological disease evaluation (within 28 days). Blood, urine, saliva and stool samples will be collected for metabolic, immunological, and microbiota analyses at scheduled time points. These procedures may also be performed at unscheduled time points if deemed clinically necessary by the investigator.

Table 2: Study Flow Chart

Tab 2a

Assessment	Screening [1]	Neoadjuvant Therapy *(Tab 2b, 2c)*	Before Surgery (2- 3 weeks from the last dose of CT)	SURGERY (within 4 weeks from the last dose of CT)	Adjuvant Pembrolizumab *(Tab 2d)*	EOT (within 4 weeks after the last Pembrolizumab administration)	FOLLOW UP [20]
Signed Informed Consent[2]	X						
Demographic data[3]	X						
Breast Tumor Core Biopsy[4]	X						X[22]
Complete Medical History [5]	X						
Physical Examination [6]	X		X			X	X
Weight, BMI and assessment of food intake[7]	X		X			X	
Vital signs[8]	X		X			X	
ECOG Performance status	X		X			X	

Hematology [9]	X		X[19]			X	X[21]
Blood chemistry [10]	X		X[19]			X	X[21]
T3, FT4 and TSH	X		X			X	X
Serology [11]	X						
Urinalysis [12]	X		X[19]			X	X[21]
Saliva and Stool samples[14]			X[19]			X	X[21]
PBMC[15] and other immunological analysis			X[19]			X	X[21]
Metabolite and growth factor assessment[15]			X[19]			X	X[21]
Breast imaging [16]	X		X				X[20] (annually)
Systemic radiological staging[17]	X		X				
Cardiac Examination (ECG and LVEF)	X		X				

Pregnancy Test[18]	X						
QoL Assessment	X		X			X	
Food diaries[22]	X[22]		*Throughout the study*[22]				
Surgery[23]					*X*		
Concomitant Medications		*Throughout the study*					
Adverse events		*Throughout the study*					

1. **Screening/Baseline Assessments**: must be completed within 28 days prior to study treatment start unless otherwise specified as shown in the schedule of events above. Study treatment start is considered day 1 of the first FLA cycle
2. **Informed Consent**: must be obtained prior to undergoing any study specific procedures. After enrollment, all patients will perform a visit (which can be a televisit) at the study Sponsor Institution (Fondazione IRCCS Istituto Nazionale dei Tumori), during which the patient will meet the multidisciplinary team responsible for the management of the FLA in all participating centers.
3. **Demographic**: date of birth.
4. For all the patients enrolled in the study, tumor specimen collected before the initiation of the experimental treatment must be submitted to the study Sponsor Institution to perform translational analyses. Preferably, a formalin-fixed, paraffin-embedded (FFPE) tumor tissue block should be provided. However, if it is not possible, the provision of at least seven (preferably 15) unstained slides with 4 μm-thick sections of the FFPE tumor tissue block is mandatory for study inclusion. An optional re-biopsy to collect fresh-frozen tumor material can be performed at the study Sponsor Institution, if technically feasible. Optionally, patients will be proposed to repeat a tumor biopsy at the study Sponsor Institution 14-21 days after the first FLA cycle completion, to collect FFPE and fresh-frozen tissue for early evaluation. A separate informed consent will be signed for optional biopsies.
5. **Medical History**: Medical history should include history of disease process other than oncology (active or resolved) and concomitant illnesses.

6. **Physical Exam:** will include an assessment for emergent toxicities or changes from prior visits. These procedures may be conducted by the Investigator or his/her designee.
7. **Weight, BMI and food intake:** weight will be measured in kilograms; BMI will be measured as weight (kg)/[height(m)]^2
8. **Vital signs:** will include blood pressure, body temperature and heart rate
9. **Hematology**: to include platelet count, hemoglobin, and WBC count with 5- part differential.
10. **Blood chemistry**: to include glucose, AST (SGOT), ALT (SGPT), serum creatinine, total bilirubin, alkaline phosphatase, LDH, chloride, uric acid, phosphorus, calcium, magnesium, potassium, sodium, BUN or urea.
11. **All patients will be tested for HBV and HCV.** HBV serology will include HBsAg, antibodies against HBsAg, total HBcAg antibody (anti-HBcAb). HBV DNA should be obtained prior to the treatment phase in the case of HBsAg and/or anti-HBc positivity. HCV serology will include HCV antibody (anti-HCV). HCV RNA should be obtained prior to the treatment phase if patient tests positive for anti-HCV.
12. **Urinalysis**: physical-chemical analyses, including ketonuria.
13. **Saliva and Stool samples:** to be centralized at the Study Sponsor Institution.
14. **PBMC analysis**: to include MDSCs, activated T cells, NK and DC cells. These analyses will be performed on samples centralized at the Study Sponsor Institution.
15. **Metabolite and growth factor assessment:** total proteins, albumin, triglycerides; total and HDL cholesterol, prealbumin, transferrin, sideremia, ferritin, cholinesterase, cortisol, insulin, ®-hydroxybutyrate, IGF-1; IGFBP-1, IGFBP-2, IGFBP-3; VEGF-A, FGF; whole blood, plasma and red blood cell membrane lipid profiling; serum amino acid quantification. These analyses will be performed on samples centralized at the Study Sponsor Institution.
16. **Local staging:** breast US is mandatory at each timepoint (screening, after PCb completion, before surgery); CE-MRI is mandatory at baseline and before surgery. Other radiological exams (mammography, contrast enhanced mammography (CESM), contrast enhanced MRI) can be performed at the discretion of the physician.
17. **Systemic radiological staging**: at screening a CE total body CT scan (preferably) or, alternatively, a 18FDG Positron Emission Tomography (PET)/CT will be performed. Images will be centralized at the study Sponsor Institution. Before surgery, optionally, patient can undergo a CE total body CT scan to evaluate body composition.
18. **In women of childbearing potential**: Serum is preferred at screening, but urine is allowed.
19. **These evaluations must be performed the day of surgery or no more than 24 hours before surgery**
20. **Follow up**: A visit will be performed every 6 months for the first 5 years, then a phone contact will be performed annually up to 10 years. Mammography will be performed annually as per local and international Guidelines.
21. **At disease recurrence** At disease recurrence, if a diagnostic biopsy of a metastatic lesion is indicated as per clinical practice, optional core biopsy of the tumor will be performed for the collection of fresh-frozen tumor material.

22. All patients will be given food diaries to annotate the type and amount of food and beverage consumed at specific timepoints, i.e., i) during 7 consecutive days before treatment initiation ii) during one single day once a week (with the exception of the week in which patients receive chemotherapy plus Pembroluzumab plus/minus FLA) during the neoadjuvant treatment phase iii) during one single day every three weeks during the adjuvant treatment phase. In addition, patients randomized to arm B will receive a specific 5-day food diary to annotate the type and amount of food and beverage consumed during each FLA cycle; patients randomized to arm A will receive at the same timepoints 5-day food diaries to annotate the type and amount of food and beverage consumed.
23. Surgical FFPE tumor specimens will be centralized to the study Sponsor Institution. Only for patients enrolled at the study Sponsor Institution, surgical fresh frozen tumor material will be collected as well.
24. After surgery, patients will receive up to a maximum of nine triweekly Pembrolizumab cycles, according to local and international guidelines.

Tab 2b

	Neoadjuvant weekly Paclitaxel plus Carboplatin (PCb) x 12 cycles in combination with triweekly Pembrolizumab									After PCb completion
Cycles	1[10], 4				7; 10			2; 3; 5; 6; 8; 9; 11; 12		
Day	-2	1	3	4	-2	1	3	1		
PCb (all arms)		X				X		X		
Pembrolizumab (all arms)		X				X				
FLA (Arm B) [1]	X				X					
Physical Examination [2]	X				X					
Weight, BMI	X				X					
Hematology [3]	X			X	X			X*		
Blood chemistry[4]	X			X	X					
Urinalysis[5]	X			X						
Saliva and Stool samples[6]	X			X						

PBMC[7] and other immunological analysis	X			X						
Metabolite and growth factor assessment[8]	X			X						
LVEF										X
Breast imaging [9]										X
QoL assessment										X
Food diaries[11]	*Throughout the study[11]*									

Tab 2c

	Neoadjuvant AC or EC x 4 cycles (q3 weeks)						
Cycles	1				2-4		
Day	-2	1	3	4	-2	1	3
AC (all arms)		X				X	
Pembrolizumab (all arms)		X				X	
FLA (arm B) [1]		X				X	
Physical Examination [2]	X				X		
Weight, BMI	X				X		
Hematology [3]	X			X	X		
Blood chemistry[4]	X			X	X		
T3, FT4 and TSH	X						

Urinalysis[5]	X			X			
Saliva and Stool samples[6]	X			X			
PBMC[7] and other immunological analysis	X			X			
Metabolite and growth factor assessment[8]	X			X			

1. **FLA Cycles** will be performed every 3 weeks, and specifically during Cycles 1, 4, 7, 10 of weekly PCb plus pembrolizumab chemo-immunotherapy and every cycle of AC/EC plus pembrolizumab chemo-immunotherapy. Each FLA cycle begins at Day -2 and lasts until day 3 of every chemotherapy cycle (day -2; day -1; day 1, corresponding to the day of chemoimmunotherapy administration; day 2; day 3). Patients randomized to arm B will receive a specific 5-day food diary to annotate the type and amount of food and beverage consumed during each FLA cycle; patients randomized to arm A will receive at the same timepoints 5-day food diaries to annotate the type and amount of food and beverage consumed.
2. **Physical Examination** will include an assessment for emergent toxicities or changes from prior visits, an evaluation of ECOG performance status, vital signs (blood pressure, body temperature and heart rate to be recorded in the sitting position after approximately 5 minutes of rest), weight, BMI, food intake. Weight will be measured in kilograms; BMI will be measured as weight (kg)/[height(m)]2
3. **Hematology**: to include platelet count, hemoglobin, and WBC count with 5- part differential (*allowed within 48 hours prior to the planned treatment administration)
4. **Blood chemistry:** to include glucose, AST (SGOT), ALT (SGPT), serum creatinine, total and conjugate bilirubin, alkaline phosphatase, LDH, chloride, uric acid, phosphorus, calcium, magnesium, potassium, sodium, BUN or urea.
5. **Urinalysis**: physical-chemical analyses, including ketonuria.
6. **Saliva and Stool samples:** to be centralized at the Study Sponsor Institution.
7. **PBMC analysis**: to include MDSCs, activated T cells, NK and DC cells. These analyses will be performed on samples centralized at the Study Sponsor Institution.

8. **Metabolite and growth factor assessment:** total proteins, albumin, triglycerides; total and HDL cholesterol, prealbumin, transferrin, sideremia, ferritin, cholinesterase, cortisol, insulin, ®-hydroxybutyrate, IGF-1; IGFBP-1, IGFBP-2, IGFBP-3; VEGF-A, FGF; whole blood, plasma and red blood cell membrane lipid profiling; serum amino acid quantification. These analyses will be performed on samples centralized at the Study Sponsor Institution.
9. Local staging: breast US is mandatory at each timepoint (screening, after PCb completion, before surgery). CE-MRI is mandatory at baseline and before surgery. Other radiological exams (mammography, contrast enhanced mammography (CESM), contrast enhanced MRI) may be performed at the discretion of the physician.
10. **An optional biopsy may be performed 14-21 days after the first PCb-pembrolizumab cycle**
11. All patients will be given food diaries to annotate the type and amount of food and beverage consumed at specific timepoints, i.e., i) during 7 consecutive days before treatment initiation ii) during one single day once a week (with the exception of the week in which patients receive chemotherapy plus Pembrolizumab plus/minus FLA) during the neoadjuvant treatment phase iii) during one single day every three weeks during the adjuvant treatment phase. In addition, patients randomized to arm B will receive a specific 5-day food diary to annotate the type and amount of food and beverage consumed during each FLA cycle; patients randomized to arm A will receive at the same timepoints 5-day food diaries to annotate the type and amount of food and beverage consumed.

Tab 2d

	Adjuvant Pembrolizumab x 9 cycles (q3 weeks)*								
Cycles	1	2	3	4	5	6	7	8	9
Weight, BMI	X			X			X		
Urinalysis[1]	X			X			X		

Saliva and Stool samples[2]	X			X			X		
PBMC[3] and other immunological analysis	X			X			X		
Metabolite and growth factor assessment[4]	X			X			X		
Food diaries[5]	*Throughout the study*[5]								

*During the adjuvant phase, local blood analysis (hematology, biochemistry, thyroid function) will be performed at discretion of the physician, as per clinical practice.

1. **Urinalysis**: physical-chemical analyses, including ketonuria.
2. **Saliva and Stool samples:** to be centralized at the Study Sponsor Institution.
3. **PBMC analysis**: to include MDSCs, activated T cells, NK and DC cells. These analyses will be performed on samples centralized at the Study Sponsor Institution.
4. **Metabolite and growth factor assessment:** total proteins, albumin, triglycerides; total and HDL cholesterol, prealbumin, transferrin, sideremia, ferritin, cholinesterase, cortisol, insulin, ®-hydroxybutyrate, IGF-1; IGFBP-1, IGFBP-2, IGFBP-3; VEGF-A, FGF; whole blood, plasma and red blood cell membrane lipid profiling; serum amino acid quantification. These analyses will be performed on samples centralized at the Study Sponsor Institution.
5. All patients will be given food diaries to annotate the type and amount of food and beverage consumed during one single day every three weeks during the adjuvant treatment phase.

9.1 Screening and baseline (DAY -28 – DAY 0)

The following procedures have to be performed at screening and/or baseline. Individual procedures are described in Section 10.

- Written informed consent
- Availability of Breast Tumor Core Biopsy (including the assessment of ER and PgR status by IHC, assessment of HER2 status by IHC and, in case, by ISH, assessment of tumor cell proliferation measurement through IHC of Ki-67, assessment tumor grade and TILs). For all the patients enrolled in the study, a tumor specimen collected before the initiation of the experimental treatment must be submitted to the study Sponsor Institution to perform translational analyses. Preferably, a formalin-fixed, paraffin-embedded (FFPE) tumor tissue block should be provided. However, if it is not possible, the provision of at least seven (preferably 15) unstained slides with 4 μm-thick sections of the FFPE tumor tissue block is mandatory for study inclusion, An optional re-biopsy of the primary tumor can be performed at the study Sponsor Institution, if technically feasible, to collect fresh-frozen tumor material before study treatment(s) initiation.
- Demographics and medical history
- Disease details and diagnosis
- Local staging: breast US is mandatory at each timepoint (screening, after PCb completion, before surgery). CE-MRI is mandatory at baseline and before surgery. Other radiological exams (mammography, contrast enhanced mammography (CESM), contrast enhanced MRI) may be performed at the discretion of the physician.
- Systemic radiological staging: a CE total body CT scan (preferably) or, alternatively, a 18FDG Positron Emission Tomography (PET)/CT will be performed. Images will be centralized at the study Sponsor Institution..
- Eligibility criteria (Inclusion/Exclusion Criteria, see Paragraphs 7.2, 7.3)
- Randomization procedure
- Clinical and physical examination, assessment of blood pressure (BP), vital signs, nutritional/body composition
- Cardiac examination: Electrocardiograph (ECG), Left ventricular ejection fraction (LVEF) by echocardiography or multi-gated scintigraphic scan (MUGA scan)
- Laboratory tests including hematology, blood chemistry, TSH, T3 and T4, and hepatitis serology
- Concomitant medications
- Serum or urine pregnancy test in women of child-bearing potential (a negative serum pregnancy test outside the 14-days window must be confirmed with another negative pregnancy test)
- Nutritional/body composition evaluation
- Quality of life (QoL) assessment

After enrollment and randomization all patients will perform a visit at the study Sponsor Institution (Fondazione IRCCS Istituto Nazionale dei Tumori), which can be also performed virtually (tele-visit), during which the patient will meet the multidisciplinary team, composed of physicians and a biologist nutritionist, who will be responsible for the management of the FLA in all participating

centers. During this visit, the patient will receive detailed information about FLA composition, and how to prevent/manage adverse events related to the experimental treatment.
In addition, each patient may undergo an optional core biopsy of the primary tumor at the study Sponsor Institution for the conduction of translational analyses in tumor samples in fresh or fresh-frozen tumor specimens (see Section 14).

9.2 On Treatment Procedures

Patients randomized to arm B will undergo 5-day FLA cycles every 3 weeks, i.e., concomitantly with cycles n.1, 4, 7 and 10 of weekly PCb chemotherapy, i.e., at those cycles corresponding to the administration of pembrolizumab, and concomitantly with each of four triweekly AC/EC plus Pembrolizumab cycles.
Before any systemic treatment administration, all patients will undergo:

- assessment of blood tests including hematology tests
- evaluation, recording and grading of treatment related-adverse events
- evaluation of concomitant medications

Before each FLA cycle, all patients will undergo:

- Physical Examination, including an assessment for arising toxicities or changes from prior visits, an evaluation of ECOG PS, vital signs, weight, BMI, food intake.
- Blood evaluations, including blood cell counts and chemistry laboratory tests in peripheral vein blood collected after at least 8-hour fasting
- Assessment and grading of treatment-related adverse events
- Evaluation of concomitant medications.

Additionally, before and after the FLA cycles associated with the first and the fourth weekly administration of PCb (i.e., first and second Pembrolizumab cycles), as well as before and after the first AC/EC plus Pembrolizumab cycle (days -2 and +3 of these cycles), the following evaluations will be performed:

- Quantification of blood metabolites and growth factors; immunological evaluations in peripheral blood after at least 8-hour fasting
- Urinalysis with urinary ketone body dosage
- Stool sampling
- Saliva sampling

After the completion of 12 PCb chemotherapy cycles (i.e., ~ after three months from treatment initiation), patients will undergo (**Table 2**):

- Breast and regional nodes assessment with breast US. Other radiological exams (mammography, contrast enhanced mammography (CESM), contrast enhanced MRI) may be performed at the discretion of the physician.
- Quality of life (QoL) assessment
- TSH, T3 and T4 measurement (it can be performed with the blood collection performed before the first AC/EC cycle)

9.3 Before Surgery

Before surgery, which will be scheduled between 2 to 4 weeks from the last dose of neoadjuvant chemotherapy**,** the following assessments should be performed:

- Physical Examination including an assessment for arising toxicities or changes from prior visits, an evaluation of ECOG PS, vital signs, weight, BMI, food intake.
- Breast US and CE-MRI are mandatory. Other radiological exams (mammography, contrast enhanced mammography (CESM), contrast enhanced MRI) may be performed at the discretion of the physician
- Not mandatory: Contrast-enhanced total body CT scan. When the exam is performed, CT scan images will be centralized at the study Sponsor Institution.
- Nutritional assessment
- QoL assessment
- LVEF evaluation

Additionally, the day of surgery, or no more than 24 hours before surgery, the following assessments will be performed:

- Blood tests including hematology and blood chemistry laboratory tests on peripheral blood after at least 8-hour fasting
- Blood metabolites, and growth factor assessment; immunological evaluations on peripheral blood after at least 8-hour fasting
- TSH, T3 and T4 measurement
- Urinalysis
- Stool sampling
- Saliva sampling

9.4 Surgery

After completion of the preoperative treatment, patients will undergo breast conserving surgery or mastectomy in line with current standards of care and according to the recommendations of the multidisciplinary team, regardless of the study arm.
Surgery must be performed within 28 days from the last administration of preoperative systemic treatment.

9.5 Adjuvant treatment

After surgery, patients will receive up to a maximum of nine triweekly Pembrolizumab cycles, according to local and international guidelines.
During this phase, all patients will undergo every three cycles (at cycle 1, 4,7):

- Quantification of blood metabolites and growth factors; immunological evaluations in peripheral blood after at least 8-hour fasting
- Urinalysis with urinary ketone body dosage
- Stool sampling
- Saliva sampling

In addition, patients will be given food diaries to annotate the type and amount of food and beverage consumed during one single day every three weeks.

Other clinical procedures (e.g., blood analyses) during this phase will be performed at the discretion of the physician as per clinical practice.

9.6 End of treatment

The end of the study for a single patient is defined as the date of definitive discontinuation of the experimental treatment. The end of the whole study is defined as the date of the last visit/phone contact, scheduled according to the study protocol.
The End of Treatment (EOT) visit must be performed 21-28 days after the administration of the last dose of post-surgical Pembrolizumab. EOT visit requirements are outlined below:

- Physical Examination, including an assessment for emergent toxicities or changes from prior visits, the evaluation of ECOG PS, vital signs, weight, BMI, food intake
- Blood tests including hematology and blood chemistry laboratory tests on peripheral blood after at least 8-hour fasting
- Blood metabolites, and growth factor assessment; immunological evaluations on peripheral blood after at least 8-hour fasting
- TSH, T3 and T4 measurement
- Urinalysis
- Stool sampling
- Saliva sampling
- Treatment-related adverse events
- Concomitant medications
- QoL assessment

9.7 Additional therapy and follow-up

Patients may receive local radiotherapy, depending on the pathological tumor response (pCR yes vs. no) and lymph node status, and according to local and international guidelines. All patients will undergo regular follow-up as per local and international guidelines.
Only per protocol patients will enter the follow up period, which will initiate the day of the EOT visit. During the first 5 years of the follow-up period, patients will undergo a visit every 3-6 months, according to the physician judgment; then a phone contact will be performed annually up to 10 years. Mammography will be performed annually as per local and international Guidelines.

9.8 Tumor recurrence

In patients undergoing disease recurrence after surgery, if a diagnostic biopsy of a metastatic lesion is indicated as per clinical practice (e.g., TNBC biology confirmation), during the same procedure an additional, optional core biopsy of the tumor will be performed for the collection of fresh-frozen tumor material that will be used for subsequent biological evaluations. In addition, blood, urine, saliva and fecal samples will be collected at the detection of disease relapse for blood cell counts, standard biochemistry, immunologic (PBMC) and metabolic evaluations.

10 STUDY PROCEDURES

The Study Flow Chart (**Table 2**) summarizes the study procedures. Individual procedures are described in detail below.

10.1 Screening procedures

Screening procedures begin once a patient potentially candidate to be enrolled in the study has provided written informed consent. The following assessments have to be performed within 28 days prior to experimental treatment initiation.

Informed consent

The Investigator must obtain the documented consent from each patient (or his/her legal representative) considered for the trial and eligible for screening prior to performing any trial-related procedures. Informed consent should be asked once the report of the diagnostic core biopsy (including HER2 status, ER and PgR status and tumor proliferation measurement and/or tumor grade) is available at the local pathology site, and once the diagnosis of TNBC is confirmed. The Investigator will explain to all candidate participants (or their legal representative) the aims and characteristics of the study, as well as the potential benefits and risks associated with participation in the study. The original consent form, signed and dated by the patient (or by the patient's legal representative) and by the person who conducted the informed consent discussion, will be retained by the Investigator, and a copy will be given to the patient before enrollment in the trial. The informed consent form, any subsequent revised version and any other written information provided to the patients have to be approved by the IRB/EC's and must adhere to applicable laws and regulations.

Inclusion/Exclusion Criteria

All inclusion and exclusion criteria must be reviewed by the Investigator to ensure that the subject qualifies for the trial.

Demographics and Medical History

A detailed medical history has to be obtained by the Investigator. Medical history should include all active conditions, as well as any previously diagnosed condition that is considered to be clinically significant by the Investigator. Details regarding the subject's tumor must be recorded separately and not listed as medical history**.**

Prior and Concomitant Medications

The Investigator will review the use of prior medications, including any protocol-specified washout requirement. Any medication taken by the participant within 28 days before the beginning of the experimental intervention should be recorded.

Randomization procedure/Assignment of Randomization Number

All eligible subjects will be allocated to one of the two experimental arms through a 1:1 random assignment, and they will be assigned a randomization number. At randomization, patients will be stratified according to a) disease stage based on TNM staging system (clinical stage II- versus clinical stage III) and b) patient BMI (i.e., ≥25 kg/m2 vs <25 kg/m2) to guarantee a balanced distribution of patients in the two experimental groups according to these two variables. Once a randomization number is assigned to a subject, it cannot be re-assigned to another subject.

After enrollment, patients will perform a visit at the study Sponsor Institution, which can be performed virtually (tele-visit). During this visit, each patient will meet the multidisciplinary team responsible for the management of the FLA in all participating centers.

10.2 Clinical procedures/assessments

Disease evaluation

The investigator, or a qualified designee, must obtain prior and current details regarding each subject's tumor. Disease evaluation assessments have to be performed at screening/baseline and at the timepoints outlined in **Table 2**, and will include the following:

- Clinical (by palpation) and radiological breast imaging. Breast US is mandatory at screening, after PCb completion, and before surgery. CE-MRI is mandatory at baseline and before surgery. Other radiological exams (mammography, contrast enhanced mammography (CESM), contrast enhanced MRI) can be performed at the discretion of the physician.
- Total Body Computerized Tomography (CT) or, alternatively, 18FDG-PET/CT scan to rule out the presence of distant metastases.
- HER2 status (based on local testing by IHC and, if necessary, FISH, CISH, or other evaluations) to exclude HER2 protein overexpression and *HER2* gene amplification status based on International Guidelines[50,51]

- Hormone receptor status, as evaluated as the percentage of tumor cells staining positive for ER and PgR by IHC analysis
- Tumor grade and/or Ki67 value or other tumor proliferation measures

Optionally, a total body CT scan may be repeated before surgery.

Multidisciplinary visit

After enrollment, all patients will perform a visit at the study Sponsor Institution (Fondazione IRCCS Istituto Nazionale dei Tumori), which can be performed virtually (tele-visit), during which the patient will meet the multidisciplinary team responsible for the management of the FLA in all participating centers. During this visit, each patient will receive detailed information and advice for a proper management of the FLA in combination with standard chemoimmunotherapy, as well as for a timely, daily reporting of FLA tolerability and compliance to the multidisciplinary INT team during each day on the experimental dietary intervention.

Physical examination and Vital signs

The investigator, or a clinical designee, will perform a complete (full) physical examination during the screening/baseline visit, and as specified in the Study Flow Chart. Clinically significant abnormal findings emerging during the screening visit should be recorded as medical history, whereas new clinically significant abnormal findings detected after the first administration of study treatment should be recorded as AEs.
Vital signs will be measured by the Investigator, or by a qualified designee, at screening visit, and as specified in the Study Flow Chart. Vital signs should include temperature, pulse and blood pressure. Height will be measured at screening only.

12-Lead Electrocardiogram and LVEF evaluation

A standard 12-lead ECG will be prescribed to all patients during the screening phase (if not performed within one month before enrollment) to exclude meaningful alterations of cardiac rhythm. Due to the potential cardiotoxic effect of the administered drugs (anthracycline), an evaluation of left ventricular ejection fraction (LVEF) by echocardiography or multi-gated scintigraphic scan (MUGA scan) will be also performed as specified in the Study Flow Chart (**Table 2**).
Additional cardiological evaluations may be performed if clinically indicated (e.g., for AEs during the treatment administration).

ECOG Performance Status

Performance status will be assessed by the Investigator according to the Eastern Cooperative Oncology Group (ECOG, WHO) PS scale as specified in the Study Flow Chart (**Table 3**).

Table 3

ECOG (WHO) PS

Grade 0	Fully active, able to carry on all pre-disease performance without restriction.
Grade 1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g. light housework, office work
Grade 2	Ambulatory and capable of all self-care but unable to carry out any work activities. Up and about more than 50% of waking hours.
Grade 3	Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.
Grade 4	Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.
Grade 5	Dead

Pregnancy assessment

Women of childbearing potential should be advised to use highly effective contraception methods while receiving the experimental treatment and for at least 6 months after the last administration of post-neoadjuvant Pembrolizumab. The same patients will undergo a serum or urine pregnancy test, before the first administration of the study treatment.

Height, weight and body mass index (BMI)

Height in centimeters (cm) and body weight (to the nearest 0.1 kilogram (kg) in indoor clothing, but without shoes), will be measured on the days outlined in **Table 2**. Patient body mass index (BMI) will be calculated at scheduled time points through the following formula: body weight (kg)/[height (m)]^2.

Nutritional evaluation and study of body composition

Nutritional status will be assessed through the evaluation of anthropometric variables (weight, BMI) and, indirectly, through specific blood evaluations (dosages of transferrin, total cholesterol, prealbumin, absolute lymphocyte count and the neutrophil/lymphocyte ratio). A nutritional counseling will be provided by the study Sponsor Institution to all patients to maintain an appropriate diet, and to actively support patients undergoing unintentional, progressive low of body weight during the study treatment.
Lean and fat mass at baseline will be estimated through CT scan evaluation, performed before the initiation of the experimental treatment and before surgery.

Food diaries

Caloric intake will be evaluated through food diaries, i.e., a self-reported account of all foods, beverages and dietary supplements consumed by a respondent over one or more days (National

Cancer Institute, 2016 https://dietassessmentprimer.cancer.gov/). Since the instrument is open-ended, there is no limit to the number of items that can be reported. Standard portion sizes will be used to help estimate serving sizes. Mean caloric intake will be determined using Food Composition Database for Epidemiological Studies in Italy ("Food Composition Database for Epidemiological Studies in Italy" *by Gnagnarella P, Salvini S, Parpinel M. Version 1.2015 Website* http://www.bda-ieo.it/). The absolute and relative intake of macronutrients (i.e., carbohydrates, proteins, lipids) and micronutrients will be extrapolated from the food diaries and related to the caloric need of every patient.

All patients will be given food diaries to annotate the type and amount of food and beverage consumed at specific timepoints, i.e., i) during 7 consecutive days before treatment initiation ii) during one single day once a week (with the exception of the week in which patients receive chemotherapy plus Pembroluzumab plus/minus FLA) during the neoadjuvant treatment phase iii) during one single day every three weeks during the adjuvant treatment phase.

In addition, patients randomized to arm B will receive a specific 5-day food diary to annotate the type and amount of food and beverage consumed during each FLA cycle; patients randomized to arm A will receive at the same timepoints 5-day food diaries to annotate the type and amount of food and beverage consumed.

Phone/e-mail assessments

During each of the five days of each FLA cycle (or during the corresponding timepoints in patients randomized to arm A), all patients will be contacted by the Investigator on a daily basis to communicate any arising toxicity, updated weight and blood pressure measurements, and specific information regarding the type and amount of food and beverage intake (according to the provided list).

10.3 Quality of Life assessments

All patients will be evaluated for quality of life at different time points as outlined in **Table 2**. Patient QoL will be evaluated through the European Organization for Research and Treatment of Cancer (EORTC) quality of life questionnaires (QLQ) C-30 version 3.0 and the EORTC QLQ - BR23. (see Appendix 1). The EORTC instruments will be scored according to the EORTC guidelines.

10.4 Laboratory Procedures/Assessments

All patients will undergo different laboratory tests as part of the screening/baseline assessment, and during the study course at pre-specified timepoints, as described in the Study Flow Chart (**Table 4**). These tests include standard blood cell counts, biochemical evaluations and additional metabolic and immunologic analyses, as outlined below. Exploratory analyses on gut microbiota will be conducted as well.

Table 4 – Hematology and blood chemistry.

These evaluations will be performed locally at each study center.

Hematology	**Blood chemistry**	
Hemoglobin	Glucose	Alkaline phosphatase
Hematocrit	Creatinine	LDH
Platelet count	BUN	Uric acid
WBC (total and differential)	Alanine aminotransferase	Potassium
Red Blood Cell Count	Aspartate aminotransferase	Sodium
Absolute Neutrophil Count	Bilirubin	Calcium
Absolute Lymphocyte Count		
Absolute Monocyte Count		

Metabolic evaluations

Metabolic assessment on patients' blood will be conducted as listed in **Table 5** on the days outlined in Table 2. To perform these analyses, samples will be centralized at the Study Sponsor Institution.

Table 5

Metabolic evaluations		
Insulin	Insulin-like growth factor 1 (IGF-1)	Cortisol
Sideremia	Ferritin	Transferrin
Total protein	Cholinesterase	Prealbumin
Total, HDL and LDL cholesterol	Triglyceride	Albumin
FGF	Serum amino-acids	Plasma fatty acids

VEGF-A	PDGF	®-hydroxybutyrate
Serum IGFBP-1	Serum IGFBP-2	Serum IGFBP-3

Hepatitis serology

At screening all patients will be tested for HBV and HCV as by local clinical practice. HBV serology will include HBsAg and antibodies directed against HbS and HbC. HBV DNA should be obtained prior to the treatment phase in the case of HBsAg and/or anti-HBc positivity. HCV serology will include HCV antibody assessment (anti-HCV). HCV RNA should be obtained prior to treatment initiation if patient tests positive for anti-HCV. These analyses will be performed locally at each study center.

TSH, T3 and T4

Thyroid function will be evaluated as indicated in **Table 2**. These analyses will be performed locally at each study center. When needed, substitutive hormonal therapy should be initiated according to local and international guidelines. Unresolved abnormal labs that are drug-related AEs should be followed until resolution.

Urinalysis

Physical and chemical urinalysis, including the evaluation of urinary ketone concentration, will be performed on the days outlined in **Table 2**. These analyses will be performed locally at each study center.

Saliva and Stool analysis

Saliva and Fecal samples will be collected on the days outlined in **Table 2.** To perform these analyses, samples will be centralized at the Study Sponsor Institution Stool sample analysis will be performed following the procedure indicated in Section 14.

Immunological analysis (including PBMCs)

To perform these analyses, samples will be centralized at the Study Sponsor Institution.
Assessments will be conducted on the days outlined in Table 2. For details see Section 14.

Evaluation of pathological complete response (pCR) in surgical specimens

pCR is defined as the absence of residual invasive breast carcinoma, with or without ductal carcinoma in situ, AND the absence of any tumor deposit in sampled axillary nodes (ypT0/ypTis, ypN0, according to AJCC classification)[52].

Other analyses in tumor samples

For all patients, diagnostic biopsy tissue and surgically removed primary tumor tissue (in the subset of patients not undergoing pCR) will be compared to assess changes in:

- proliferation index (percentage of Ki67-positive cells on IHC);
- tumor biology, including expression of estrogen and progesterone receptors by IHC, HER2 protein by IHC (and, in case of equivocal value, by in situ hybridization analysis);

Additional biological evaluations will be also performed, as outlined in Section 14.

10.5 Withdrawal from study participation

In accordance with the current revision of the Declaration of Helsinki and other applicable regulations, every patient has the right to withdraw from the study at any time and for any reason without prejudice to his or her future medical care by the physician or at the institution.
Patients may discontinue the study protocol for any of the following reasons:

- Adverse event(s)
- Occurrence of an unacceptable toxicity
- Loss to follow-up
- General or specific changes in patient condition, that are considered unacceptable for further treatments in the judgment of the Investigator
- Pregnancy
- Protocol deviation
- Technical problems
- Patient decision
- Lack of compliance to the experimental treatment

At the time of study withdrawal, the Investigator should schedule the End of Treatment and Follow-up visits in agreement with the patient.

11 ADVERSE EVENTS (AEs)

An Adverse Event (AE) is defined as any new untoward medical occurrence or worsening of a preexisting medical condition in a patient who is administered a study treatment; the event does not necessarily need to be causally related with that treatment. An AE can therefore be any unfavorable and unintended sign (such as an abnormal laboratory finding), symptom, or disease that occurs after a patient has signed an informed consent and that is temporally associated with the use of the study treatment (regardless of the treatment arm), whether or not considered related to the study therapy.
AEs include the following:

- All suspected FLA-related adverse reactions
- All chemo-immunotherapy-related adverse reactions
- Apparently unrelated illnesses, including the worsening of a pre-existing illness
- Injury or accidents. Note that if a medical condition is known to have caused an injury or accident (e.g., a fracture due to a fall secondary to dizziness), the medical condition (dizziness) and the injury (fracture) should be reported as two separate adverse events
- Laboratory abnormalities and/or physical examination findings requiring clinical interventions (e.g., therapeutic measures, schedule changes) or further investigations (beyond ordering a repeat [confirmatory] test) or that are considered clinically significant by the investigator

As far as possible, each adverse event should be evaluated to determine:

1. The severity grade (CTCAE Grade 1-4)
2. Its duration (Start and end dates)
3. Its relationship to the study intervention (Reasonable possibility that AE is related: No, Yes)
4. Action taken with respect to study treatment (none, dose adjusted, temporarily interrupted, permanently discontinued, unknown, not applicable)
5. Whether medication or therapy was given (no concomitant medication/non-drug therapy, concomitant medication/non-drug therapy)
6. Outcome (not recovered/not resolved, recovered/resolved, recovering/resolving, recovered/resolved with sequalae, fatal, unknown)
7. Whether it is serious. Each AE has to be classified by the Investigator as serious or non-serious. AEs reporting procedures are defined according to the classification of the gravity of the event.

11.1 Serious Adverse Events (SAEs)

The definition of seriousness is based on the patient/event outcome or action criteria associated with events that pose a threat to patient's life or functioning.

A ***Serious Adverse Event (SAE)*** is any untoward medical occurrence that at any dose meets one or more of the following definitions:

- Results in death
- Is life-threatening (defined as an event in which the participant was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe)
- Requires in-patient hospitalization or prolongation of existing hospitalization (see **NOTE** below)
- Results in persistent or significant disability/incapacity
- Is a congenital anomaly/birth defect
- Suspected transmission of an infectious agent (e.g., pathogenic or nonpathogenic) via the study drug is a SAE.

Important AEs not resulting in death, not being life-threatening, or not requiring hospitalization, can still be considered serious when, based on the Investigator's medical judgment, they may jeopardize the patient or require medical or surgical intervention to avoid one of the unfavorable clinical outcomes listed above. Examples of such events include, but are not limited to, intensive treatment

in an emergency room or at home for allergic bronchospasm; blood dyscrasias or convulsions that do not result in hospitalization.

NOTE: Hospitalizations are not considered SAEs if they fulfill the following criteria:
- are elective and planned before entry into the study
- are admissions as per protocol for a planned medical/surgical procedure
- are emergency hospitalizations but do not result in overnight hospitalization, unless considered an important medical or life-threatening event

11.2 Adverse Event Intensity and Relationship to Study Intervention

All AEs, including SAEs, will be graded according to the NCI Clinical Toxicity Criteria [NCI-CTCAE version 5.0].
The causal relationship to study drug is determined by a physician and should be used to assess all adverse events. The casual relationship can be one of the following:
- Related: There is a reasonable causal relationship between the study intervention and the AE
- Not related: There is not a reasonable causal relationship between study intervention and the AE

The expression "reasonable causal relationship" is meant to convey in general that there are facts (e.g., evidence such as de-challenge/re-challenge) or other arguments to suggest a positive causal relationship.

11.3 AEs collection and reporting

AEs can be spontaneously reported or elicited during open-ended questioning, examination, or evaluation of a patient. In order to prevent reporting bias, patients should not be questioned regarding the specific occurrence of one or more AEs.

All observed AEs must be reported in the CRF, regardless of treatment arm or suspected causal relationship to the study treatment.
All AEs must be graded according to the NCI Common Terminology for Adverse Events (CTCAE), version 5.0. Any adverse event which changes CTCAE grade over the course of a given episode will have each change of grade recorded on the adverse event CRF/worksheets.
For all AEs, the Investigator must obtain information that is adequate to determine the outcome of the AE, and to assess whether the AE meets the criteria for classification as a SAE.
If known, the diagnosis of the underlying illness or disorder, rather than its individual symptoms, should be recorded. The following information should be captured for all AEs: onset, duration, intensity, seriousness, relationship to study intervention, action taken, and treatment required. If any treatment for the management of the AE was required, it should be recorded on the appropriate CRF page. Completion of supplemental CRFs may be requested for AEs and/or laboratory abnormalities that are reported/identified during the course of the study.

SAE collection and reporting

Following the Patient's written consent to participate in the study, all SAEs must be collected, including those thought to be associated with protocol-specified procedures and those occurring within 30 days of study treatment discontinuation. The investigator should notify any SAE occurring

after this time period, if they are believed to be related to study drugs or protocol-specified procedures.
Any SAE, either related or unrelated to study treatment, must be recorded on the SAE page of the CRF and reported to the Pharmacovigilance Unit of the study sponsor Institution (Fondazione IRCCS Istituto Nazionale dei Tumori) by the Investigator or a delegate member of the investigational staff within 24 hours of their knowledge of the event.
The initial report should be followed by submission of a more detailed adverse event communication within 5 calendar days after the Investigator first became aware of the serious event.
A SAE report should be completed for any event where doubt exists regarding its status of seriousness. If the investigators believe that a SAE not directly related to the administration of the investigational product(s) could be potentially caused by other conditions related to the study (such as withdrawal of previous therapy, or a complication of a study procedure), the relationship should be specified in the narrative section of the SAE page of the CRF.
If an ongoing SAE changes in its intensity or relationship to study treatment, a follow-up SAE report should be completed within 24 hours. All SAEs should be followed until resolution or stabilization.

Non-serious Adverse Events collection and reporting

The collection of non-serious AE information should begin at initiation of the study treatment.
If an ongoing non-serious AE worsens in its intensity, or its relationship to the study intervention changes, a new non-serious AE entry for the event should be reported.
Patients who have an ongoing study treatment−related adverse event upon study completion or at discontinuation from the study will be followed until the event has resolved to baseline grade, the event is assessed as stable by the investigator, new anti-cancer treatment is initiated, the patient is lost to follow-up, the patient withdraws consent, or it has been determined that study treatment or participation is not the cause of the adverse event.
Non-serious AEs should be reported as SAEs if they become serious.
All identified non-serious AEs must be recorded and described on the appropriate non-serious AE page of the CRF.

11.4 Laboratory test abnormalities

Laboratory abnormalities that constitute an AE on their own (i.e., that are considered as clinically significant, or inducing clinical signs or symptoms, or requiring concomitant therapy or changes in study treatment) should be recorded on the Adverse Events CRF. Whenever possible, a diagnosis, rather than a symptom, should be provided (e.g., “anemia” instead of “low hemoglobin”). Laboratory abnormalities that meet the criteria for AEs should be followed until they have returned to normal, or until an adequate explanation of the abnormality is found. When an abnormal laboratory test result corresponds to a sign/symptom of an already reported adverse event, it is not necessary to separately record the lab/test result as an additional event.
Laboratory abnormalities that do not meet the definition of an AE should not be reported as AEs. A Grade 3 or 4 event (severe) as per CTCAE does not automatically indicate a SAE unless it meets the definition of serious as previously defined and/or as per the investigator’s discretion. Any laboratory abnormality requiring the discontinuation or the interruption of a study drug, or requiring the subject to receive specific corrective therapy, represents an AE and must be reported as such.

12 COMPLIANCE TO THE EXPERIMENTAL TREATMENT

12.1 Food diary during the FLA periods

Patients randomized to arm B (experimental arm) will be given a food diary to annotate the type and amount of each food and beverage consumed during each of the 5-day FLA period.
Patients' compliance to the FLA will be evaluated through the analysis of these diaries. Quantitative variation within +10-50% of the allowed total daily calorie content will be considered minor deviations. Consuming non caloric food/beverages that are not contained in the prescribed scheme will be considered a minor deviation. Consuming caloric foods/beverages that are not contained in the prescribed scheme will be considered a major deviation, independently from the amount of the consumed food/beverage. Two minor deviations will define a major deviation as well. The number of minor and major deviations will be used to assess patients' compliance to the prescribed FLA scheme.
Patients randomized to the control arm (A) will be asked to fill the same food diaries for the recording of foods and beverages consumed during the same periods. The analysis of these diaries will be important to be sure that patients in the control arm undergo a regular diet, i.e., a normo-caloric diet that is based on international guidelines for cancer patients and survivors.

12.2 Food diary outside the FLA periods

All patients will be given food diaries to annotate the type and amount of food and beverage consumed at specific timepoints, i.e., i) during 7 consecutive days before treatment initiation ii) during one single day once a week (with the exception of the week in which patients receive chemotherapy plus Pembroluzumab plus/minus FLA) during the neoadjuvant treatment phase iii) during one single day every three weeks during the adjuvant treatment phase.

This information may be useful to interpret long-term effects of the experimental treatment on metabolic parameters and clinical outcomes.
Patients randomized to the control arm will be asked to fill the same food diaries for the recording of foods and beverages consumed during the same periods. The analysis of these diaries will be important to be sure that patients in the control arm undergo a regular diet, i.e., a normo-caloric diet that is based on international guidelines for cancer patients and survivors.

13 DISCONTINUATION/MODIFICATION OF THE EXPERIMENTAL TREATMENT

13.1 Discontinuation of chemo-immunotherapy

All patients undergoing overt radiological or clinical disease progression, deterioration of clinical conditions or death during the study treatment will permanently discontinue all study-related treatments and procedures. The study treatment will be permanently discontinued in case of SAEs

that, according to the judgment of the physician, could compromise patient general health status/conditions or contraindicate/delay curative surgery.
Patients who are not are able for any reason to maintain an adequate chemotherapy dose intensity, e.g., when chemotherapy administration needs to be delayed by more than three weeks for reasons strictly related to treatment tolerability (i.e., excluding infections, accidents or other reasons, which will be evaluated individually by the treating physician), will discontinue study treatments, and they will undergo anticipated surgery within 2-4 weeks after the last dose of chemotherapy. Patients who will need temporary or permanent discontinuation of Pembrolizumab for any reason (e.g., treatment tolerability, Pembrolizumab-related severe AEs or SAEs) will continue to receive chemotherapy, with or without cyclic FLA.

During the first part of the combination therapy (PCb plus Pembroluzumab plus/minus FLA), if one or more than one chemotherapy drug needs to be discontinued due to toxicity, patients may continue treatment as follows:

· If paclitaxel is permanently discontinued at any time and for any reason according to the physician judgment, also discontinue the other treatment of the first part of neo-adjuvant chemotherapy (carboplatin plus/minus FLA); then, start and complete AC or EC regimen plus Pembrolizumab plus/minus FLA as per protocol, then proceed with surgery.

·If only carboplatin is discontinued for any reason according to the physician judgment: continue with weekly paclitaxel (up to the completion of 12 cycles) plus triweekly Pembrolizumab plus/minus FLA followed by AC or EC plus Pembrolizumab plus/minus FLA as planned per protocol, then followed by surgery

During the second part of the combination therapy, if doxorubicin (or epirubicin) or cyclophosphamide need to be permanently discontinued for any reason, proceed with anticipated surgery. If only Pembrolizumab is discontinued, continue AC/EC for the remaining cycles as planned per protocol, and then proceed with surgery.

Arm B patients, who should permanently discontinue chemotherapy for any reason, and undergoing anticipated surgery, will also discontinue the FLA.

Note: if Pembrolizumab is permanently discontinued during the neoadjuvant treatment phase because of unacceptable Pembrolizumab-related toxicities, no Pembrolizumab will be administered after surgery.

13.2 Modification of chemotherapy administration

The dosage or schedule of chemotherapy can be modified in case of AEs according to clinical practice and physician's judgment. The following suggestions are provided to support clinical decisions for adverse events of particular interest.

Granulocyte colony stimulating factor for primary or secondary prophylaxis of neutropenia may be used in selected cases, and based on the physician judgment and according to International Guidelines.

Dose modifications for paclitaxel and carboplatin

Recommendations for dose interruptions/modifications in case of specific treatment-emergent AEs are provided in the following sections.
As a general rule, if dose reduction is necessary, the dose should be reduced by one dose level (see **Table 6**), and the subject should be monitored for 10 to 14 days at each dose level. If toxicity does not resolve during this monitoring time, drug delivery may need to be discontinued with continued monitoring for an additional 10-14 days.
Any treatment-related non-hematological toxicity of grade higher than or equal to 2 (except for G2 alopecia, peripheral neuropathy and fatigue, for which resolution is not necessarily needed) requires the treatment to be delayed until the adverse event has resolved to ≤ Grade 1.

Once the dose has been reduced no re-escalation is allowed.

Table 6:

Paclitaxel Dose Levels	
Dose Level	**Paclitaxel (mg/m^2)**
0 (starting dose)	80
-1	75
Carboplatin Dose Levels	
Dose Level	**Carboplatin (AUC)**
0 (starting dose)	1.5
-1	1.1

Patients experiencing any of the following toxicities during the previous cycle should have their chemotherapy reduced for all subsequent cycles by 1 dose level as outlined in **Table 6.**

Table 7:

	Dose Modifications for paclitaxel alone or with carboplatin[(a)]

Toxicity		**Adjustment for treatment component believed to be associated with specific toxicity. Continue other treatment component as per protocol (see Paragraph 13.1).**
Neutropenia	**≥1000 x 10^9/L (G1/G2)**	No change to paclitaxel and/or carboplatin
	<1000 x 10^9/L (G3)	Withhold paclitaxel and/or carboplatin until ANC≥1000 x 10^9/L. G-CSF may be used at discretion of the investigator. Resume paclitaxel and/or carboplatin based on time of recovery: ● ≤1 week: No change to paclitaxel and/or carboplatin ● 1 but ≤3 weeks: decrease paclitaxel and/or carboplatin dose (-1 dose level) ● ≥3 weeks: Discontinue paclitaxel and/or carboplatin
	Febrile neutropenia (≥ 38.3°C in a single temperature reading or a sustained temperature of ≥38°C for at least one hour associated with ANC < 1.0 x 10^9/L)	Withhold paclitaxel and/or carboplatin until resolution. G-CSF may be used at discretion of the investigator. Resume paclitaxel and/or carboplatin based on number of episodes: ● First episode: No change to paclitaxel and carboplatin dosages ● Second episode: decrease paclitaxel and/or carboplatin dose (-1 dose level) ● Third episode: Discontinue paclitaxel and/or carboplatin

Thrombocytopenia	**Grade 1**	Withhold paclitaxel and/or carboplatin until ≥100,000/mm3. Resume paclitaxel and/or carboplatin based on timing of recovery: · ≤1 week: no change in paclitaxel and carboplatin dosage and schedule. · >1 but <3 weeks: Reduce paclitaxel and/ or carboplatin (-1 dose level). · ≥3 weeks: Discontinue paclitaxel and /or carboplatin
	Grade ≥2 (<75000) or in the presence of significant bleeding or requiring blood transfusion	Withhold paclitaxel and/or carboplatin until ≥100,000/mm Decrease 1 dose level[b] Discontinue paclitaxel and/or carboplatin if the event lasts for ≥ 3 weeks
Anemia	**All grades**	No change to paclitaxel and carboplatin. Iron studies should be done and iron supplementation/ RBC transfusion can be given at the physician's discretion
Sensory neuropathy	**Grade 3**	Withhold paclitaxel and/or carboplatin. If neuropathy improves to ≤ Grade 2, treatment may be resumed with 1 dose level reduction. If neuropathy does not improve to ≤ Grade 2 within 3 weeks, discontinue treatment.
	Grade 4 sensory neuropathy	Withhold paclitaxel and/or carboplatin. If neuropathy improves to ≤ Grade 2 within 3 weeks, treatment may be resumed with 1 dose level reduction. If neuropathy does not improve to ≤ Grade 2 within 3 weeks, discontinue treatment.
Abnormal bilirubin value	Grade 1	Re-test bilirubin every week, continue study treatment

	Grade 2 or 3	Withhold treatment until improvement to Grade 1. Re- start treatment at the same dose level
	Grade 4	Discontinue treatment; liver ultrasound should be performed immediately
Abnormal AST/ALT values	Grade 1	Continue study treatment
	Grade 2 or 3	Withhold treatment until improvement to Grade 1. Re- start treatment at the same dose level
	Grade 4	Discontinue treatment; liver ultrasound should be performed immediately
Other Grade ≥ 3 toxicities[c]		Adjust dose or discontinue therapy as medically indicated.

[a]Despite adequate/maximal medical intervention and/or prophylaxis.
[b]Platelets have to recover to ≥ 100 x 10^9/L (and neutrophils have to be ≥ 1000/mm3) before the start of the next cycle.
[c]Except Grade 3 fatigue, transient joint or muscle pain for which no dose modifications are required.

Hypersensitivity Reactions to Paclitaxel or Carboplatin
Hypersensitivity reactions rarely occur. If they do occur, minor symptoms such as flushing, skin reactions, dyspnea, lower back pain, hypotension, or tachycardia may require temporary discontinuation of the infusion. In case of mild hypersensitivity reactions, at the investigators' discretion, chemotherapy can be resumed with an implementation of prophylactic drugs (i.v. antihistamine and 12 mg of dexamethasone), together with an infusion time prolongation. However, severe reactions, such as hypotension requiring treatment, dyspnea requiring bronchodilators, angioedema or generalized urticaria, mandate immediate discontinuation of study drug administration and aggressive symptomatic therapy. Patients who experience severe allergic reactions should not be re-challenged.

Dose modifications for doxorubicin/epirubicin and cyclophosphamide

Any treatment-related non-hematological toxicity of grade higher than or equal to 2 (except for G2 alopecia and fatigue for which resolution is not needed) requires the treatment to be delayed until the adverse event has resolved to ≤ Grade 1.
Recommendations for dose interruptions/modifications in case of specific treatment-emergent AEs are provided in the following sections.

Once the dose has been reduced no re-escalation is allowed.

Table 8

Anthracyclines and Cyclophosphamide Dose Levels			
Dose Level	**Doxorubicin (mg/m^2)**	**Epirubicin (mg/m^2)**	**Cyclophosphamide (mg/m^2)**
0 (starting dose)	60	90	600
-1	48 (-20%)	72 (-20%)	480 (-20%)

Patients experiencing any of the following toxicities during the previous cycle should have chemotherapy dose reduced for all subsequent cycles by 1 dose level as outlined in **Table 8**.

Table 9

	Dose Modifications for anthracyclines and cyclophosphamide [(a)]	
Toxicity		**Adjustment**
Neutropenia	**≥1000 x 10^9/L (G1/G2)**	No changes to AC or EC
	<1000 x 10^9/L (G3)	Withhold AC or EC until ANC≥1000 x 10^9/L. G-CSF may be used at discretion of the investigator. Resume treatment based on time of recovery: ● ≤1 week: No change ● 1 but ≤3 weeks: decrease -1 dose level ● ≥3 weeks: Discontinue AC or EC, and plan surgery

	Febrile neutropenia (≥ 38.3°C in a single temperature reading or a sustained temperature of ≥38°C for at least one hour associated with ANC < 1.0 x 10^9/L)	Withhold AC or EC until resolution. G-CSF may be used at discretion of the investigator. Resume AC or EC based on number of episodes: ● First episode: No change to AC or EC ● Second episode: reduce AC or EC (-1 dose level) ● Third episode: Discontinue AC or EC, and plan surgery
Thrombocytopenia	**Grade 1**	Withhold AC or EC until ≥100,000/mm3, Resume paclitaxel and/or carboplatin based on timing of recovery: · ≤1 week—no change · >1 but <3 weeks: reduce 1 dose level. · ≥3 weeks: Discontinue AC or EC, and plan surgery
	Grade ≥2 (<75000) or in the presence of significant bleeding or requiring blood transfusion	Withhold treatment until Plt≥100,000/mm3 Decrease 1 level[b] Discontinue AC or EC if treatment withheld for ≥ 3 weeks; plan surgery
Anemia	**All grades**	No change to AC or EC Iron studies should be done and iron supplementation/ RBC transfusion can be given at the physician's discretion
Abnormal bilirubin value	Grade 1	Re-test bilirubin every week, continue study treatment
	Grade 2 or 3	Withhold treatment until improvement to Grade 1. Re- start treatment at the same dose level

	Grade 3	Repeat biochemical tests every two days; liver ultrasound should be performed immediately. Discontinue AC or EC.
Abnormal AST/ALT values	Grade 1	Continue study treatment
	Grade 2 or 3	Withhold treatment until improvement to Grade 1. Re- start treatment at a lower dose level
	Grade 4	Discontinue treatment; liver ultrasound should be performed immediately. Discontinue AC or EC.
Mucositis and dysphagia		May occur during anthracycline-cyclophosphamide administration. Temporarily withhold anthracycline if these side effects are moderate to severe (≥ grade 2), but restart full dose once they resolve
Cystitis		May occur with cyclophosphamide administration. Temporarily withhold anthracycline -cyclophosphamide if cystitis is moderate to severe. Encourage the patient to drink large amounts of water; if urine culture is positive, antibiotics will be given
Cardiac toxicity		Maximum recommended cumulative dose of anthracyclines is 450 mg/m^2 for doxorubicin and 900 mg/m^2 for epirubicin Below this cumulative dose cardiac effects are not expected. Anthracyclines will be discontinued if: - congestive heart failure arises - persistent arrhythmia (including sinus tachycardia with no demonstrable cause) arises - asymptomatic decrease of LVEF below 45%

	Other Grade ≥ 3 toxicities[c]	Adjust dose or discontinue therapy as medically indicated

[a]Despite adequate/maximal medical intervention and/or prophylaxis.
[b]Platelets have to recover to ≥ 100 x 10^9/L (and neutrophils have to be ≥ 1000/mm3 before the start of the next cycle.
[c]Except Grade 3 fatigue, transient joint or muscle pain for which no dose modifications are required.

13.3 Pembrolizumab discontinuation

No dose reductions of Pembrolizumab are allowed; Pembrolizumab will be withheld or discontinued to manage adverse reactions as described in Table 10.

Table 10

Immune-related adverse reactions	**Severity**	**Treatment modification**
Pneumonitis	Grade 2	Withhold until adverse reactions recover to Grades 0-1*
	Grades 3 or 4, or recurrent Grade 2	Permanently discontinue
Colitis	Grades 2 or 3	Withhold until adverse reactions recover to Grades 0-1*
	Grade 4 or recurrent Grade 3	Permanently discontinue
Nephritis	Grade 2 with creatinine > 1.5 to ≤ 3 times upper limit of normal (ULN)	Withhold until adverse reactions recover to Grades 0-1*
	Grade ≥ 3 with creatinine > 3 times ULN	Permanently discontinue
Endocrinopathies	Grade 2 adrenal insufficiency and hypophysitis	Withhold treatment until controlled by hormone replacement

	Grades 3 or 4 adrenal insufficiency or symptomatic hypophysitis Type 1 diabetes associated with Grade ≥ 3 hyperglycaemia (glucose > 250 mg/dL or > 13.9 mmol/L) or associated with ketoacidosis Hyperthyroidism Grade ≥ 3	Withhold until adverse reactions recover to Grades 0-1* For patients with Grade 3 or Grade 4 endocrinopathies that improved to Grade 2 or lower and are controlled with hormone replacement, if indicated, continuation of pembrolizumab may be considered after corticosteroid taper, if needed. Otherwise treatment should be discontinued.
	Hypothyroidism	Hypothyroidism may be managed with replacement therapy without treatment interruption.
Hepatitis	Grade 2 with aspartate aminotransferase (AST) or alanine aminotransferase (ALT) > 3 to 5 times ULN or total bilirubin > 1.5 to 3 times ULN	Withhold until adverse reactions recover to Grades 0-1*
	Grade ≥ 3 with AST or ALT > 5 times ULN or total bilirubin > 3 times ULN	Permanently discontinue
Skin reactions	Grade 3 or suspected Stevens-Johnson syndrome (SJS) or toxic epidermal necrolysis (TEN)	Withhold until adverse reactions recover to Grades 0-1*
	Grade 4 or confirmed SJS or TEN	Permanently discontinue

Other immune-related adverse reactions	Based on severity and type of reaction (Grade 2 or Grade 3)	Withhold until adverse reactions recover to Grades 0-1*
	Grades 3 or 4 myocarditis Grades 3 or 4 encephalitis Grades 3 or 4 Guillain-Barré syndrome	Permanently discontinue
	Grade 4 or recurrent Grade 3	Permanently discontinue
Infusion-related reactions	Grades 3 or 4	Permanently discontinue
*If treatment-related toxicity does not resolve to Grades 0-1 within 6 weeks after last dose of pembrolizumab, or if corticosteroid dosing cannot be reduced to ≤ 10 mg prednisone or equivalent per day within 6 weeks, pembrolizumab should be permanently discontinued.		

Pembrolizumab should be permanently discontinued for Grade 4 or recurrent Grade 3 immune-related adverse reactions, unless otherwise specified in Table 10.

Patients experiencing disease progression that precludes definitive surgery or unacceptable toxicity related to Pembrolizumab as neoadjuvant treatment will not receive Pembrolizumab monotherapy as adjuvant treatment.

13.4 FLA discontinuation (arm B)

Patients may permanently discontinue the FLA because of unacceptable toxicity.
In particular, patients undergoing ≥ grade 3 hypoglycemia, or symptomatic ≥ grade 2 hypoglycemia during the FLA will immediately interrupt that specific FLA cycle. If the same toxicity occurs more than one time, the FLA will be permanently discontinued. No modifications of the dietary scheme are allowed on the basis of occurring toxicities. Patients for which the FLA is interrupted or permanently discontinued due to an AE, or because of the occurrence of clinically significant changes in laboratory parameters, will continue to be treated with chemoimmunotherapy.

Table 11

Discontinuation guidelines for FLA-related toxicities		
Adverse event	**Toxicity (grade)**	**Action**
Hypotension	Grade 1	Continue the FLA
	Grade 2	Continue the FLA. If the toxicity re-occurs despite optimal supportive care, permanently discontinue study treatment
	Grade 3 or 4	Permanently discontinue the FLA
Hypoglycemia	Grade 1 (LLN - 55 mg/dL; LLN - 3.0 mmol/L)	Continue the FLA
	Grade 2 (55 - 40 mg/dL; 3.0 - 2.2 mmol/L) without clinically relevant neurological or cardiac symptoms	Continue the FLA
	Grade 2 (55 - 40 mg/dL; 3.0 - 2.2 mmol/L) with clinically relevant symptoms, including diplopia, tremor, convulsions, drop/loss of consciousness, arrhythmias requiring medical treatment, myocardial infarction.	Interrupt that specific FLA cycle. Then the FLA can be re-initiated after three weeks, i.e., according to the pre-defined schedule. If the same toxicity occurs again, permanently discontinue the FLA.
	Grade 3	Interrupt that specific FLA cycle. Then the FLA can be re-initiated after three weeks, i.e., according to the

		pre-defined schedule. If the same toxicity occurs again, permanently discontinue the FLA.
	Grade 4	Permanently discontinue the FLA
Headache	Grade 1 or Grade 2	Continue the FLA
	Grade 3	Permanently discontinue the FLA
Fatigue	Grade 1 or Grade 2	Maintain dose level
	Grade 3	Permanently discontinue the FLA

Criteria for FLA discontinuation based on weight loss

Mild-to-moderate weight loss (about 3-6 % of baseline body weight) is expected at the end of each 5-day FLA cycle. After re-feeding, almost complete (>70%) restoration of pre-FLA weight is also expected in the majority of patients. However, some patients may fail to regain weight comparably to pre-FLA period, thus undergoing progressive weight loss.
Specific guidelines for continuing/discontinuing the experimental treatment on the basis of body weight loss and regain are reported in **Table 12**.

Table 12

Guidelines for treatment discontinuation due to weight-loss	
Weight loss before initiation of a new FLA cycle	**Action**
Weight loss < 5% from last FLA cycle **and** BMI ≥ 19 kg/m^2	Continue the FLA
Weight loss < 5% from last FLA cycle **and** BMI < 19 kg/m^2	Withhold FLA up to the next scheduled chemotherapy cycle, then re-evaluate weight and BMI. If BMI is still < 19 Kg/m^2 three weeks after the initial FLA delay, permanently discontinue the FLA. Otherwise, i.e., weight loss is <5% and BMI>19 kg/ m^{2,} resume the FLA

Weight loss 5-10% from last FLA cycle **and** BMI ≥ 22 kg/m^2	Continue the FLA
Weight loss 5-10% from last FLA cycle **and** BMI 19-22 kg/m^2	Withhold the next scheduled chemotherapy cycle, then re-evaluate weight and BMI. If the AE is confirmed, permanently discontinue study treatment. Otherwise, continue the FLA.
Weight loss 5-10% from last FLA cycle **and** BMI < 19 kg/m^2	Permanently discontinue the FLA
Weight loss > 10% from last FLA cycle with any BMI	Permanently discontinue the FLA

14 BIOLOGICAL EVALUATIONS

Tumor tissue and blood samples collected at prespecified timepoints (see Table 2) will be used to perform different biological and molecular analyses, including genomic, gene expression, proteomic, immunologic and metabolomic evaluations (see paragraphs below).
Since the knowledge of biomarkers and the development of new analytic procedures is rapidly evolving, after the completion of the planned assessments, remaining sample materials (e.g., aliquots of blood or tumor samples) may be used for further biological assessments.

14.1 Blood analyses

Patient blood samples (total volume: approximately 50 ml) will be taken at scheduled time points (see **Table 2**) to perform immunological, metabolic and other biochemical analyses.
With the exception of blood samples for blood cell count and standard blood chemistry, which will be analyzed at each participating center, blood-derived plasma and serum samples will be shipped to and stored at the study Sponsor Institution for the analyses detailed below. Samples will be stored for up to 10 years after study closure, with the additional option of further long-term storage. They will be stored under the Sponsor responsibility.
Biological evaluations will be performed in the laboratories of the Department of Diagnostic Pathology and Laboratory at Fondazione IRCCS Istituto Nazionale dei Tumori.

For sampling procedures and shipment see instructions in the appropriate sections of the Laboratory Manual.

Immunological parameters (20 ml of peripheral blood required)

Analyses of the impact of FLA on frequency, phenotype, function and metabolism of innate and adaptive antitumor immune cell populations will be performed. Our studies will include 13-color FACS analysis of counts and activation status of circulating inflammatory monocytes (CD11b+CD33+CD14+PD-L1+), polymorphonuclear myeloid-derived suppressive cells (PMN-MDSCs: CD11b+CD33+CD15+HLA-DRneg), monocytic MDSCs (M-MDSCs: CD11b+CD33+CD14+ and CD11b+CD33+CD14+HLA-DRneg), dendritic cells (DCs), natural killer (NK: CD3negCD16+CD56dimCD25+Ki67+ perforin+), B cells, regulatory T cells (CD4+CD25highFoxp3+CD127+ Ki67negCTLA4+Lag3+IL- 10+) and antitumor T cells, including the study of cell polarization (Th1, Th2 and Th17/Th22) and the quantification of exhausted/anergic effector T cells (CD3+, CD8+, CD25dimPD-1highLag3+TIM3+Ki67neg) versus differentiated/activated T cells (CD3+, CD8+, CD25+PD-1dimPerforin+Ki67+).
Plasma of individual patients will be analyzed to measure the concentration of pro-inflammatory (e.g. IL-1β, IL-2, IL-6, IL-8, IL-12, IL-17, IFN-gamma, TNF-α and chemokines MIP-1beta and MCP-1) and immunosuppressive cyto/chemokines (e.g. IL-4, IL-10, IL-13, TGF-beta). Serum MIF and the soluble part of its receptor sCD74 will be also measured.
Global gene expression profiling will be performed to assess the transcription of metabolic genes, including those encoding glucose and amino acid transporters, or enzymes involved in glycolysis, tricarboxylic acid (TCA) cycle, fatty acid and cholesterol biosynthesis, amino acid and nucleotide synthesis or catabolism.

For each immunological parameter average measurements will be performed at scheduled time points, and comparisons between the experimental arms at different time points will be calculated.

Blood cell count (8 ml of peripheral blood required)

For each patient and each scheduled time point, the number of blood cells (red blood cells, neutrophils, platelets, lymphocytes) will be measured. Average values in the two treatment arms will be compared at different time points.

Standard blood chemistry (12 ml of peripheral blood required)

Patient blood will be collected at baseline and at different time points after therapy initiation. Measured parameters include: fasting glycemia; markers of renal (blood levels of creatinine, urea and uric acid) and liver (AST, ALT, total and conjugated bilirubin, alkaline phosphatase) function; potassium, calcium, phosphate, magnesium; lactate dehydrogenase (LDH).

Quantification of plasma amino acids (5 ml)

Plasma amino acids will be quantified at scheduled time points through the AccQ Tag Ultra Derivatization kit (Wates). Ultra-Performance Liquid Chromatography (UPLC) analysis will be performed to measure the concentration of all amino acids, and in particular, glutamine, glutamate, leucine, tryptophan, methionine, arginine.

Lipid profile of whole blood, plasma and red blood cell membranes (3 ml)

Blood samples (4 ml) will be collected into heparin tubes. Whole blood, purified RBC cell membranes and plasma will be used for lipid assessment. Cell membranes of RBCs (ghosts) will be prepared by lysis with hypotonic buffer and washed to eliminate hemoglobin. Phospholipids and neutral lipids (free, ester and oxy-cholesterol, triglycerides) of RBCs will be purified and quantified by HPLC-ELSD system.
Ghost and plasma lipids will be extracted with different chloroform/methanol mixtures. Gas-chromatographic analysis will be used to determine FA composition of whole blood, plasma, membrane phospholipids, as well as of each phospholipid or neutral lipid species from RBC membranes and plasma. The FA methyl esters will be obtained after derivatization with sodium methoxide in methanol 3.33% w/v and injected into gas chromatograph (Agilent Technologies 6850 Series II). SP, SP1P and cholesterol-derived oxysterols, such as 7α-, 7β-, 7-keto-, 20-, 25-hydroxy-cholesterol, cholesterol-5,6-epoxide and the sterol metabolite 27-hydroxy-cholesterol, will be measured by UPLC-MS.

Metabolic evaluations and Growth factor analyses (3 ml)

Plasma will be separated from patient blood samples (4 ml) collected at scheduled time points and frozen at -80o C. Total proteins, albumin, triglycerides; total and HDL cholesterol, prealbumin, transferrin, sideremia, ferritin, cholinesterase, cortisol, insulin, β-hydroxybutyrate, IGF-1; IGFBP-1, IGFBP-2, IGFBP-3; VEGF-A, FGF will be dosed through specific immunoradiometric or ELISA assays.

14.2 Urine analyses

Urine samples obtained at scheduled time points will be collected to perform chemical and physical analyses at each participating center. In particular, urine ketone bodies will be dosed as a measure of patient systemic ketosis.

14.3 Saliva and Stool analyses

Stool samples collected at the pre-specified timepoints will be shipped to the study Sponsor Institution.

Oral and Fecal Microbiota analysis

Saliva and Fecal samples will be collected through the ORAGENE DNA Saliva and **OMNIgene GUT** (DNA Genotek, Ottawa, Canada) collection method.

Metagenomic DNA will be extracted using the Powersoil DNA Isolation kit (Mo Bio Laboratories), and the variable regions of 16S ribosomal RNA gene will be amplified and then sequenced using the Illumina MiSeq technology platform. Sequencing data will be analyzed using the QIIME pipeline or Mothur program with Greengenes or Ribosomal Database Project (RDP) databases to obtain bacterial composition data down to the genus level.

Bacterial metabolites in feces

SCFAs will be extracted from fecal samples and will be analyzed by ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry (UPLC-HRMS).

14.4 Biological evaluations on tumor tissue samples

Biological evaluations on tumor tissue sample will be performed on tumor specimens obtained from patients' baseline core biopsies (available as FFPE and, optionally, fresh frozen specimens), and surgical specimens from the same patients, depending on tissue availability.

For all the patients enrolled in the study, a Formalin-Fixed Paraffin-Embedded (FFPE) tumor specimen collected before the initiation of the experimental treatment must be shipped to the study Sponsor Institution. Optionally, patients enrolled in the study may undergo an additional core biopsy of the primary tumor study Sponsor Institution for the collection of fresh-frozen tumor material for different types of biological analyses (see below).

Optionally, patients will be proposed to repeat a tumor biopsy at the study Sponsor Institution 14-21 days after the first FLA cycle completion, to collect FFPE and fresh-frozen tissue for early evaluation.

Surgical FFPE tumor specimens will be centralized to the study Sponsor Institution. Only for patients enrolled at the study Sponsor Institution, surgical fresh frozen tumor material will be collected as well.

In patients undergoing disease recurrence, if a diagnostic biopsy of a metastatic lesion is indicated as per clinical practice (e.g., TNBC biology confirmation), an optional core biopsy will be proposed to the patient for the collection of fresh-frozen tumor material that will be used for subsequent biological evaluations.

In particular, the analyses will be performed at genomic and transcriptomic level to correlate tumor genomic and gene expression profiles with the efficacy of the experimental arms and to evaluate the effect of the administered experimental treatments on gene transcription.
Results of these analyses will be used *a posteriori* to select few proteins of interest whose expression will be analyzed on tissue samples with IHC or mass spectrometry.

Moreover, hematoxylin and eosin (H&E) staining on tumor tissue samples will be performed to evaluate the proportion of tumor-infiltrating lymphocytes (TILs).
Finally, we will perform metabolomic evaluations on tumor tissue samples. Metabolites will be extracted from tumor cells lysates and analyzed by mass spectrometry.

These analyses will be performed in the Pathology Unit of Fondazione IRCCS Istituto Nazionale dei Tumori (INT)

For sampling procedures and shipment see instructions in the appropriate sections of the Laboratory Manual.

Gene expression analysis

Whole-transcriptome analysis with global RNA sequencing (RNA-Seq, Illumina technology) will be performed in the Pathology Unit of Fondazione IRCCS Istituto Nazionale dei Tumori (INT).
Specifically, our interest will be focused on studying expression profiles of genes involved in

- Gene involved in autophagy and endosomal trafficking (e.g., *ULK1*, *ATG5*, *BECN1* and *BECN2*)
- Genes encoding glucose and amino acid transporters *(*e.g., *GLUT1* and *GLUT4*), enzymes involved in glucose metabolism (e.g., hexokinase*,* phosphofructokinase, pyruvate kinase, lactate dehydrogenase), mitochondrial oxidative phosphorylation (e.g., isocitrate dehydrogenase, fumarate hydratase, succinate dehydrogenase), tricarboxylic acid (TCA) cycle, fatty acid and cholesterol metabolism, amino acid and nucleotide synthesis or catabolism.
- Endoplasmic reticulum (ER) stress and unfolded protein response (UPR)-related genes, including *GRP78*, *ATF6*, *IRE1a* and *ATF4*.
- DNA repair systems genes, including *BRCA1* and *2, ATM, ATR, CHK1/2, TP53, PRKDC, RAD51, RPA, TPR, ATRIP, NBS1, MATR3, SUN1, SUN2, NESPRIN1-7, EMERIN.*
- Genes encoding surface and secreted proteins expressed by non-cancerous cells within the tumor microenvironment. In particular, we will focus on transcription of genes expressed by different immune cells (including *CD8*, *CD3*, *IFN*, perforin, granzyme, *CD14*, *PDL1*) in order to define specific immunological profiles.

In addition, to evaluate the spatial distribution of pre-defined metabolic and immune biomarkers in cancer cells and in the surrounding cells in the tumor, we will perform spatial transcriptomic analyses through a a high-plex, high-throughput spatial profiling platform, the GeoMx® Digital Spatial Profile (DSP).

In a subset of patients undergoing additional biopsy at baseline and after one treatment cycle for the collection of fresh material, we will also perform single cell RNA-seq (scRNA-seq) analyses to follow the clonal evolution of cancer cell subsets, as well as of cells in the tumor microenvironment, during the experimental treatment, as well as to assess the modulation of crucial metabolic pathways in subsets of tumor cells and cells of the immune infiltrate. ScRNA-seq analyses will be performed in fresh tumor material after immediate separation of single cells.

Genomic assays

We will perform next generation sequencing (NGS) analysis to evaluate the mutational status of around 400 genes. The panel will include, but will not be limited to, genes involved in, DNA repair systems, mTORC 1 (e.g., LKB1, mTORC1, PI3K, AMPK) and autophagy (e.g. BECN1) pathways.

Peritumoral adipose tissue analysis

In fresh breast samples collected at the time of surgery (regardless of pCR status) in patients undergoing surgery at the Study Sponsor Site, we will isolate peritumoral adipose tissue to perform transcriptomic analysis and for the isolation of peritumoral adipocytes. In patients undergoing pCR (i.e., for whom no residual viable tumor cells can be found at surgery), peritumoral adipose tissue will be identified based on the position of the magnetic seed (inserted inside the tumor before treatment initiation). In patients not undergoing pCR (i.e., those with residual tumor cells), adipocytes that are close to the residual tumor will be collected. In both cases, RNA will be extracted from peritumoral adipocytes to assess differences in adipose tissue transcriptomic profiles between patients undergoing or not undergoing pCR. In addition, living adipocytes will be separated and cultured under different metabolic conditions to study the possibility to reprogram adipocyte biology.

15 STATISTICAL CONSIDERATIONS AND ANALYTICAL PLAN

15.1 Definition of study populations

The per-protocol (PP) population will include all enrolled patients receiving at least one full 21-day cycle of experimental treatment with no major violations of the eligibility criteria during study conduction. Moreover, to be included in the PP population, patients should undergo breast surgery with curative intent in order to evaluate the achievement, or lack of achievement, of pCR.
Therefore, patients randomized to arm A, who will have received at least one full 21-day cycle of chemo-immunotherapy, will be evaluated for arm A; patients randomized to arm B, who will have received at least one full 21-day cycle of chemo-immunotherapy plus FLA, will be evaluated for arm B.
The population in which the compliance will be evaluated will include all enrolled patients with no major violations of the eligibility criteria during study conduction.
The safety population will include all enrolled patients who received at least one dose of study treatment, whether withdrawn prematurely or not, and had at least one safety follow-up.

15.2 Statistical plan and sample size calculation

Information on demographics and baseline characteristics of patients satisfying the definition of the study populations will be provided. The number of patients withdrawing from the study, not meeting the eligibility criteria, and violating the protocol will also be described.
Patients enrolled in this study will be randomized in a 1:1 ratio to one of two experimental arms.
Based on the assignment cohort, each enrolled patient will receive one of two experimental treatments as preoperative therapy before surgery of the breast and draining lymph-nodes. At randomization, patients will be stratified on the basis of disease clinical stage (II vs. III) and patient BMI to guarantee a proper balance between patients in the two treatment arms.

Data from the KN522 trial indicate that the rate of pathological complete response (pCR) after neo-adjuvant anthracycline-taxane-carboplatin based chemotherapy in combination with Pembrolizumab is 64.8% (95% CI, 59.9 to 69.5). In this study, we will test the hypothesis that the experimental treatment is able to improve the pCR from 65% to 85%.
Specifically, by assuming a one-sided α error of 5% and a β error of 15% (power = 85%), 132 patients (66 for each arm) will be needed. Finally, incorporating around 10% of patient drop-out, a total of 145 patients will be enrolled. Allocation of a patient to a cohort will be based on a randomization list.

15.3 Statistical analyses

Primary endpoint analysis

Treatment effect will be expressed as absolute difference in pCR rates between treatments and 95% confidence intervals will be calculated. The analysis will be stratified by clinical disease stage according to TNM classification at enrollment (stage II vs. stage III), as well as by patient BMI (≥25 kg/m2 vs <25 kg/m2). Pathological complete response is defined as absence of invasive disease in breast and nodes (ypT0/ypTis, ypN0). The impact of known prognostic factors (age, BMI, disease stage, node status, Tumor Infiltrating Lymphocytes (TILs), Grade and proliferation rate (Ki-67)) on the rate of pCR will be first assessed using logistic regression models. Covariates significantly associated with pCR will be included in a multivariate analysis to assess their independent association with pCR.

Secondary efficacy analyses

Secondary analyses on efficacy endpoints will be conducted on the PP population. EFS, DFS, DMFS and OS will be analyzed by the Kaplan-Meier (KM) method. Survival differences between the two treatment arms will be tested by a log-rank test. A Cox proportional hazard model will be used to obtain hazard ratios and 95% confidence intervals for the treatment effect on EFS, DFS, DMFS and OS, and stratified by disease stage at enrollment. The impact of known prognostic factors (age, BMI, disease stage, node status, Tumor Infiltrating Lymphocytes (TILs), Grade and proliferation rate (Ki-67)) on patient survival (in terms of EFS, DFS, DMFS and OS) will be first assessed at univariate analysis. Covariates significantly associated with survival outcome will be included in a Cox

proportional hazard model (multivariate analysis) to assess their independent association with survival.
Biologic biomarkers (including metabolic, genomic, gene expression biomarkers) modulation will be analyzed using generalized linear and logistic models according to the variable considered.

Safety analyses and unacceptable toxicity monitoring

Safety analyses will be conducted on the safety population. All safety parameters will be analyzed and presented in terms of listings and summary tables. The assessment of safety will be based mainly on the frequency and nature of severe (G3/G4) AEs or serious adverse events (SAEs). Number and percentage of patients having any severe AEs or SAEs as well as the system/organ class involved in the severe AE will be presented. Any other information collected (e.g., severity or suspected relationship to study medication) will be listed as appropriate.
Drug-discontinuing AEs will be closely monitored throughout the study in order to promptly close the experimental arm in case of unacceptable toxicity. No formal safety interim analyses are planned for this study. Periodic safety reviews will be conducted and any outcome affecting the study conduct will be promptly communicated by the investigators for notification to the Ethics Committees (ECs)

16 ETHICAL AND ADMINISTRATIVE CONSIDERATIONS

16.1 Independent Ethics Committee (IEC)

Before initiating the trial, the Investigators must obtain a written favourable opinion from the IEC for the study protocol, the written informed consent form, the subject recruitment procedures and any other written information to be provided to subjects. All the correspondence with the IEC must be retained in the Investigator Site File.
Before implementing any protocol amendment, the IEC written approval must be obtained. The only circumstance in which an amendment may be initiated prior to IEC approval is where the change is necessary to eliminate apparent immediate hazards to the Patients. In that event, the IEC must be notified in a written form as soon as possible.

16.2 Ethical conduct of the trial

The study will be performed in accordance with the general principles of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, the Declaration of Helsinki and applicable local regulatory requirements and laws.

16.3 Informed Consent

It is the responsibility of the Investigators to give each patient (or to the patient's representative) full and adequate verbal and written information regarding the objective and procedures of the trial and the potential risks for taking part in the study. The patient must be informed about his/her right to

withdraw from the trial at any time. The patient should have time and opportunity to enquire about details of the trial and to decide whether to participate in the trial.
Written Patient information must be approved by the IEC and must be given to each patient before any trial-related procedure is undertaken.
It is the responsibility of the Investigators to obtain informed consent signed and dated by the patient or her/his legal representative or by an impartial witness, when applicable, and by the medical person conducting the informed consent discussion, before undertaking any trial-related procedure. One copy of the signed and dated Informed Consent Form should be given to the patient. The originally signed document should be archived in the Investigator Site File.
The approved Patient Information sheet must not be changed without prior approval by the IEC.
Whenever new study information becomes available during the trial conduct, the patients still on treatment must be informed and a new Informed Consent form or an addendum to the already signed Informed Consent form must be signed and dated by each patient, or her/his legal representative or by an impartial witness, when applicable.
If a subject unexpectedly becomes incapable during the course of the trial, legally acceptable representative authorization should be obtained for the patient to continue trial participation.

16.4 Confidentiality of information

Information about study patients will be kept confidential and managed under the applicable laws and regulations, which require a signed patient authorization providing the following information:

- What protected health information (PHI) will be collected from the patient enrolled in the study
- Who will have access to that information and for what purpose
- Who will use or disclose that information
- The right to revoke authorization for disclosure of His/Her own PHI.

If a patient revokes authorization to collect or use PHI, the Investigator, by regulation, retains the ability to use all information collected prior to the revocation of patient authorization.
The data collection system for this study exploits built-in security features to encrypt all data, preventing unauthorized access to confidential participant information.
Patient names will not be included on any forms, reports, publications, or in any other disclosure, except when required by laws.
Patient names, address, birth date and other identifiable data will be replaced by a numerical code.
All information regarding study treatment is privileged and represents confidential information. The Investigator agrees to keep all study results in confidence. Such information shall not be disclosed to third parties except to regulatory authority(ies), when requested. Individual Investigators may present results of the study at scientific meetings.

17 QUALITY CONTROL

17.1 Responsibilities of the investigators

The Investigators undertake to perform the study in accordance with Good Clinical Practice. The Investigators are required to ensure their compliance to the procedures required by the protocol. The Investigators agree to provide all information requested in the Case Report Form in an accurate and legible manner according to the instructions provided. The Investigators have responsibilities to take all reasonable steps to ensure the proper conduct of the study as regards ethics, protocol adherence, integrity and validity of the data recorded on the Case Report Forms.

17.2 Study Monitoring

Monitoring activities will be done in order to verify that the data are authentic, accurate, and complete; that the safety and rights of the subject are being protected; and that the study is conducted in accordance with the currently approved protocol, GCP, and all applicable regulatory requirements. At regular intervals during the study, each participating center will be contacted by a representative of the Sponsor (internal and/or external staff authorized and duly appointed by the Sponsor), through site visits and/or telephone calls, to review the study progress, the investigators and subjects adherence to protocol requirements.
The following points will be scrutinized:

- subject informed consent
- subject recruitment and follow-up
- study drug allocation
- subject compliance to the study treatment
- study treatment accountability
- adverse event documentation and reporting

According to the guidelines on ICH Good Clinical Practice, the monitor of the study will check the Case Report Form entries against the source documents.
Data will be collected by eCRF and data will be systematically checked by representative of the Sponsor (internal and/or external staff authorized and duly appointed by the Sponsor) for consistency, completeness and accuracy.

17.3 Audits and inspections

Authorized representatives of a regulatory authority or Ethics Committee may perform audits or inspections at the study centers, including source data verification. The purpose of an audit or inspection is to systematically and independently examine all study-related activities and documents, to determine whether these activities were conducted, and data were recorded, analyzed, and accurately reported according to the protocol, GCP, guidelines of the ICH, and any applicable regulatory requirement.

17.4 Retention of Records

Records and documents pertaining to the conduct of this study, including eCRFs, ICFs, Investigator Site File (ISF) must be retained by the Principal Investigator for at least 7 years after completion or discontinuation of the study, or for the length of time required by relevant national or local regulations, whichever is longer. After that period, the documents may be destroyed, according to local regulations.

18 STUDY CONDUCTION

18.1 Administrative Structure

This study is sponsored by the Fondazione IRCCS Istituto Nazionale dei Tumori di Milano.
The study Sponsor will allocate qualified personnel to the present trial. All the figures involved in the study design, management and conduct are qualified according to Italian laws and regulations concerning clinical trials, and specifically trained on the objectives, procedures and instruments of this trial.

18.2 Training of study site personnel

The Principal Investigator will ensure that appropriate training relevant to the study is given to all investigational staff, and that any new information relevant to the performance of this study is forwarded to the staff involved.

18.3 Study timetable and end of study

The end of the study is defined as the last visit of the last patient undergoing the study.
The study is expected to start in January 2023 and patient enrollment should be completed by January 2025 (expected total study duration 36 months). The study may terminate prematurely if concerns for safety arise. All patients will receive follow-up care in accordance with standard local clinical practice.
Any SAE or non-serious AE that is ongoing at the time of this data cut-off must be followed up to resolution unless the event is considered unlikely to resolve by the investigator, or the patient is lost to follow-up.

18.4 Data Handling

Source documents are where data is first recorded. These include, but are not limited to, hospital records, clinical and office charts, laboratory and pharmacy records, diaries, microfiches, radiographs, and correspondence.
Direct access to source data will be granted to authorized representatives to permit trial-related monitoring, audits and inspections.

18.5 Publication of Data

The protocol will be registered in a publicly accessible database such as clinicaltrials.gov.
This trial is intended for publication, even if terminated prematurely. Publication may include any or all of the following: posting of a synopsis online, abstract and/or presentation at a scientific conference, or publication of a full manuscript. In addition, after study completion and finalization of the study report, results of this trial will be submitted for publication and posted in a publicly accessible database of clinical trial results (e.g. Clinicaltrials.gov).

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APPENDIX I : Quality of Life questionnaires