I.R.C.C.S. Ospedale San Raffaele

CLINICAL STUDY PROTOCOL

Study Code: ONSET

Title:

ON-treatment Single-cell analysis for the identification of Early Tumor response biomarkers on prospective collected serial tumor biopsies in Triple Negative Breast Cancer patient during standard neoadjuvant chemo-immunotherapy

Principal Investigator: Dr.ssa Giulia Viale

CLINICAL STUDY PROTOCOL

Title:

ON-treatment Single-cell analysis for the identification of Early Tumor response biomarkers on prospective collected serial tumor biopsies in Triple Negative Breast Cancer patient during standard neoadjuvant chemo-immunotherapy

Study Identifier	ONSET
RSO number	NA
Other Protocol Codes	NA
Version	1.1
Date	10/Feb/2026

Sponsor	IRCCS Ospedale San Raffaele
Authorized Sponsor Representative	Prof. Michele Reni. MD Director UO Medical Oncology IRCCS Ospedale San Raffaele
Funding Source(s)	Medical Oncology Unit internal funding (Eredità Buffa)
Principal Investigator	Giulia Viale, MD Medical Oncology IRCCS Ospedale San Raffaele

DISCLAIMER

VERSION HISTORY

Protocol version n.	Reason of changes	Date issued
1.0	First Version submitted to the Ethics Committee (EC)	15 Dec 2025
1.1	Updated according to EC revision	10 Feb 2026

PROTOCOL SIGNATURE PAGE

Study Title:

ON-treatment Single-cell analysis for the identification of Early Tumor response biomarkers on prospective collected serial tumor biopsies in Triple Negative Breast Cancer patient during standard neoadjuvant chemo-immunotherapy

Study Code: *ONSET*

Protocol Version and Date: v 1.1 Datet 10 Feb 2026

The undersigned has read and understood all the aspects of the protocol detailed within this document and agrees to supervise and conduct the study in accordance with the protocol, the Declaration of Helsinki, Guideline for Good Clinical Practice ICH E6 (R3), and all applicable regulatory requirements.

		Medical Oncology Unit IRCCS Ospedale San Raffaele	
Authorized Sponsor Representative Name Michele Reni	**Signature**	**Affiliation**	**Date**
		Medical Oncology Unit IRCCS Ospedale San Raffaele	
Principal Investigator Name Giulia Viale	**Signature**	**Affiliation**	**Date**

TABLE OF CONTENTS

1. KEY STUDY CONTACTS

Sponsor	IRCCS Ospedale San Raffaele Via Olgettina, 60 20132 – Milano, Italy
Authorized Sponsor Representative	Prof Michele Reni, MD Director UO Medical Oncology IRCCS Ospedale San Raffaele Via Olgettina, 60 – 20132 – Milano, Italy Tel 02-26436529 Email: reni.michele@hsr.it
Principal Investigator	Giulia Viale, MD Medical Oncology IRCCS Ospedale San Raffaele Via Olgettina, 60 – 20132 – Milano, Italy Tel 02-26436530 Email: viale.giulia@hsr.it;
Study Clinical Unit	Medical Oncology Unit IRCCS Ospedale San Raffaele Via Olgettina, 60 – 20132 – Milan, Italy Tel 02-26436530 Email: viale.giulia@hsr.it ; licata.luca@hsr.it
Participant Clinical Units	San Raffaele Telethon Institute for Gene Therapy, Milan Cancer Research Institute, Cambridge, UK
Funding source(s)	Medical Oncology Unit internal funding (Eredità Buffa)
Clinical Trial Center	Email: ctc.firstcontact@hsr.it; ctc.trialstartup@hsr.it; ctc.datamanagement@hsr.it; ctc.quality@hsr.it

2. SYNOPSIS

Study Identifier	ONSET
Study Title	ON-treatment Single-cell analysis for the identification of Early Tumor response biomarkers on prospective collected serial tumor biopsies in Triple Negative Breast Cancer patient during standard neoadjuvant chemo-immunotherapy
Protocol Version and Date	V 1.1 10-Feb-2026
Sponsor	IRCCS Ospedale San Raffaele

Funding Source(s)	Medical Oncology Unit internal funding (Eredità Buffa)
Principal Investigator	Giulia Viale, MD Medical Oncology IRCCS Ospedale San Raffaele
Study Description	This exploratory translational study investigates early breast cancer (EBC), focusing on triple-negative (TNBC) and high-risk luminal (ER+/HER2-) subtypes. By integrating single-cell RNA sequencing, using the 10x Genomics platform (or similar technologies if it will be not available at the time of experiment), and spatial imaging analyses from serial tumor biopsies, the project aims to characterize tumor–immune interactions and identify predictive biomarkers of response or resistance to neoadjuvant chemo-immunotherapy. We hypothesize that early molecular and spatial features, when detected at the single-cell level, can predict treatment sensitivity or resistance. This would enable adaptive therapeutic strategies that minimize overtreatment while maximizing efficacy in EBC. Our goal is to reveal, for the first time, the cellular heterogeneity and spatial interactions that drive therapy resistance and disease progression. The analysis will be performed on tumor biopsies collected before the start of treatment, after C1D1 and on surgical material. The only tissue sample collected specifically for the study is the biopsy after C1D1.
Study Design	This is an interventional translational, single-center study with a prospective, longitudinal cohort design based on serial tumor tissue collection and analysis for the identification of early tumor response biomarkers. Tumor samples will be obtained at three predefined time points: (i) prior to initiation of treatment (baseline biopsy), (ii) after Cycle 1 Day 1 (C1D1) of therapy, and (iii) at the time of surgery (surgical specimen). Among these, the only biopsy performed specifically for study purposes is the post-C1D1 biopsy; the baseline and surgical samples are collected as part of standard clinical management with no differences expected among the procedures. Analyses will focus on temporal molecular and histopathological changes across serial samples to characterize treatment-related tumor evolution.

Primary Objective To characterize the dynamic changes in tumor and immune cell populations in TNBC and to prospectively collect serial samples from high-risk luminal (ER+/HER2-) breast cancer patients.	**Primary Endpoint** Gene expression levels per single-cell and relative cell clustering (%)	**Timepoints** Pre-treatment, on-treatment (after first cycle of therapy), and post-treatment (surgery or residual disease)
Exploratory Objectives To correlate single-cell gene expression data with pCR and RD to identify predictive signatures of therapy response	**Other** pCR and RD	**Timepoints** Across all collected timepoints
Study Population	Adult female patients (≥18 years) with early breast cancer, triple negative or high-risk luminal (ER+/HER2) subtypes, candidate to receive SOC neoadjuvant chemo/immunotherapy	
Inclusion Criteria	1. Female patients aged ≥18 years. 2. ECOG performance status 0–1. 3. Histologically confirmed early breast cancer with one of the following molecular profiles: • TNBC: ER and PR *negative* (IHC <10%) and HER2 *negative* (IHC 0–1+ or FISH non-amplified). • High-risk luminal (ER+/HER2-): ER *positive* (IHC ≥10%) HER2 *negative* (IHC 0–1+ or FISH non-amplified), with high-risk features (e.g., Grade 3, PR-negative, high proliferation, high TILS). 4. Clinical indication for neoadjuvant treatment according to standard practice: • TNBC: cT1c and/or cN positive, or cT2 (>2 cm) and/or cN positive (stage II,III). • High-risk luminal: features as defined above (Grade 3, PR-negative, high proliferation, ER low). 5. Ability to understand and sign written informed consent for participation in the study, approved by the local Ethics Committee.	
Exclusion Criteria	1. HER2-positive tumors.	

	2. Multifocal tumors — exclusion if a single index lesion cannot be identified and sampled; otherwise allowed if a representative lesion can be biopsied. 3. Known metastatic disease. 4 Clinical contraindications to the planned neoadjuvant therapy. 5. Decision for upfront surgery as determined by the multidisciplinary team. 6. Inability to provide informed consent. 7. Pregnancy or breastfeeding. 8. Prior systemic therapy (chemotherapy, immunotherapy, or endocrine therapy) for the current breast cancer before baseline biopsy 9. On-treatment biopsy clinically not feasible or controindicated
Intervention(s)	
Patients will receive standard-of-care neoadjuvant chemotherapy according to sequential anthracycline and taxane regimens. In addition, patients with triple-negative breast cancer (TNBC) will receive immunotherapy with pembrolizumab as per the KEYNOTE-522 schema. No modifications to the SOC treatments need to be reported. Patients enrolled in the study will undergo a single on-treatment biopsy after the first cycle of neoadjuvant therapy, that is not required as per SOC, and two biopsies (at diagnosis and at surgery) already planned for clinical practice to assess biomarkers predictive of early response/progression with single-cell sequencing technique.	
Intervervention for Biomarker identification	The study will analyze fresh biopsies collected from TNBC patients receiving standard neoadjuvant chemo-immunotherapy at OSR. The samples will be collected at three timepoints (pre-treatment, on-treatment (after the first cycle), and after-treatment (in cases with presence of viable tumor cells in the post-treatment/surgical tissue specimen as Residual Disease). In particular, single-cell RNA sequencing (scRNA-seq) of TNBC biopsies will be performed, and the scRNA-seq data will be analyzed to characterize the dynamic molecular landscape of the tumor and immune system using the 10x Genomics platform (or similar technologies if it will be not available at the time of experiment).
Safety Criteria	The breast biopsy performed for study purposes does not pose any safety concerns, as it follows standard clinical procedures with minimal risk to participant and being minimally invasive

Efficacy Criteria	The potential clinical utility of on-treatment biopsy to further inform clinical management of the patient is an exploratory objective of this project
Sample Size	We plan to prospectively enroll 55 patients (20 TNBC and 35 Luminal BC). For the cohort of TNBC, we will collect biopsies from 20 patients at 3 different timepoints (baseline, after C1, surgery). For the cohort of Luminal BC, we expect to collect tissue samples from 35 patients at 3 different timepoints (baseline, after C1, surgery)
Statistical Design	Given the translational and exploratory nature of this study, which primarily aims to understand tumor–immune interactions and identify potential predictive biomarkers, a formal statistical plan and power calculation are not applicable. Descriptive statistics and exploratory analyses will be performed, applying appropriate methods to account for multiple testing where relevant. These analyses will include the identification of transcriptional programs, cellular states, and spatially resolved cellular distributions associated with treatment response. Multi-modal data integration approaches (e.g., clustering, dimensionality reduction, and machine-learning–based modeling) will be applied to derive candidate predictive signatures. Correlations between molecular, spatial, and clinical variables will be assessed to explore potential biological mechanisms of sensitivity or resistance to therapy. The resulting exploratory signatures or models will be evaluated for their association with treatment response and relevant clinical outcomes (pCR).
Duration of the Study	Duration of enrollment: From January 2026 to December 2028 Duration of treatment: about six months Duration of subject participation: 1 year Duration of total study period: 5 years

3. ABBREVIATIONS AND DEFINITIONS

3.1. Abbreviations.

CRF	Case Report Form
CRO	Contract Research Organization
CTC	Clinical Trial Center
DPIA	Data Protection Impact Assessment
EC	Ethics Committee
GCP	Good Clinical Practice
ICF	Informed Consent Form
ICH	International Conference on Harmonization
LVLP	Last Visit of Last Patient
OSR	Ospedale San Raffaele
PI	Principal Investigator
ADCs	Antibody-drug Conjugates
AIRC	Associazione Italiana per la Ricerca sul Cancro
BC	Breast Cancer
eBC	Early Breast Cancer
ER	Estrogen Receptor
HER2	Human Epidermal growth factor Receptor 2
pCR	Pathologic Complete Response
PgR	Progestinic Receptor
RD	Residual Disease
sTILs	Stromal Tumor-Infiltrating Lymphocytes
TNBC	Triple-Negative Breast Cancer
scRNA-seq	Single Cell RNA sequencing

3.2. Definitions

RD	Presence of viable tumor cells in the post-treatment tissue specimen
pCR	Absence of viable tumor cells

4. BACKGROUND AND RATIONALE

Over recent decades, prognosis and cure rates in early breast cancer (eBC) have improved due to new treatments, including immunotherapy, antibody-drug conjugates (ADCs), and targeted therapies such as CDK4/6 and PARP inhibitors. However, most were introduced empirically, often leading to unnecessary treatments and associated toxicities, while a significant proportion of patients still experience recurrence.

In triple-negative breast cancer (TNBC), pembrolizumab added to neoadjuvant chemotherapy (KEYNOTE-522)[1] increased pathological complete response (pCR) rates and event-free survival, now forming a standard of care. Patients without pCR may receive adjuvant capecitabine or continued immunotherapy, although optimal post-neoadjuvant strategies remain under investigation. For patients with germline BRCA1/2 mutations, adjuvant olaparib (OlympiA)[2] improves disease-free and overall survival, supporting PARP inhibitor–based tailored strategies in TNBC and high-risk luminal eBC (CPS+EG ≥3). In hormone receptor-positive (HR+) breast cancer, adding CDK4/6 inhibitors to adjuvant endocrine therapy (MonarchE, NATALEE)[3,4] improves invasive disease-free survival in high-risk patients. Ongoing studies also explore immunotherapy in the neoadjuvant setting (CheckMate-7FL, KEYNOTE-756)[5,6], reflecting a trend toward intensifying treatment in biologically high-risk luminal disease. ADCs, which selectively deliver cytotoxic agents to tumor cells, are being evaluated in both neoadjuvant and adjuvant settings. Trials such as TROPION-Breast03 and TROPION-Breast04 are investigating datopotamab deruxtecan in TNBC to optimize treatment intensity[7,8].

The dual challenge of avoiding overtreatment in low-risk patients while optimizing outcomes in high-risk patients has led to a growing emphasis on refining treatment strategies through biologic and molecular stratification. Prognostic and predictive information can be derived from pre-treatment and on-treatment core biopsies, as well as from residual disease (RD) after neoadjuvant therapy. In triple-negative breast cancer (TNBC), we already have data of an extensive molecular characterization of pre-treatment, on-treatment (after the first cycle of therapy) and post-treatment samples from patients enrolled in the NeoTRIP trial[9-12], a randomized Phase III trial that compared neoadjuvant carboplatin and nab-paclitaxel with or without the anti-PD-L1 atezolizumab[9]. These data showed that stromal tumor-infiltrating lymphocytes (sTILs) and several tumor and immune-related biomarkers were predictive of pathologic complete response (pCR) and RD. Particularly, response to therapy was best predicted by combining tissue features before and on-treatment, pointing to a role for early biopsies in guiding adaptive therapy. However, bulk mRNA expression does not consider intratumor heterogeneity in terms of single cell expression and spatial tissue organization. In a recent publication in Nature, we performed an analysis by imaging mass cytometry demonstrating the relevance of spatial organization and type and functional state of immune cells in precision immunology. Single-cell analysis by RNA-seq may contribute to better characterize these tumor and immune cell populations, providing insights into mechanisms of

response and resistance to therapy and guiding the development of the next generation of chemo/immune combinations[13,14]. In addition, in luminal breast cancer (ER+/HER2-) we have recently identified a distinct molecular subset characterized by high proliferation and low ER-related signaling with unique molecular features that may have important therapeutic implications[15]. In particular, biomarker analyses and clinical data suggest a potential role for immunotherapy in this group.

Given these data in the present study we will enroll a cohort of patients with stage II-III early triple negative breast cancer, candidate to receive neoadjuvant chemo-immunotherapy and a cohort of patients with High-risk luminal (ER positive/HER2 negative) breast cancer with high-risk features (Grade 3, PR-negative, high proliferation, high TILS).The aim is to identify in these specific populations of patients useful predictive and prognostic biomarkers potentially useful in clinical practice to tailor therapy dynamically based on individual tumor biology and response, balancing escalation and de-escalation of therapies.

We plan to prospectively enroll a total of 55 patients, comprising 20 patients with TNBC and 35 patients with high-risk luminal breast cancer. For the cohort of TNBC, we will collect biopsies from 20 patients at 3 different timepoints (baseline, after C1, surgery). For the high-risk luminal cohort, we expect to collect tissue samples from 35 patients at 3 different timepoints (baseline, after C1, surgery)

5. RISK/BENEFIT ASSESSMENT

This is an interventional clinical study in which all diagnostic and therapeutic procedures are part of standard clinical practice, except for one additional research-related procedure: an on-treatment tumor biopsy. The study therefore carries minimal incremental risk for participants

5.1. Known Potential Risks

The only additional procedure beyond routine clinical management is the on-treatment tumor biopsy, performed for translational research purposes. Potential risks are therefore limited to those commonly associated with standard biopsy procedures, as reported in the literature and routine clinical practice.

- Immediate risks: Local pain, mild bleeding, bruising, and risk of infection at the biopsy site. In rare cases, more significant complications such as hematoma or delayed wound healing may occur. These risks are minimized by performing the biopsy under sterile conditions by experienced personnel, with appropriate post-procedural monitoring.
- Long-term risks: Long-term adverse effects are not expected. Scarring at the biopsy site may persist but is usually minimal and clinically insignificant.

- Alternative procedures: No less invasive alternatives could provide the same type of tissue material necessary for the planned molecular and histopathological analyses. Peripheral blood or imaging data cannot fully replace the biological information derived from tumor tissue. Therefore, the on-treatment biopsy is justified as the only additional procedure needed to achieve the study objectives.

5.2. Known Potential Benefits

Participants are not expected to derive direct therapeutic benefit from study participation, as the protocol does not modify clinical treatment or management and the purpose is translational and exploratory. However, there are potential indirect benefits for participants:

- Immediate potential benefits: participants may receive additional clinical assessments and closer monitoring at the time of biopsy, which may incidentally contribute to improved documentation of treatment response.
- Long-term potential benefits: The information obtained from the molecular and histopathological analyses of the on-treatment biopsy may improve scientific understanding of treatment response and resistance mechanisms. These results could contribute to the development of more effective and personalized therapeutic strategies of early escalation/descalation for future patients with the same disease.

5.3. Assessment of Risks and Benefits ratio

The study exposes participants only to the minimal incremental risk associated with one additional biopsy, a procedure commonly performed in oncology with a low complication rate. All reasonable measures have been implemented to minimize risk, including strict adherence to sterile technique and safety procedures, performance by trained medical staff, exclusion of patients for whom biopsy poses a medical contraindication, and immediate post-biopsy clinical observation.

Given the limited and well-controlled risk, and the high potential value of the biological data to advance knowledge on treatment mechanisms, the overall risk-benefit ratio is considered favorable. The anticipated scientific and societal benefits clearly outweigh the minimal additional risk to individual participants.

6. OBJECTIVES AND ENDPOINTS

Primary Objective	Primary Endpoint	Timepoints

To characterize the dynamic changes in tumor and immune cell populations in TNBC and to prospectively collect serial samples from high-risk luminal (ER+/HER2-) breast cancer patients	Gene expression levels per single-cell and relative cell clustering (%)	Pre-treatment, on-treatment (after first cycle of therapy), and post-treatment (surgery or residual disease)
Exploratory Objectives To correlate single-cell gene expression data with pCR and RD to identify predictive signatures of therapy response	**Other** pCR and RD	**Timepoints** Across all collected timepoints

7. STUDY DESIGN

This exploratory interventional translational study investigates early breast cancer (EBC), focusing on triple-negative (TNBC) and high-risk luminal (ER+/HER2-) subtypes. By integrating single-cell RNA sequencing and spatial imaging analyses from serial tumor biopsies, the project aims to characterize tumor–immune interactions and identify predictive biomarkers of response or resistance to neoadjuvant chemo-immunotherapy[9-12].

The hypothesis of this study is that early molecular and spatial features identified at the single-cell level can predict therapeutic response or resistance in patients with early breast cancer, enabling adaptive, personalized treatment strategies that minimize overtreatment while maximizing efficacy. To test this hypothesis, we will conduct a translational study with a prospective longitudinal cohort design. The study is based on the existing retrospective experience with TNBC samples from the NeoTRIP trial[9]. Moreover we will prospectively collecte serial tumor samples from high-risk luminal (ER+/HER2-) patients supported by the evidences already reported in the background. Two main patient cohorts will be included, TNBC and high-risk luminal breast cancer, and each participant will be followed from baseline (pre-treatment biopsy) through on-treatment (after the first cycle) to surgery (post-treatment or residual disease). The expected duration of participant involvement is approximately six months, according to the neoadjuvant treatment schedule. The study is monocentric, conducted at Ospedale San Raffaele, with collaborative contributions from SR-TIGET and Cambridge University for advanced single-cell and spatial analyses. No formal interim analysis is planned

due to the exploratory and hypothesis-generating nature of the study, and data will be analyzed descriptively, with integrative analyses performed as samples become available. Data will be collected through serial tumor biopsies processed for single-cell RNA sequencing and spatial imaging technologies, and clinical and pathological outcomes including pCR (defined as absence of tumor infiltrating cells in the surgical sample) and residual disease status (defined as presence of viable tumor cells in the post-treatment tissue/surgical specimenwill be recorded from medical records. This approach allows high-resolution, multi-modal characterization of tumor and immune landscapes and is essential to address the study objectives. Any future research on previously stored biological material will require approval by the Ethics Committee prior to initiation, in accordance with local and international regulations.

7.1. Study duration

Duration of enrollment: 3 years
Duration of treatment: about six months
Duration of total follow-up: one year
Duration of total study period: 5 years

8. STUDY POPULATION

8.1. Study Participants

We plan to prospectively enroll a total of 55 patients, comprising 20 patients with TNBC and 35 patients with high-risk luminal breast cancer. For the cohort of TNBC, we will collect biopsies from 20 patients at 3 different timepoints (baseline, after C1, surgery). For the high-risk luminal cohort, we expect to collect tissue samples from 35 patients at 3 different timepoints (baseline, after C1, surgery)

8.2 Inclusion Criteria

Patients eligible for the present study would have the following inclusion criteria:

1. Female patients aged ≥18 years.
2. ECOG performance status 0–1.
3. Histologically confirmed early breast cancer with one of the following molecular profiles:
   - TNBC: ER and PR *negative* (IHC <10%) and HER2 *negative* (IHC 0–1+ or FISH non-amplified).
   - High-risk luminal (ER+/HER2-): ER *positive* (IHC ≥10%) HER2 *negative* (IHC 0–1+ or FISH non-amplified), with high-risk features (e.g., Grade 3, PR-negative, high proliferation, high TILS).
4. Clinical indication for neoadjuvant treatment according to standard practice:
   - TNBC: cT1c and/or cN positive, or cT2 (>2 cm) and/or cN positive (stage II, III).

- High-risk luminal: features as defined above (Grade 3, PR-negative, high proliferation, ER low).

5. Ability to understand and sign written informed consent for participation in the study, approved by the local Ethics Committee.

8.3 Exclusion Criteria

Patients will be excluded from the study if they meet one or more of the following criteria:

1. HER2-positive tumors.
2. Multifocal tumors — exclusion if a single index lesion cannot be identified and sampled; otherwise allowed if a representative lesion can be biopsied.
3. Known metastatic disease.
4 Clinical contraindications to the planned neoadjuvant therapy.
5. Decision for upfront surgery as determined by the multidisciplinary team.
6. Inability to provide informed consent.
7. Pregnancy or breastfeeding.
8. Prior systemic therapy (chemotherapy, immunotherapy, or endocrine therapy) for the current breast cancer before baseline biopsy
9. On-treatment biopsy clinically not feasible or controindicated

8.4 Screening Failures

Participants who provide written informed consent to participate in the study but are found not to meet one or more eligibility criteria during the screening procedures will be considered screen failures. For transparency and accurate reporting, minimal information will be collected for all screen failure participants, including demographic data, details of the screening process, and the specific eligibility criteria that were not met. This information will help ensure proper documentation of the study population and support the interpretation of study results.

9. STUDY INTERVENTIONS

Patients will receive standard-of-care neoadjuvant chemotherapy according to sequential anthracycline and taxane regimens as per standard guidelines. In addition, patients with triple-negative breast cancer (TNBC) will receive immunotherapy with pembrolizumab as per clinical practice (as the KEYNOTE-522 schema). The prescription of these treatments is entirely independent of the decision to include the patient in the study, and all medications are used within their approved indications and as part of routine clinical practice. The study includes one additional research-related procedure (the on-treatment biopsy after C1D1) beyond standard

clinical practice: an on-treatment tumor biopsy. No difficulties are anticipated in performing the on-treatment biopsy; however, should the procedure not be feasible for any reason, the patient will be considered a screen failure.
All therapeutic indications and diagnostic assessments, including baseline imaging, laboratory tests, and follow-up evaluations, are part of the patient's routine clinical management and are conducted according to institutional guidelines and the treating physician's discretion.

Follow-up visits correspond to routine care or national and international guideline requirements.

9.1. Intervention description

The on-treatment biopsy consists of a tissue sampling performed during the ongoing systemic therapy, after one cycle of neoadjuvant therapy. The procedure will be carried out under local anesthesia by experienced clinicians, using standard sterile techniques and imaging guidance if required (e.g., ultrasound). No comparator, placebo, or sham procedure is foreseen. The study is non-randomized and all patients continue to receive standard-of-care therapy as per clinical indications.

9.2. Treatment Schedule

Patients will receive standard-of-care neoadjuvant chemotherapy according to sequential anthracycline and taxane regimens as per standard guidelines. In addition, patients with triple-negative breast cancer (TNBC) will receive immunotherapy with pembrolizumab as per clinical practice (as the KEYNOTE-522 schema).

9.3. Method for Assigning Subjects to Treatment/Intervention Groups

Not applicable

9.4. Blinding of treatment/procedure

Not applicable

9.5. Preparation, Administration and Accountability of the study Treatments

This study does not include the administration of any investigational medicinal product or experimental therapy. All systemic anticancer treatments are prescribed and administered according to standard clinical practice and current institutional or national guidelines. The treating physician remains fully responsible for therapeutic decisions and management.

9.6. Compliance with study treatment

Not applicable

9.7. Concomitant Medication

Information regarding concomitant medications will be collected at baseline and updated at each relevant study visit . The following data will be recorded in the source documents and Case Report Forms (CRFs):

- name of the medication or therapy,
- indication and therapeutic class,
- route of administration,
- start and stop dates,
- dosage and frequency, and
- whether the treatment was ongoing at the time of the biopsy.

All standard-of-care treatments are permitted. No specific restrictions apply, provided that clinical conditions allow the safe performance of the on-treatment biopsy.
Medications that may interfere with coagulation (e.g., anticoagulants, antiplatelet agents, non-steroidal anti-inflammatory drugs) will be carefully evaluated prior to the biopsy procedure; temporary discontinuation may be requested in accordance with institutional safety policies to minimize bleeding risk.

9.8 Other Interventions

Not Applicable

10. EFFICACY AND SAFETY CRITERIA

The study does not evaluate therapeutic efficacy in terms of clinical outcomes, since systemic treatments follow standard-of-care protocols. The objective of this project is exploratory and translational in nature.

10.1. Efficacy Criteria

The efficacy of the study procedure will be defined in relation to the technical and biological success of the on-treatment biopsy (.especially in terms of sample adequacy)
No clinical efficacy endpoints are foreseen.

10.2.Safety Criteria

Safety evaluation will focus on potential adverse events (AEs) related to the biopsy procedure, since no investigational drug or device is used in this study. All AEs will be collected from the time of the biopsy until the next scheduled clinical evaluation or up to 30 days post-procedure.

11. STUDY PROCEDURES

Procedures / Day	Enrollment	Screening	Baseline	C1	Biopsiy post C1D1	C2-C8**	Surgery	Final Study Visit
		-28 (+/-3)	0 (-5)	0 (+/-2)	3-18 (+/-2)	Q3W (+/-2)	+168 (+/-56)	+190 (+15)
Informed consent	R							
Demographics	C							
Medical history	C							
Eligibility assessment	R							
Pregnancy test		C				C	C	C
Physical examination (including height and weight)	C	C	C	C		C	C	C
Vital signs	C	C	C	C		C	C	C
Performance status	C	C	C	C		C	C	C
Laboratory test (hematology and Biochemistry)		C	C	C		C	C	C
Study procedure: biopsy	C*				R		C*	
Radiologic/Imaging assessment (breast ultrasound and/or mammography and/or breast MRI)		C	C			C	C	
Adverse event assessments	C	C	C	C		C	C	C

*Done for clinical practice

**As per clinical practice

C= clinical standard of care activities; R: research activities (activities done only for the participation in the study)

11.1. Informed Consent

All participants will provide written informed consent prior to any study-specific procedures or data collection, in compliance with ICH GCP and applicable regulations. The investigator or a designated team member will explain the study objectives, procedures, potential risks and benefits, and participants' rights in terms they can understand, answering any questions. Participants will have time to review the consent form, discuss it with family or surrogates, and consider their decision. Participation is voluntary, and participants may withdraw at any time without affecting their medical care. Written consent, including the date, will be documented in the source records, and a copy of the signed form will be given to the participant.

11.2. Subject Recruitment and Screening

Participants will be identified through outpatient clinics at Ospedale San Raffaele and approached by the study team. Screening will include verification of inclusion/exclusion criteria, review of medical history, concomitant medications, physical examination, performance status (ECOG), and relevant laboratory or diagnostic tests. Recruitment is expected to be completed over approximately 36 months, with all participants enrolled at the single study site.

11.3. Subject Identification

Participants are considered enrolled once informed consent is signed by the patient or their legal representative. Each participant will receive a unique study code (e.g., ON_TN_XX; where ON_TN is the abbreviation of the Protocol Code, XX is the progressive subject number in increasing order starting from 01) for tracking, and a confidential list linking codes to identities will be maintained at the study site.

11.4 Randomization and Blinding

Not applicable

11.5 Baseline Assessments

At baseline, all participants will undergo assessments necessary to meet the study objectives and endpoints. These include verification of eligibility criteria, medical history, physical examination, performance status (ECOG), laboratory tests, imaging as indicated, and collection of tumor tissue for molecular and immune profiling. Baseline biopsies will be obtained prior to the initiation of neoadjuvant therapy, as per standard care.

11.6 Visits and Follow Up

Participants will be evaluated at defined study visits:

Visit 0 (Enrollment): Screening, informed consent, eligibility confirmation, medical history, physical examination
Neoadjuvant Phase: Clinical evaluation, on-treatment tumor biopsy for molecular analyses after first cycle of standard therapy
Surgery: Clinical assessment
Final study visit (20-45 days after surgery): As per standard clinical practice, participants will be followed for safety and outcome assessments. Clinical evaluation, collection of surgical specimen, evaluation of pCR.

11.7 Definition of End of Study

According to the study design, the end of the study is defined as the date on which the last patient completes the last visit.

11.8 Premature termination or suspension of a study

This study may be temporarily suspended or prematurely terminated by the Principal Investigator or the authorized Sponsor representative if there is reasonable cause. Possible reasons include insufficient compliance with protocol requirements, incomplete or unevaluable data, determination that the primary endpoint has been met, or futility of the study. In such cases, written notification documenting the reason will be provided to study participants, the Ethics Committee, and relevant regulatory authorities

12 DISCONTINUATION AND WITHDRAWAL

Participants are free to withdraw from the study at any time, for any reason, without any impact on their medical care.

12.1 Discontinuation of study Intervention

Discontinuation from <study intervention> does not mean discontinuation from the study, and remaining study procedures should be completed as indicated by the study protocol. This may happen for a number of reasons, including but not limited to:

The occurrence of what the participant perceives as an intolerable AE.

Inability to comply with study procedures

Participant decision

Participants may choose to stop treatment and/or study assessments but may remain on study follow-up.

If a clinically significant finding is identified (including, but not limited to changes from baseline) after enrollment, the investigator or qualified designee will determine if any change in participant management is needed. Any new clinically relevant finding will be reported as an adverse event (AE).

13 SAMPLE HANDLING

All samples collected in this study consist exclusively of tumor tissue and are obtained as part of routine clinical practice (baseline tumor biopsy or surgical sample) or as per protocol (on treatment biopsy). All tissue samples used for research analyses, including molecular and immune profiling, are fully integrated into the participants' clinical management. After the routine analysis of the Pathologic Unit, only the necessary tumor material from biopsies for the experiment will be transferred to CRB for temporaneal storage and will be completely used for analysis of the study by SR-tiget No residual material is expected to remain. In the event of any leftovers, it will be destroyed by our collaborator.

The SR-TIGET will performing single-cell RNA-seq of TNBC specimens obtained on pre-treatment, on-treatment and after-treatment (in cases with RD) from patients receiving neoadjuvant chemo-immunotherapy at OSR.

The laboratory of Raza Ali, in Cambridge UK will characterizing the comprehensive tumor/immune molecular landscape dynamic by analyzing scRNA-seq and integrating these information with those already available and in particular with spatially resolved single cell analysis. They will not receive any biological material from tumor biopsies, but only raw experimental data obtained by single-cell RNA-seq.

14 PATIENT SAFETY

All procedures performed in this study are part of standard clinical practice. No additional interventions or experimental treatments are being introduced beyond routine care; therefore, no additional safety risks are anticipated for study participants.

14.1 Safety profile of the procedure

Safety evaluation will focus on potential adverse events (AEs) related to the biopsy procedure, since no investigational drug or device is used in this study.

14.2 Adverse Event Definitions

Adverse Event (AE)	Any untoward medical occurrence in a patient or clinical investigation subject treated with the investigational treatment and which does not necessarily have a causal relationship with this treatment. An adverse event (AE) can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the treatment, whether or not related to the tratment. Reference: adapted from GCP ICH E6(R2)
Serious Adverse Event (SAE)	Any untoward medical occurrence that at any dose: – results in death, – is life-threatening, – requires inpatient hospitalization or prolongation of existing hospitalization, – results in persistent or significant disability/incapacity, or – is a congenital anomaly/birth defect. Reference: GCP ICH E6(R2)
Adverse Event of Special Interest AESI	An adverse event of special interest (serious or non-serious) is one of scientific and medical concern specific to the sponsor's product or program, for which ongoing monitoring and rapid communication by the investigator to the sponsor may be appropriate. Such events may require further investigation in order to characterize and understand them. Depending on the nature of the event, rapid communication by the study sponsor to other parties may also be needed (e.g., regulators). Reference: CIOMS VI
The AEs will be coded using the current version of Medical Dictionary for Regulatory Activities (MedDRA; refer to https://www.meddra.org).	

14.3 Adverse Event Severity Grading Scale

Grade 1	Mild	Asymptomatic or mild symptoms; clinical or diagnostic observations only; no intervention indicated
Grade 2	Moderate	Minimal, local, or noninvasive intervention indicated; limiting age-appropriate instrumental activities of daily living (ADL)
Grade 3	Severe	Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self-care ADL
Grade 4	Life-threatening	Life-threatening consequences; urgent intervention indicated
Grade 5	Death	Death related to AE
Reference: Common Terminology Criteria for Adverse Events (CTCAE) version 5.0		

14.4 AE Attribution Scale

RELATIONSHIP	ATTRIBUTION	DESCRIPTION
	Unrelated	The AE is ***clearly NOT*** related to the intervention.

Unrelated to investigational agent/intervention	Unlikely	The AE is ***doubtfully related*** to the intervention.
Related to investigational agent/intervention	Possible	The AE ***may be related*** to the intervention.
	Probable	The AE is ***likely related*** to the intervention.
	Definite	The AE is ***clearly related*** to the intervention.
Reference: NCI Guidelines: Adverse Event Reporting Requirements		

14.5 Reporting procedures for AE

Given that the only study-specific procedure is an on-treatment biopsy — a well-established diagnostic technique with a well-known and limited safety profile — a risk-adapted approach will be adopted for AE reporting. Only biopsy-related adverse events (AEs) will be collected and reported in the study Case Report Form (CRF).
Routine, non-related AEs associated with anticancer treatment or underlying disease will not be systematically recorded, as they are considered part of standard clinical management and will already be documented in the patient's medical record.

14.6 Reporting Procedures for SAE/ AESI

All Serious Adverse Events (SAEs) deemed related or possibly related to the on-treatment biopsy must be reported promptly to the Sponsor/Authorized Representative and the Ethics Committee, following institutional and regulatory timelines. Examples of biopsy-related SAEs that require immediate reporting include:

- severe bleeding requiring transfusion or surgical intervention,
- biopsy-related infection requiring hospitalization or intravenous antibiotics,
- any event leading to prolonged hospitalization, permanent damage, or death.

SAEs clearly attributable to the underlying malignancy or standard anticancer therapy (e.g., disease progression, treatment-related toxicities, hospitalization for chemotherapy complications) will not be reported as study SAEs, as they are outside the scope of this protocol.

No AESIs have been predefined for this study, as no investigational product is used and the procedure's risk profile is well established.

14.7 Follow-up of AE / SAE / AESI

The Investigator should take all appropriate measures to ensure the safety of the patients. Notably, they should follow up on the outcome of any event (clinical signs, laboratory values, etc.) until resolution or until the event is considered stable.
In the case of any Serious Adverse Event, the patient must be followed up until clinical recovery is complete and laboratory results have returned to normal, or until progression has been stabilized. This may imply that follow-up will continue after the patient has left the study and that the Ethics Committee may request additional investigations.

14.8 Unblinding Procedures

Not applicable

15 DATA MANAGEMENT

15.1 Definition of source data and source documents

In this study, source documents consist exclusively of the participants' standard clinical records, including hospital charts and medical files, which are used to evaluate patient history and therapies received. The only additional study-specific source documents is the on-treatment biopsy procedure report. All data recorded in the Case Report Forms (CRFs) will be derived from these clinical records. Patient screen failures will also be documented in the source records, including the reason for ineligibility.

15.2 Documentation of data in Case Report Forms (CRFs)

All relevant study data for enrolled participants will be recorded in the CRFs (RedCap) by the principal investigator or an authorized delegate as soon as possible after collection. The investigator will verify completeness, accuracy, and compliance with ICH guidelines and institutional SOPs, signing and dating each CRF. Any missing data will be explained. Completed CRFs will remain at the study site, and for electronic CRFs, a copy of all pages will be securely stored locally.

15.3 Data Recording and Record Keeping

The investigator shall arrange for the retention of Essential Documents for the Conduct of a Clinical study (e.g., patient files, other source data, and the Trial Master File/Investigator Site File) after the completion or discontinuation of the study according to institutional procedures and applicable laws.

15.4 Data Protection

The Investigator undertakes to:
- use the data only for the foreseen analyses and within the limits established by the study and approved by the competent EC;
- store data in a secure network system;
- prohibit unauthorised third parties from accessing, even partially, data.
- guarantees to limit access to and processing of data only to its employees and collaborators who, upon appointment as an authorized person:
    1. need to process the data to carry out their work in relation to the study;
    2. have undertaken to maintain the confidentiality of the Data and any information deriving from it or that is communicated to them.

If, within the context of the Study, the Investigator needs to make use of its own suppliers, the latter undertakes to appoint these subjects as Data Processors/Responsabili dei Trattamenti with a specific agreement or other legal act suitable for this, before the start of any data processing by them, according to Regulation (EU) n. 2016/679, art. 28.
The Investigator also undertakes to adopt suitable measures to facilitate the exercise of the rights of the subject provided for by Regulation (EU) n. 2016/679, art. 15 – 22, including the rights of access, rectification, cancellation, limitation, opposition, and portability, within 30 days of receiving the relevant request. The Investigator will communicate the data outside OSR (the Data Controller/Titolare) in pseudonymized form, the Investigator will refrain from carrying out any activity that could identify the subjects to whom such data refer. In the event, however, that the data cannot be communicated in pseudonymized form by the Data Controller, the Investigator undertakes to adopt all necessary security and organizational measures aimed at protecting the confidentiality of the Data Subject.

16 STATISTICS

16.1 Given the translational and exploratory nature of this study, which is primarily focused on understanding the interactions between biology and clinical outcomes and on identifying potential predictive biomarkers employing high-throughput molecular and immunological profiling approaches, a formal statistical plan and power calculation are not applicable. The study is not designed for confirmatory hypothesis testing but for hypothesis generating. The number of participants has been defined to ensure scientific validity in high-throughput assays as those described, while minimizing patient burden and the use of biological material, in accordance with the principles of proportionality and ethical research conduct.Description of Statistical Methods

Given the exploratory and translational nature of this study, which focuses on understanding tumor and immune dynamics and identifying potential predictive biomarkers, no formal

sample size calculation or hypothesis testing will be performed. Descriptive statistics will be used to summarize patient characteristics, sample collection, and molecular/immune profiling results. Continous variables will be described using mean, median, range, and standard deviation, while categorical variables using frequency and percentage. To test association between categorical variables, chi-squared test will be applied while differences in continuous variables will be evaluated between different timepoints with t-test or Wilcoxon-Mann-Whitney, as appropriatewith adjustments for multiple testing where relevant. Endpoints will be assessed at baseline, on-treatment, and at surgery, according to the time points specified in the study design. All analyses will be conducted using validated statistical software andall statistical tests, $p < 0.05$ will be considered statistically significant.

16.2 Sample Size Determination

The sample size has been determined based on practical considerations and the objectives of generating hypothesis-generating data.We plan to prospectively enroll a total of 55 patients, including 20 with TNBC and 35 with high-risk luminal breast cancer to conduct an exploratory proof-of-concept study. Based on the expected pCR rate at surgery and the early response to treatment at cycle 1, This project will leverage collaborations with Cambridge University (UK) for imaging mass cytometry (IMC) analyses and SR-TIGET for single cell-RNA sequencing experiments, which may allow expansion of the case series. Descriptive statistics and exploratory analyses will be conducted, applying appropriate methods to account for multiple testing and to summarize patient characteristics and molecular data.

All patients data and biological samples that need to be shared with collaborators will be pseudo-anonymized according to Institutional procedures and will be shared only after a specific agreement between San Raffaele Hospital and the research center.

16.3 Analysis Populations

All enrolled participants who meet inclusion and exclusion criteria and provide tissue samples at the defined time points will be included in the analyses. Participants who withdraw consent or whose samples are unsuitable will be excluded from molecular and immune profiling, but will remain in descriptive summaries of patient characteristics. No additional selection criteria will be applied.

This study is exploratory and primarily descriptive. Analyses will summarize patient characteristics, sample collection, and molecular/immune profiling results using descriptive statistics. Exploratory analyses of primary and secondary endpoints will be conducted with appropriate statistical methods, including adjustments for multiple testing. No formal hypothesis testing or sample size calculation will be performed, reflecting the translational nature of the study.

16.4 Interim Analysis

Not applicable

16.5 Stopping Rules

Not applicable

17 ETHICAL AND REGULATORY CONSIDERATIONS

This clinical study will be conducted in accordance with the principles laid down by the 18th World Medical Assembly (Helsinki, 1964) and all applicable amendments established by the World Medical Assemblies, and the ICH guidelines for Good Clinical Practice.
This clinical study will be conducted in compliance with all international laws and regulations; national laws and regulations of the country in which the clinical study is performed; as well as any other applicable guidelines.

17.1 Responsibilities of the Investigator(s)

The Investigator(s) undertake(s) the responsibility to perform the study in accordance with this Protocol, Good Clinical Practice, and the applicable regulatory requirements. The Investigator is required to ensure compliance with the investigational product schedule, visits schedule, and procedures required by the protocol. The Investigator agrees to provide all information requested in the Case Report Form (CRF) in an accurate and legible manner. The investigator may implement a deviation from, or a change of, the protocol to eliminate an immediate hazard(s) to study subjects without prior EC approval/favorable opinion. As soon as possible, the implemented deviation or change, the reasons for it, and, if appropriate, the proposed protocol amendment(s) should be submitted. The investigator must have available an adequate number of qualified staff and adequate facilities for the foreseen duration of the study to conduct the study properly and safely.

17.2 Ethics Committee (EC) Approvals

This clinical study protocol as well as the Informed Consent are to be submitted to the appropriate Ethics Committee, and it is mandatory to obtain the written and dated approval, signed by the chairman with Ethics Committee(s) composition.
The clinical study the documents reviewed, the list of voting members and their qualifications, and the date of the review should be clearly stated on the written Ethics Committee approval.

17.3 Other Ethical Considerations

NA

18 GENDER MEDICINE IN RESEARCH PROTOCOL

This study is focused on early breast cancer, a condition that predominantly affects women; therefore, sex- and gender-based analyses are not applicable in this context.

19 QUALITY ASSURANCE AND QUALITY CONTROL

19.1 Monitoring

Monitor Regular monitoring will be performed, according to the Monitoring Plan. Data will be evaluated for compliance with the protocol and accuracy in relation to source documents. Following written standard operating procedures, the monitors will verify that the clinical study is conducted and data are generated, documented, and reported in compliance with the protocol, GCP, and the applicable regulatory requirements.

19.2 Deviation from study protocol

A deviation from the protocol is an unintended departure from the procedures or processes described in the protocol and approved by the EC.
The Investigator or designee must document and explain in the patient's source documentation any deviation from the approved protocol.

19.3 Data and Safety Monitoring Board

NA

19.4 DSMB roles and responsibilities

NA

20 FINANCE AND INSURANCE

20.1 Funding

This is a non-profit study aimed at advancing translational research in early breast cancer. Financial support for the study is provided by the "Eredità Buffa" fund, which contributes to the conduct of molecular and immune profiling analyses and other research-related activities. No commercial sponsor is involved, and the study is not intended for profit. All resources will be used solely to achieve the scientific objectives described in the protocol.

20.2 Patient Insurance

A specific insurance certificate will be issued to provide coverage for the on-treatment biopsy procedure..

21 END OF CLINICAL STUDY

In accordance with applicable regulation, ICH GCP and SOPs, the PI shall notify the end of the clinical study within 15 days from the end of the clinical study and the reasons for such action.

21.1 Summary of the results of the clinical study

Irrespective of the outcome of a clinical study, within one year from the end of a clinical study, the PI shall submit a summary of the results of the clinical study.

22 INTELLECTUAL PROPERTY

IRCCS San Raffaele Hospital and the Principal Investigator is the sole owner of data, without prejudice to the provisions of the regulations in force regarding the publication of the data.

23 PUBLICATION POLICY

Study results will be disseminated through peer-reviewed scientific journals and presentations at national and international conferences. Authorship will follow the guidelines of the International Committee of Medical Journal Editors (ICMJE), with contributors acknowledged appropriately based on their level of involvement. All manuscripts or abstracts derived from the study will undergo internal review by the principal investigator and relevant collaborators prior to submission.

24 REFERENCES

1. Schmid P, Cortes J, Pusztai L, et al. Pembrolizumab for Early Triple-Negative Breast Cancer. N Engl J Med. 2020;382:810-821. doi:10.1056/NEJMoa1910549.
2. Tutt ANJ, Garber JE, Kaufman B, et al. Adjuvant Olaparib for Patients with BRCA1- or BRCA2-Mutated Breast Cancer. N Engl J Med. 2021;384:2394-2405. doi:10.1056/NEJMoa2105215.
3. Johnston SRD, Harbeck N, Hegg R, et al. Abemaciclib Combined With Endocrine Therapy for the Adjuvant Treatment of HR+, HER2-, Node-Positive, High-Risk, Early Breast Cancer (monarchE). J Clin Oncol. 2020;38:3987-3998. doi:10.1200/JCO.20.02514.
4. Hortobagyi GN, Lacko A, Sohn J, et al. A phase III trial of adjuvant ribociclib plus endocrine therapy versus endocrine therapy alone in patients with HR-positive/HER2-negative early breast cancer: final invasive disease-free survival results from the NATALEE trial. Ann Oncol. 2025;36:149-157. doi:10.1016/j.annonc.2024.10.015.

5. Loi S, Salgado R, Curigliano G, et al. Neoadjuvant nivolumab and chemotherapy in early estrogen receptor-positive breast cancer: a randomized phase 3 trial. Nat Med. 2025;31:433-441. doi:10.1038/s41591-024-03414-8.
6. Cardoso F, O'Shaughnessy J, Liu Z, et al. Pembrolizumab and chemotherapy in high-risk, early-stage, ER+/HER2- breast cancer: a randomized phase 3 trial. Nat Med. 2025;31:442-448. doi:10.1038/s41591-024-03415-7.
7. Bardia A, Pusztai L, Albain K, et al. TROPION-Breast03: a randomized phase III global trial of datopotamab deruxtecan ± durvalumab in patients with triple-negative breast cancer and residual invasive disease at surgical resection after neoadjuvant therapy. Ther Adv Med Oncol. 2024;16:17588359241248336. doi:10.1177/17588359241248336.
8. McArthur HL, Tolaney SM, Dent R, et al. TROPION-Breast04: a randomized phase III study of neoadjuvant datopotamab deruxtecan (Dato-DXd) plus durvalumab followed by adjuvant durvalumab versus standard of care in patients with treatment-naïve early-stage triple negative or HR-low/HER2- breast cancer. Ther Adv Med Oncol. 2025;17:17588359251316176. doi:10.1177/17588359251316176.
9. Bianchini G, Viale G, Dugo M, et al. Analysis of event-free survival (EFS) of stromal tumor infiltrating lymphocytes (sTILs), PD-L1 expression, and their early dynamics in the NeoTRIP trial. JCO. 2025.
10. Licata L, Mariani M, Rossari F, et al. Tissue- and liquid biopsy-based biomarkers for immunotherapy in breast cancer. Breast. 2023;69:330-341. doi:10.1016/j.breast.2023.03.014.
11. Gianni L, Huang CS, Egle D, et al. Pathologic complete response (pCR) to neoadjuvant treatment with or without atezolizumab in triple-negative, early high-risk and locally advanced breast cancer: NeoTRIP Michelangelo randomized study. Ann Oncol. 2022;33(5):534-543. doi:10.1016/j.annonc.2022.02.004.
12. Bianchini G, Huang CS, Egle D, et al. LBA13 Tumour infiltrating lymphocytes (TILs), PD-L1 expression and their dynamics in the NeoTRIPaPDL1 trial. Ann Oncol. 2020;31:S1145-S1146.
13. Wang XQ, Danenberg E, Huang CS, et al. Spatial predictors of immunotherapy response in triple-negative breast cancer. Nature. 2023;621:868-876. doi:10.1038/s41586-023-06498-3.
14. Dugo M, Huang CS, Egle D, et al. The Immune-Related 27-Gene Signature DetermaIO Predicts Response to Neoadjuvant Atezolizumab plus Chemotherapy in Triple-Negative Breast Cancer. Clin Cancer Res. 2024;30(21):4900-4909. doi:10.1158/1078-0432.CCR-24-0149.
15. Licata L, Barreca M, Galbardi B, et al. Breast cancers with high proliferation and low ER-related signalling have poor prognosis and unique molecular features with implications for therapy. Br J Cancer. 2023;129:2025-2033. doi:10.1038/s41416-023-02477-7.

25 APPENDIX A: AMENDMENT HISTORY

Not Applicable

26 APPENDIX B: LIST OF CLINICAL SERVICES / LABORATORIES

Name	Institution	Responsible person	Activity/service	Notes
Medical Oncology	OSR	Dugo Matteo, Galbardi Barbara	Biostatistician, elaboration of data	
Cambrige Cruk UK	Cambrige Cruk UK	Raza Ali	External collaborators, spatial trascriptomic analysis, elaboration of data	
Medical Oncology	OSR, UniSR	Bianchini Giampaolo, Naldini Matteo Maria	Medical Oncology Support in accrual, elaboration of data	
SR-TIGET	OSR	Naldini Matteo Maria	scRNA-seq experiments	