Official Title:

Dissecting the Role of miR-3916 and miR-3613-5p in Breast Cancer and Developing a Metastases Predictor PORTENT Algorithm

This document is a translation from the original Italian Clinical Study Protocol and Statistical Analysis Plan.

Ethical Committee Approvals

Latest Approval from Ethical Commettee: December 19th, 2024

(Original documents available in Italian only)

Study Protocol and Statistical Plan

1. INTRODUCTION

1.1 State of the Art

Metastasis development is the leading cause of mortality in female breast cancer patients. In clinical practice, prognostic markers based on immunohistochemical evaluation and certain expression profiles are used. These biomarkers are highly relevant for breast cancer subtyping and guiding therapy but are less effective in identifying the risk of disease progression. Indeed, about 30% of patients will develop metastases regardless of tumor subtype (1-3).

1.2 Rationale

MicroRNAs (miRNAs) are small non-coding RNAs now recognized for their active involvement in tumor development and progression (4-9). In previous studies using an internal cohort (CSS_1 cohort), we identified two miRNAs, miR-3916 and miR-3613-5p, differentially expressed in tumors that developed distant metastases within 5 years of follow-up compared to disease-free cases over the same period. Furthermore, including expression levels of these two miRNAs significantly improved the performance of a multivariate logistic regression model that incorporates currently used clinical prognostic markers. Specifically, the clinical model's AUC (95% CI) improved from 0.76 (0.68-0.84) to 0.85 (0.79-0.91) (delta AUC (p-value) = 0.001, DeLong test) when adding the combined logarithmic expression values of miR-3916 and miR-3613-5p. Based on these data, we developed an algorithm that integrates clinicopathological data and miRNA expression to assess the risk of metastasis development in women with breast cancer (PORTENT → Prediction Of bReasT cancEr distaNt meTastases).

The predictive multivariate logistic model is as follows:

Linear Predictor (LP) = -2.498 + (b1×V1) + (b2×V2) + (0.018×V3) + (-0.915×V4) + (0.731×V5)

V1: Stage 7th edition AJCC staging system
V2: Histological grade
V3: Ki67 (%)
V4: Natural logarithm of miR3916 expression
V5: natural logarithm of miR3613-5p expression

Using the inverse formula ("expit" function), the individual probability (%) of developing distant metastases can be calculated.

2. STUDY OBJECTIVES

The primary objective of the study is the clinical validation of the PORTENT metastasis risk assessment algorithm in women with breast cancer. Secondary objectives are:

- To evaluate the association of miR-3916 and miR-3613-5p with overall survival (OS).

- To assess the association between the expression of the target genes of miR-3916 and miR-3613-5p and clinicopathological parameters (hormone receptors, lymph node status, Ki67-Mib1, etc.), as well as disease-free survival (PFS), metastasis-free survival (MFS), and overall survival (OS).

- To evaluate the performance of the PORTENT algorithm in molecular subgroups identified by PAM50 classification.

3. STUDY DESIGN

This is a retrospective observational study conducted on three cohorts of patients with confirmed breast cancer diagnosis.

3.1 Patient Selection

3.1.1 CSS-cohort_2: This cohort will include 350 cases with at least 5 years of follow-up, selected from a prospectively recruited cohort between 2014 and 2019 within the TRANSCAN-BREMIR Study (Protocol References: Prot 116/CE dated 30/09/2014; Prot 140/CE dated 28/10/2014; Prot 150/CE dated 24/10/2018), carried out at the Oncology Laboratory of Fondazione "Casa Sollievo della Sofferenza", IRCCS. Residual tumor tissue must be available at the Pathology Unit, Fondazione "Casa Sollievo della Sofferenza" – IRCCS.

3.1.2 External_cohort: Patients will be retrospectively enrolled at the centers listed in Table 1. At least 450 patients will be selected with available residual tumor tissue at the respective Pathology Units. Each center will enroll at least 75 patients: 60 disease-free after at least 5 years of follow-up and 15 with metastatic progression after at least 3 years.

Table 1. List of recruiting centers for the External_cohort:

IRCCS Oncological Referral Center of Basilicata, Rionero in Vulture (PZ) (CROB): Onco-Hematology Department (Dr. Michele Aieta)
National Cancer Institute IRCCS- Fondazione Pascale, Naples (INT-Nap): Experimental Clinical Oncology of Senology (Dr. Michelino De Laurentis)
HUMANITAS Cancer Center, Milan (HUM): Oncology and Hematology Department (Dr. Rita De Sanctis)
IRCCS Policlinico San Matteo Foundation, Pavia (SM-PAV): Medical Oncology Unit (Dr. Elisa Ferraris)
Cancer Institute “Giovanni Paolo II” – IRCCS, Bari (IT-Bari): Molecular Diagnostics and Pharmacogenetics (Dr. Stefania Tommasi, Dr. Francesco Giotta)
National Cancer Institute “Regina Elena” – IRCCS, Rome (INT-Roma): Phase IV Clinical Trials Unit (Dr. Patrizia Vici)

3.1.3 INT_Milan cohort: This cohort will include 300 cases of Luminal A breast cancer treated with neoadjuvant therapy, with residual tumor material available from pre-treatment biopsy and, if available, surgical specimens. This cohort is available at the National Cancer Institute of Milan (INT_Milan), Department of Applied Research and Technological Development (Contact: Dr. Serena Di Cosimo).

3.2 Inclusion Criteria

1. Willingness to participate in the study through acceptance and signature of Informed Consent and Privacy Notice.
2. Age ≥ 18 years.
3. Tumor stage I, with or without lymph node involvement; or stage II, with or without lymph node involvement; or stage III without lymph node involvement.

3.3 Exclusion Criteria

1. Age < 18 years.
2. Tumor stage IV at diagnosis.
3. Failure to accept and sign Informed Consent and Privacy Notice.

3.4 Study Duration

According to the original GANTT chart (see page 16 of the file ApplicationSubmission_Parrella_Paola_IG_2021), patient enrollment was initially planned within the first 24 months following Clinical Protocol approval by the recruiting centers, with a deadline of 31 December 2024. However, due to delays in recruitment at participating centers, the Ethics Committee of Foggia, now the competent authority for the coordinating institution following recent changes in Italian law (art. 2, D.Lgs. 52/2023), granted an extension of the patient recruitment period until 31 June 2026. Consequently, patient recruitment can continue under the same protocol, ensuring compliance with ethical and regulatory requirements.

4. PROCEDURES

4.1 Collection of Clinical History

The Principal Investigator or a designated delegate will collect patient medical history and enter relevant clinicopathological data (age, histology, receptor status, lymph node status, etc.) and follow-up information (presence or absence of metastasis) into a dedicated platform. Follow-up updates (metastasis status, patient alive/deceased) are planned by month 48 of the project (31/12/2025).

4.2 Laboratory Analysis

Pathology Units of each center will prepare sections from residual tumor tissue to be sent to the Oncology Laboratory of Fondazione “Casa Sollievo della Sofferenza”, IRCCS for RNA extraction according to standard experimental procedures. Expression of miR-3916, miR-3613-5p, and miRNA target genes will be analyzed by quantitative real-time PCR (RT-qPCR) at the Oncology Laboratory. Protein expression of miRNA target genes will be assessed by immunohistochemistry. The extracted RNA will also be analyzed at the Gerobiomics and Exposomics Laboratory of the Romagna Institute for Tumor Studies “Dino Amadori” – IRST - IRCCS (Contact: Dr. Bravaccini) to evaluate gene expression involved in molecular classification of female breast cancer.

4.3 Biological Material Storage

Biological material will be stored for 15 years at the Oncology Laboratory of Fondazione IRCCS “Casa Sollievo della Sofferenza” and at the Gerobiomics and Exposomics Laboratory of IRST IRCCS (Contact: Dr. Bravaccini). Unused material after this period will be destroyed.

5. STATISTICAL METHODS

Study data will be analyzed at the Biostatistics Unit of Fondazione “Casa Sollievo della Sofferenza” IRCCS. Disease progression is defined by local recurrence or distant metastases assessed by mammography, CT, PET-CT, MRI, or bone scintigraphy.

5.1 PORTENT Algorithm Validation

The prognostic ability of the PORTENT algorithm will be tested on CSS_cohort_2, External_cohort, and INT_Milan cohort for internal and external validation, verifying robustness and generalizability (11). The algorithm will calculate individual risk probabilities for distant metastasis separately for each cohort. Discriminatory power will be assessed by comparing the Area Under the ROC Curve (AUC) of predicted probabilities, including 95% confidence intervals (DeLong et al., 12). Calibration will be evaluated by calibration curves and Integrated Calibration Index (ICI) (13). Differences in AUCs between the original and independent cohorts will be tested by Gönen et al.’s modification of DeLong’s test (14). A p-value < 0.05 will be considered statistically significant.

5.2 Descriptive Analysis and Survival Correlations

Clinicopathological characteristics will be summarized as mean ± SD for continuous variables and frequencies/percentages for categorical variables. Data distribution will be tested by Kolmogorov-Smirnov and Shapiro-Wilk tests. Non-normally distributed variables will be log-transformed. miRNA and target gene expression will be correlated with clinicopathological features and follow-up data. Group comparisons will use chi-square for categorical and t-tests for continuous variables. Pearson correlation coefficients will assess correlations among continuous variables. $p<0.05$ is significant. Survival analyses will use Cox proportional hazards models reporting Hazard Ratios (HR) and 95% confidence intervals.

5.3 Software

Analyses will be performed using SAS Release 9.4 (SAS Institute, Cary, NC, USA). Graphs will be generated with R Foundation for Statistical Computing (version 4.04, packages: pROC, rms, gweissman/gmish).

6. ADMINISTRATIVE REQUIREMENTS

The study will be conducted according to ethical principles derived from the Declaration of Helsinki and in compliance with Good Clinical Practice and applicable regulations on observational studies.

6.1 Informed Consent

Prior to participation, the written informed consent form must be personally signed and dated by the participant or legally authorized representative and by the person conducting the informed consent discussion.

6.2 Consent Copy

Before participation, the participant or representative will receive a dated and signed copy of the informed consent form.

REFERENCES

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4. Cao R, et al. miR-3613-5p enhances the metastasis of pancreatic cancer by targeting CDK6. Cell Cycle 2020; 19(22):3086-3095. PMID: 33073681.
5. He T, et al. MicroRNA-3613-5p Promotes Lung Adenocarcinoma Cell Proliferation through a RELA and AKT/MAPK Positive Feedback Loop. Mol Ther Nucleic Acids 2020; 22:572-583. PMID: 33230458.
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14. Gönen M. Analyzing Receiver Operating Characteristic Curves with SAS. Cary, NC: SAS Institute Inc, 2007.

INFORMED CONSENT FORM FOR PARTICIPATION IN A RESEARCH STUDY

Study Title: Dissecting the Role of miR-3916 and miR-3613-5p in Breast Cancer and Developing a Metastases Predictor PORTENT Algorithm. (for brevity, referred to as the "Study" or "Project")

Italian Title: Studio del ruolo dei miR-3916 e miR-3613-5p nel Tumore al Seno e sviluppo dell'algoritmo di predizione delle metastasi PORTENT*

Coordinating Centre: Fondazione "Casa Sollievo della Sofferenza" – Opera di San Pio da Pietrelcina

Scientific Institute for Research, Hospitalization and Healthcare (IRCCS)

Viale Cappuccini s.n.c., 71013 – San Giovanni Rotondo (FG), Italy

Principal Investigator:

Dr. Paola Parrella

Oncology Laboratory – U.O.C. of Oncology

E-mail: pparrella@operapadrepio.it

Funder: Italian Association for Cancer Research (AIRC) – Investigator Grant 25758

Participating Centre: [INSERT NAME AND ADDRESS]

Local Principal Investigator: [INSERT NAME, TITLE, CONTACTS]

This document is a translation from the original Italian Privacy and Informed Consent FORM

Latest Approval from Ethical Committee: December 19th, 2024

(Original documents available in Italian only)

INFORMATION FOR THE PARTICIPANT

Dear Madam,

You are invited to participate in a research project entitled: “Dissecting the Role of miR-3916 and miR-3613-5p in Breast Cancer and Developing a Metastases Predictor PORTENT Algorithm.”

This study will be conducted at the Oncology Laboratory – U.O.C. of Oncology of the Fondazione Casa Sollievo della Sofferenza and is funded by the Italian Association for Cancer Research (AIRC).

Our research aims to identify molecular mechanisms involved in breast cancer. Specifically, we are studying microRNAs, small RNA molecules that play important roles in tumor development and progression. Preliminary studies have identified two candidates of interest: miR-3916 and miR-3613-5p.

The purpose of this study is to determine how these two microRNAs may influence tumor progression and whether they could serve as biomarkers for identifying breast cancer patients at higher risk of developing metastases.

1. Purpose of the Study

Your biological material will be used exclusively for the objectives described above.

Molecular analyses will be performed to assess variations in miR-3916 and miR-3613-5p expression and that of their target genes, along with other genes involved in breast cancer.

The ultimate goal is to develop diagnostic tools enabling personalized, patient-specific treatments.

2. Type of Biological Material Collected

If you agree to participate, a portion of your archived tumor tissue (previously collected during your diagnostic surgery) may be used for research analyses.

Samples will be reviewed by a pathologist, ensuring sufficient material remains for diagnostic and clinical purposes.

Selected samples will be sent to:

Fondazione Casa Sollievo della Sofferenza (Coordinating Centre)

IRCCS Istituto Romagnolo per lo Studio dei Tumori “Dino Amadori” (IRST)

Both institutions will store and use your material solely for this study.

3. Use of Samples for Other Purposes

Samples will be used only within this project, unless you provide explicit written consent for future studies.

4. Voluntary Participation

Participation is entirely voluntary.

You are free to refuse or withdraw at any time without providing a reason.

Refusal or withdrawal will not affect your clinical care, treatment, or relationship with your doctors.

5. Potential Benefits

While you may not receive direct personal benefit, your contribution may help improve breast cancer diagnosis and treatment in the future, benefiting other patients.

6. Potential Risks

No physical risk is associated with this study.

Only material already collected for your diagnosis will be used, and only if sufficient tissue remains.

7. Storage of Biological Material

Samples will be securely stored for 15 years at:

the Oncology Laboratory, Fondazione Casa Sollievo della Sofferenza, and

the IRST "Dino Amadori" IRCCS.

After this period, any unused material will be destroyed.

8. Withdrawal of Consent

You may withdraw your consent at any time without any consequence to your medical care.

Upon withdrawal, any remaining samples will be destroyed, but analyses already completed will remain valid.

9. Data Protection and Privacy

Your biological material and related data will be pseudonymized, meaning your identity will be replaced by a unique code known only to your Participating Centre.

Only authorized study staff will access the link between your code and your identity.

All data and samples will be handled under strict confidentiality and security measures.

Researchers will only access coded data, ensuring your privacy.

10. Access to Your Data

You may access your personal data at any time and request correction or deletion.

Access may be temporarily restricted while the study is ongoing to maintain its integrity, but will be granted once the study is completed.

11. Transfer and Sharing of Data

Your pseudonymized data may be shared with the Coordinating Centre, IRST, and other scientific collaborators.

For publications or presentations, data will be fully anonymized, ensuring that no individual can be identified.

12. Use of Research Results

Results derived from your biological samples may be used for:

scientific publications (in anonymous, aggregated form);

development of new diagnostic or therapeutic approaches;

further research consistent with the objectives of this project.

PROCESSING OF PERSONAL DATA

(in compliance with EU Regulation 2016/679 – GDPR)

The Participating Centre [INSERT NAME], the Coordinating Centre, and other sites are joint data controllers responsible for processing your personal and health data only within the limits necessary for this study.

Your written consent is the legal basis for this processing.

Data will be collected, coded, and transmitted in pseudonymized form to the Coordinating Centre.

Authorized staff, auditors, or regulatory bodies may access data only under confidentiality to verify compliance with ethical and scientific standards.

The study physician may, with your consent, contact your general practitioner to collect relevant medical information.

You may also choose to receive updates on study results or be contacted for potential future studies.

At the end of the study, your data will be stored for the legally required period.

Data may be included in aggregated statistical analyses or publications, but no identifying information will ever appear.

You have the right to:

access, rectify, restrict, or erase your data;

withdraw consent at any time;

contact the Data Protection Officer (DPO) of your Centre at [INSERT EMAIL];

lodge a complaint with the Italian Data Protection Authority (https://www.garanteprivacy.it).

Your data will be securely stored and handled according to GDPR and Good Clinical Practice (ICH-GCP) principles.

CONSENT FORM

Participant Information

Name and Surname: .....

Date of Birth: ..... Tax Code: .....

Address: .....

City/Province: ..... Telephone: .....

☐ I am signing on my own behalf

☐ I am signing as Legal Guardian / Curator / Representative of the Participant

Participant's Name (if applicable): .....

Declarations

I hereby declare that:

1. I have read and understood this information sheet and consent form.

2. I have had the opportunity to ask questions and received satisfactory answers.

3. I have received a copy of this document, signed and dated.

4. I understand that participation is voluntary and that I may withdraw at any time without affecting my care.

5. I consent to the collection and processing of my personal and sensitive data.

6. I consent to participate in the study described above.

7. I consent to the storage and use of my biological material for this study only.

Optional Authorizations

☐ I wish / ☐ I do not wish to be informed of study results by phone.

☐ I wish / ☐ I do not wish my general practitioner Dr. .....

to be informed of my participation and results.

☐ I wish / ☐ I do not wish to be contacted for potential participation in future research studies.

City: ..... Date: ____ / ____ / ____

Participant's Signature (or Legal Representative): .....

SECTION FOR INDEPENDENT WITNESS (if applicable)

I, the undersigned .....,

certify that Dr. ..... explained the content of this document to the participant, who has agreed verbally to participate.

City: ..... Date: ____ / ____ / ____

Signature of Independent Witness: .....

SECTION FOR STUDY PHYSICIAN

I confirm that I have explained to the participant the study objectives, procedures, risks, and benefits, and that they have understood this information.

City: ..... **Date:** ____ / ____ / ____

Physician's Name and Signature: .....