Study Title: **BREMIR MicroRNAs methylation and expression profiling for identification of breast cancer patients at high risk to develop distant metastases.**

CLINICAL PROTOCOL and STATISTICAL PLAN

Protocol for the Collection, Preservation, and Use of Biological Material from Breast Cancer Patients to Identify Epigenetic Alterations Associated with Prognosis.

English translation from the original document in Italian approved by the Ethical Commettee on October 26th 2018.

Rationale

Breast cancer is the most common malignancy and the second leading cause of death after lung cancer in women. The mortality associated with breast cancer is primarily due to metastatic spread to other organs, making the ability to predict, identify, and eliminate metastases one of the major challenges in the treatment of this disease (1). Recent studies have shown that the propensity of tumors to develop metastases is driven by genetic and epigenetic alterations that occur early during carcinogenesis (2). Among these alterations, an important role appears to be played by a class of small non-coding RNAs known as microRNAs (miRNAs) (3-9).

Study Objective

The primary objective of this study is to identify and validate a panel of miRNAs whose expression/methylation can predict the development of distant metastases (Metastasis-Free Survival, MFS). Secondary objectives include: a) evaluating the association between miRNA expression/methylation and overall survival (OS); b) assessing the use of non-invasive methods to monitor minimal residual disease during follow-up; c) evaluating methodologies that allow the implementation of miRNA expression/methylation analysis in clinical practice.

Experimental Design

The study consists of four experimental phases to be conducted over three years:

Phase I: Identification in a retrospective cohort of patients (training cohort) of a panel of miRNAs with an expression/methylation profile associated with the development of distant metastases. The study will focus on 21 miRNAs for expression alterations and 22 miRNAs for methylation state alterations. The retrospective cohort will consist of tumor and normal tissue obtained from 352 breast cancer patients, stored at -80°C in the Oncology Laboratory. Expression analysis will be performed on RNA extracted from normal and pathological tissues using RT-qPCR at the Oncology Laboratory of the IRCCS Casa Sollievo della Sofferenza. Methylation analysis will be conducted using pyrosequencing in collaboration with the Bellvitge Biomedical Research Institute (IDIBELL), Barcelona, Spain.

The molecular analysis results will be correlated with pathological data provided by the Pathology Unit and clinical data provided by the Breast Surgery Unit and the Oncology Unit. The primary endpoint of the study is the identification of miRNAs whose expression/methylation is correlated with Metastasis-Free Survival (MFS), while the assessment of associations with Overall Survival (OS) and treatment response will be secondary endpoints. RT-qPCR and pyrosequencing data will be compared between groups using the non-parametric Wilcoxon rank test. The association between endpoints will be evaluated using univariate and multivariate COX regression models. Model

calibration, i.e., the evaluation of the agreement between observed outcomes and predictions, will be determined using the Hosmer-Lemeshow survival test. The models' ability to discriminate between subjects who will develop an event and those who will not will be assessed using the modified C-statistic method for censored data. The added prognostic value of the miRNA panel to already defined biomarkers will be measured using Integrated Discrimination Improvement (IDI) and the increase in C-statistic. The ability of the miRNA panel to improve glioma classification will be quantified using the survival-based Net Reclassification Index (NRI), followed by the application of a method based on the Kaplan-Meier test (10-13).

Phase II: Validation of the prognostic and predictive value of the miRNA panel identified in Phase I in a prospective cohort (validation cohort). In this phase, the association between the miRNA expression profiles and the clinical course of the disease identified in Phase I will be confirmed in an independent prospective cohort. Both normal and tumor tissues of the patients, as well as plasma collected before surgery and at each follow-up time point, will be analyzed.

Based on the annual number of surgeries performed at the Breast Surgery Unit, we estimate that the prospective cohort will consist of about 500 patients recruited over three years. To participate in the study, patients will need to confirm their willingness to donate biological material for molecular studies by signing an informed consent form. During the pre-operative visit, 2.5 ml of whole blood in Sodium Citrate will be drawn and processed within 1 hour for plasma recovery. After surgical removal of the lesion, the reference pathologist will evaluate the biological sample and, if it is adequate in quantity and quality to ensure a pathological diagnosis, will proceed with the identification and collection of normal and tumor tissue, which will be immediately frozen in liquid nitrogen and stored at -80°C. For each patient, 2.5 ml of whole blood in Sodium Citrate will be collected for plasma recovery at four subsequent time points: I) at the time of study enrollment; II) at the first outpatient visit; III) at the end of treatment; IV) at any disease recurrence. After surgical resection, the Breast Surgery staff will send the fresh surgical specimen to Pathology, where the pathologist will assess the possibility of sampling, and if positive, will proceed with the isolation of tumor tissue and normal tissue at least 2 cm from the neoplasm. The materials will be immediately collected in cryogenic tubes labeled with a unique reference code, immersed in liquid nitrogen, and stored at -80°C until use. The pathological and clinical data of the patients will be collected respectively by the Pathology Unit, the Breast Surgery Unit, and the Oncology Unit and stored in a database. Statistical analyses will be conducted as described in Phase I of the project.

Phase III: Analysis of miRNA expression/methylation in Circulating Tumor Cells (CTCs). Approximately 40 patients undergoing treatment at the Oncology Unit of our institution with a diagnosis of metastatic breast cancer will be recruited. Before the start of therapy and at each follow-up time point, 20 ml of blood will be drawn in Sodium Citrate. DNA and RNA obtained from tumor

tissue and CTCs will be analyzed for the expression/methylation of the microRNAs selected in Phases I and II of the project.

Phase IV: Evaluation of new methodologies for miRNA expression analysis in routine clinical practice. The possibility of applying the in situ hybridization (ISH) method as an alternative to real-time RT-qPCR to analyze miRNA expression in paraffin-embedded tissues will be evaluated. Paraffin-embedded material from a subgroup of 40 patients from the retrospective cohort available at our institution's Oncology Laboratory will be provided by the Pathology Unit. Paraffin-embedded tissues will be analyzed using the ISH method to evaluate the expression of miRNAs selected in Phase I of the study. For each case, the concordance between RT-qPCR performed on fresh tissue and ISH performed on paraffin-embedded tissue will be assessed.