UNIVERSITÀ DEGLI STUDI DI CATANIA

DIPARTIMENTO DI MEDICINA CLINICA E SPERIMENTALE
AZIENDA OSPEDALIERO-UNIVERSITARIA
POLICLINICO "G. RODOLICO-SAN MARCO" CATANIA
*PRESIDIO "GASPARE RODOLICO"*
U.O.C.ClinicaPsichiatrica
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**Prenotazioni Prime visite:**
Tel. 0941055190 (ore 8.00 - 18.00)
Dal Lunedi al Venerdì

**Visite di Controllo:**
Rivolgersi ai relativi strutturati

# PROTOCOL: Gene Expression Analysis of Non-Coding RNAs in Psychiatric Disorders
# (Study Code: PsiRNA25 Ver.1; 02/07/2025)

## Introduction

The majority of human genetic material is composed of genes that do not encode proteins. In fact, less than 3% of gene sequences are transcribed into messenger RNA. Non-coding RNAs (ncRNAs) represent a large and functionally heterogeneous class of the eukaryotic transcriptome.[1] In recent years, many of these biomolecules have been structurally and functionally characterized, and some of them have been shown to play a fundamental role in the complex processes related to cellular development and homeostasis.[2] Furthermore, evidence from the scientific literature highlights the importance of ncRNAs, showing that their dysregulation, by altering the physiological processes involved in gene expression modulation, contributes to the development of several pathological conditions, including neuropsychiatric disorders.[3]

In the mammalian brain, a vast number of ncRNAs are expressed with high topographic selectivity and in a cell-specific manner, and the role of several ncRNAs in neurogenesis and synaptic plasticity is now well established. Several studies in the literature indicate ncRNAs, particularly the aberrant expression of miRNAs and lncRNAs, as possible key elements in the etiopathogenesis and pathophysiology of numerous neurodegenerative conditions, as well as some of the major psychiatric disorders, including schizophrenia, bipolar disorders, and major depressive disorder.[4]

Despite the widespread prevalence of these conditions, little is still known about the exact pathophysiological mechanisms underlying them. Furthermore, the lack of sensitive and reliable biomarkers often leads to delays in diagnosis, with clear repercussions on patients' prognosis. In this context, several authors have identified ncRNAs among the possible molecules of interest for research into psychiatric disorders, since their polymorphisms or alterations in gene expression levels may reflect the immunological or central nervous system abnormalities underlying the aforementioned pathological manifestations.

Among the ncRNAs most strongly implicated in psychiatric disorders are several microRNAs (miRNAs) selectively expressed within the CNS, as well as several long non-coding RNAs (lncRNAs) associated with apoptosis.[4]

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In light of the above, the aim of this project is to evaluate the gene expression levels of several ncRNAs in groups of patients hospitalized in inpatient, day-service, or day-hospital settings who are classified as drug-naive or drug-free (T0), and subsequently after at least 5 months of treatment (T1). No treatments will be prescribed for the purposes of the study other than those that would normally be prescribed within routine clinical practice.

In addition to the patient group, gene expression levels of the same ncRNAs will also be evaluated in a group of healthy volunteer controls. These assessments will be performed on peripheral blood samples (blood serum), since it is now well established that ncRNAs, including those of neuronal or glial origin, are packaged into exosomal vesicles and are therefore detectable in body fluids.[5]

The samples will be used for the extraction of the RNAs of interest, which, after reverse transcription into cDNA, will be analyzed quantitatively by real-time polymerase chain reaction (RT-PCR). The analyses performed may allow the evaluation of possible differences in expression levels among the various groups, highlighting potential dysregulation of ncRNAs in acute psychiatric patients compared with healthy subjects, as well as the effects of pharmacological treatment in the same subjects after 5 months of therapy.

Furthermore, this analysis may provide insights relevant to the identification of biomarkers useful for diagnosis and monitoring treatment efficacy in the disorders under investigation, as well as to a better understanding of the neurophysiopathological mechanisms involved in the pathogenesis of major psychiatric disorders, such as schizophrenia spectrum disorders, bipolar disorders, and depressive disorders.

1.Wang, S., Mao, C. & Liu, S. Peptides encoded by noncoding genes: Challenges and perspectives. Signal Transduction and Targeted Therapy (2019) doi:10.1038/s41392-019-0092-3.

2.Mercer, T. R., Dinger, M. E. & Mattick, J. S. Long non-coding RNAs: Insights into functions. Nat. Rev. Genet. 10, 155–159 (2009).

3.Esteller, M. Non-coding RNAs in human disease. Nat. Rev. Genet. 12, 861–874 (2011).

4.Yoshino, Y. & Dwivedi, Y. Non-Coding RNAs in Psychiatric Disorders and Suicidal Behavior. Frontiers in Psychiatry (2020) doi:10.3389/fpsyt.2020.543893.

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5.Tellez-Gabriel, M. & Heymann, D. Exosomal lncRNAs: The newest promising liquid biopsy. Cancer Drug Resist. 2, 1002–1017 (2019).

## Methods

*Study Design*

Multicenter interventional study without investigational drugs or medical devices, comparing a group of drug-naive/drug-free patients diagnosed with Bipolar Disorder (type I or type II), Panic Disorder, Major Depressive Disorder, Obsessive-Compulsive Disorder or Schizophrenia at baseline (T0) with a group of healthy controls (CTRL), with sequential recruitment of patients hospitalized in inpatient, Day-Service, or Day-Hospital settings; a group of drug-naive/drug-free patients diagnosed with Bipolar Disorder (type I or type II), Panic Disorder, Major Depressive Disorder, Obsessive-Compulsive Disorder, or Schizophrenia, at baseline (T0) and the same group of patients after 5 months of treatment (T1); and a group of healthy controls (CTRL) and patients after 5 months of treatment (T1).

The healthy control group will consist of regular blood donors recruited from the transfusion services of the University Hospital "G. Martino" of Messina, the University Hospital "G. Rodolico" of Catania, and the University Hospital "P. Giaccone" of Palermo.

Primary Objective of the Study

The aim of this study is to evaluate possible abnormalities in the expression of a panel of non-coding RNAs selected ad hoc for the psychiatric disorders mentioned above, based on the known pathophysiology of these conditions. Such evaluations may allow the identification of biomarkers useful for diagnosis and monitoring treatment efficacy, as well as provide a better understanding of the neurophysiopathological mechanisms involved in the pathogenesis of major psychiatric disorders, including schizophrenia spectrum disorders, bipolar disorders, and depressive disorders.

Secondary Objectives

1. Evaluation of possible differences in gene expression of ncRNAs involved in the modulation of synaptogenesis, synaptic pruning, glial activation, and systemic stress response between T0 patients and healthy controls (CTRL);

2. Evaluation of possible modifications in ncRNA expression induced by treatment through analysis of ncRNA gene expression levels in T1 patients;

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3. Evaluation of possible differences in ncRNA gene expression between T0 patients during manic episodes and T0 patients during depressive episodes among patients affected by Bipolar Disorder.

Study Setting

- Psychiatry Unit, University Hospital "G. Rodolico – San Marco" of Catania, Via Santa Sofia 78. Director: Prof. A. Petralia;
- Psychiatry Unit, University Hospital "G. Martino" of Messina, Via Consolare Valeria 1. Director: Prof. M.R.A. Muscatello;
- Psychiatry Unit, University Hospital "P. Giaccone" of Palermo, Via del Vespro 129. Director: Dr. G. Marrazzo;
- SPDC Psychiatry Unit, "Umberto I" Hospital of Enna, Contrada Ferrante snc. Director: Dr. A. Messina.

*Participants*

Patients Inclusion Criteria:

- Age range between 18 and 55 years;
- Male sex or female sex in the mid-luteal phase of the menstrual cycle;
- Consistency between the clinical-symptomatic presentation and the diagnostic criteria for Bipolar Disorder (type I or type II), Panic Disorder, Major Depressive Disorder, Obsessive-Compulsive Disorder or Schizophrenia;
- First diagnosis (drug-naive) or absence of ongoing psychopharmacological treatment for at least 6 months (drug-free).

Patients Exclusion Criteria:

- Current immunosuppressive, antibiotic, or hormone replacement therapy;
- Significant comorbid conditions (autoimmune diseases or major internal medicine disorders);
- Active infectious diseases or infections resolved less than 3 weeks prior to enrollment;
- Significant psychiatric comorbidities;

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- Ongoing psychopharmacological treatment or discontinuation of treatment less than 6 months prior to enrollment.

Control healthy subjects with the following characteristics will be recruited:

- Age range between 18 and 55 years;
- Male sex or female sex in the mid-luteal phase of the menstrual cycle;
- Not receiving psychotropic medications;
- Complete absence of clinical elements supporting a diagnosis of Bipolar Disorder (type I or type II), Panic Disorder, Major Depressive Disorder, Obsessive-Compulsive Disorder or Schizophrenia;
- Absence of clinical or laboratory findings suggestive of ongoing internal medicine or infectious diseases;
- Absence of autoimmune diseases;
- Hamilton Depression Rating Scale (HAM-D) score < 8;
- Mania Rating Scale (MRS) score < 11;
- Yale-Brown Scale (YBS) score < 7;
- Brief Psychiatric Rating Scale (BPRS) score = 18;
- Absence of stressful life events during the previous 6 months (e.g., bereavement, traumatic events, divorce, etc.).

All participants, including both patients and healthy controls, will be required to provide written informed consent prior to enrollment in the study. Participation in the study will not modify routine clinical practice in any way, nor will it involve diagnostic or therapeutic procedures other than those already included within standard clinical care.

*Assessors*

The psychometric instruments will be administered by physicians trained in their administration. Gene expression levels of ncRNAs will be evaluated by the team of molecular biologists of the Molecular Biology Laboratory of the University Hospital of Messina.

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*Variables*

1.Demographic Variables

- Age
- Sex
- Educational level
- Marital status and children
- Employment status
- Smoking habits (tobacco and/or e-cigarettes)

2.Clinical Variables

- Diagnosis
- Substance use
- Detailed pharmacological treatment (which medications are being taken, duration of treatment, and dosage). The overall dosage will be converted into equivalent doses.
- Duration of illness
- Number of previous hospitalizations

3.Main Rating Scales

- Hamilton Depression Rating Scale (HAM-D)
- Mania Rating Scale (MRS)
- Yale-Brown Scale (YBS)
- Brief Psychiatric Rating Scale (BPRS)

*Clinical Procedure*

The patient population from which clinicians will recruit participants will consist of patients hospitalized in inpatient, Day-Service, or Day-Hospital settings at the Psychiatry Units of the University Hospitals of Catania, Messina, and Palermo, as well as at the Psychiatry Unit of the "Umberto I" Hospital in Enna. The clinician will

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collect anamnestic data and perform a clinical evaluation to confirm the patient's eligibility for participation in the study using standardized instruments. Approximately 25 minutes are estimated for this phase.

The patient will then be introduced to the study, including a brief synopsis describing the study procedures. Questions from the patient will be addressed, followed by the formal request for written informed consent. Approximately 10 minutes are estimated for this phase.

Subsequently, a peripheral venous blood sample will be collected as part of the routine laboratory investigations performed during hospitalization. Fifteen milliliters of residual peripheral venous blood from routine sampling will be used for the purposes of the study. This procedure is expected to require less than 5 minutes.

Therefore, the entire procedure is estimated to last approximately 40 minutes, corresponding to the duration of a standard routine clinical visit.

Healthy controls will consist of healthy volunteer subjects recruited from the transfusion services of the University Hospital "G. Martino" of Messina, the University Hospital "G. Rodolico" of Catania, and the University Hospital "P. Giaccone" of Palermo. Specifically, subjects meeting the inclusion criteria described above will be selected.

Eligible subjects will be introduced to the study through a brief synopsis describing the study procedures. Questions from the donor will be addressed, followed by the formal request for written informed consent. Approximately 10 minutes are estimated for this phase.

Subsequently, the Hamilton Depression Rating Scale, Mania Rating Scale, Yale-Brown Scale, and Brief Psychiatric Rating Scale will be administered. Approximately 15 minutes are estimated for this phase. Afterwards, the biological sample will be collected.

Under no circumstances will donors' personal data be used in this study. The only information retained for statistical analysis purposes will include age, sex, educational level, marital status, employment status, and smoking habits.

Once collected, samples obtained from patients or healthy controls will be immediately centrifuged in order to obtain blood serum, which will then be stored at −80 °C. Samples will subsequently be transferred on dry ice to the Cellular and Molecular Biology Laboratories of the Department of Clinical and Experimental

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Medicine of the University of Messina (5th floor, Biological Tower, University Hospital "G. Martino", Via Consolare Valeria 1).

*Laboratory Analysis*

Total RNA will be extracted from 400 µL of each serum sample using RNAzol (Genecopoeia, MD, USA), following the manufacturer's protocol instructions. After the addition of 1 mL of RNAzol (lncRNA extraction), samples will remain at room temperature for 5 minutes. After 3 minutes, samples will be centrifuged at 12,000 rpm for 15 minutes. Following centrifugation, an aqueous phase will be obtained and subsequently transferred into new tubes.

After the addition of 1 mL of isopropanol, RNA precipitation will occur. Following a 10-minute incubation on ice, samples will be centrifuged at 12,000 rpm for 11 minutes. The resulting pellets will be washed with 75% ethanol and centrifuged again at 7600 g for 5 minutes. The same procedure will be repeated once more.

After allowing the tubes to dry under a hood for 5 minutes, RNA will be resuspended in nuclease-free $H_2O$. RNA concentration (ng/µL) and purity (OD260/OD280 ratio) will be assessed using a NanoDrop spectrophotometer.

For reverse transcription of mature miRNAs, complementary DNA (cDNA) will be obtained using the All-in-One miRNA qRT-PCR Detection Kit 2.0 by GeneCopoeia, which includes Poly A Polymerase to extend RNA nucleotide sequences with poly(A) tails and an oligo-dT adaptor primer serving as the reverse transcription primer.

For lncRNAs, the High Capacity cDNA Reverse Transcription Kit by Applied Biosystems (USA) will be used. Both procedures will be performed according to the respective manufacturers' protocols.

The cDNAs obtained following reverse transcription will be used to analyze non-coding RNA expression levels using the Applied Biosystems 7500 Real-Time PCR System. PowerUp SYBR Green Master Mix (Applied Biosystems, USA) will be used for amplification and quantification of lncRNAs.

For miRNAs, the All-in-One miRNA qRT-PCR Detection Kit 2.0 will be employed, which also uses SYBR Green chemistry and includes a Universal Adapter Primer recognizing the oligo-dT adaptor primer used during reverse transcription.

To normalize serum miRNA expression levels, miR-39 will be used as a spike-in external control (Norgen Biotech, Canada), according to the literature.[1] For lncRNAs, GAPDH will be used in accordance with published studies.[2]

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After Real-Time PCR reactions, the threshold cycle (Ct) will be considered the reference parameter for target gene and housekeeping gene expression levels. Each sample will be analyzed in duplicate.

Expression levels for each sample will be quantified using the delta-delta CT method (2^-ΔΔCT). Initially, the average of duplicate samples will be calculated for each target gene and each control. Subsequently, delta CT (ΔCT) will be calculated using the following formula:

ΔCT = average CT (target) – average CT (housekeeping)

Once normalized, ncRNA levels will be quantified according to the 2^-ΔΔCT algorithm (relative analysis), using an arbitrary calibrator with a delta Ct value higher than that of all tested samples. This step will be performed in order to obtain histograms with consistently positive values and therefore more easily interpretable visually.

ΔΔCT = ΔCt (Sample) – ΔCt (Calibrator)

1. Marabita, F. et al. Normalization of circulating microRNA expression data obtained by quantitative real-time RT-PCR. Brief. Bioinform. (2016) doi:10.1093/bib/bbv056.

2. Iempridee, T. et al. Identification of reference genes for circulating long noncoding RNA analysis in serum of cervical cancer patients. FEBS Open Bio (2018) doi:10.1002/2211-5463.12523.

*Sample Size*

Sample size was determined through power analysis using G*Power 3.1 software. Based on an expected effect size of 0.5 (Cohen's d, medium effect), an alpha error (α) of 0.05, and a statistical power (1 − β) of 80%, a minimum of 64 subjects per group was estimated for comparisons between independent groups (e.g., patients vs. controls).

Regarding within-subject comparisons (e.g., pre- and post-intervention clinical changes in treated patients), the minimum required sample size was estimated at 34 participants, considering the same statistical power and effect size.

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**Prenotazioni Prime visite:**
Tel. 0941055190 (ore 8.00 - 18.00)
Dal Lunedi al Venerdì

**Visite di Controllo:**
Rivolgersi ai relativi strutturati

Sample size calculation was performed assuming the use of two-tailed independent sample t-tests (between-group comparisons) and paired sample t-tests (within-group comparisons), in accordance with the study design.

The estimate does not account for potential dropouts or loss to follow-up; therefore, an oversampling of 10–15% is recommended.

*Study Duration*

In order to achieve the planned sample size, participant recruitment will continue until September 1, 2027.

*Expression Analysis and Statistical Methods*

In order to evaluate differences in expression levels, fold changes of individual RNAs will be calculated using the Livak and Schmittgen 2^-ΔΔCt formula [1], where:

• ΔCt = Ct(target gene) – Ct(normalizer)

• ΔΔCt = mean ΔCt(sample) – mean ΔCt(control)

Using ΔCt values, independent two-tailed Welch-corrected t-tests will be performed to compare expression levels between T0 patients and healthy controls (CTRL). A p value < 0.05 will be considered statistically significant.

To compare expression levels between T0 and T1, paired t-tests will be performed.