MEDICAL UNIVERSITY OF PLOVDIV

DEPARTMENT OF PSYCHIATRY AND MEDICAL PSYCHOLOGY

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TRANSLATIONAL CROSS-VALIDATION OF NEUROIMAGING AND MOLECULAR BIOMARKERS IN THE DIFFERENTIAL DIAGNOSIS OF UNIPOLAR AND BIPOLAR DEPRESSION

ABSTRACT

OF DISSERTATION THESIS FOR ACQUISITION OF EDUCATIONAL AND SCIENTIFIC DEGREE "DOCTOR OF PHILOSOPHY"

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The dissertation is written on 122 pages and is illustrated with 11 tables and 16 figures. 246 literature sources have been cited.

The dissertation was approved and directed for defence by an extended departmental council of the Department of Psychiatry and Medical Psychology at the Medical University of Plovdiv.

The public defence of the dissertation thesis before the scientific jury will take place at ..... from ..... at ..... Auditorium of the Medical University of Plovdiv, "Vasil Aprilov" 15A Blvd, Plovdiv.

The materials for the defence are available at the Department of Science and Research at the Medical University of Plovdiv, "Vasil Aprilov" 15A Blvd. and on the website of the Medical University of Plovdiv www.meduniversity-plovdiv.bg.

Content

Abbreviations used:

ACC – Anterior Cingulate Cortex

AI – Anterior insula

BD – Bipolar disorder

FC – Functional connectivity

fMRI – functional Magnetic Resonance Imaging

HAM-A - Hamilton Anxiety Rating Scale

HC – Healthy controls

IAPS - International Affective Picture System

l- - left

lncRNA – long non-coding RNA

MADRS - Montgomery–Åsberg Depression Rating Scale

MDD – Major depressive disorder

MFG – Medial Frontal Gyrus

P - Patients

r- - right

SPL – Superior Parietal Lobule

SSC – Somatosensory cortex

WBC – White blood cells

YMRS - Young Mania Rating Scale

Introduction

According to the World Health Organization, the incidence of mental illness is increasing worldwide (5.1% for severe cases and 17% for moderate and mild cases). This trend is particularly evident in the affective disorders major depressive disorder (MDD) and bipolar disorder (BD), which are socially significant illnesses and affect around 300 million and 45 million people respectively worldwide. And although recent decades have been marked by important breakthroughs in the pharmacotherapy of these disorders, no significant change in statistical indicators, including social burden, has been found. Therefore, the ongoing study of the etiopathogenesis and the diagnostic and therapeutic process in MDD and BD remains a topical scientific issue.

The influence of many different factors on the etiopathogenesis of both illnesses has been demonstrated. Examples of such factors are: genetic, epigenetic, immunological, oxidative and nitrosative stress, neurobiological, etc. The data accumulated so far are extremely heterogeneous. However, most of the findings relate to changes in the activity and connectivity of structures involved in the regulation of several domains, namely: cognition, perception and processing of emotional stimuli, reward-processing, and decision making.

One of the common and frequent symptoms in affective disorders is a transient cognitive deficit. Altered prefrontal cortex (PFC) activity is associated with this symptom. Deactivation of the medial PFC in healthy individuals is associated with better executive functions. Yet, patients with MDD and BD in remission demonstrate low performance on working memory tasks, which is manifested by significantly less inhibition of the medial PFC in contrast to healthy individuals. In addition, both MDD and BD are associated with structural and functional changes in the fronto-limbic network. Reduced cerebral gray matter volume was found in the left anterior cingulate cortex (l-ACC) and in the right hippocampus (r-HIP) in both disorders compared to healthy controls (HC). Furthermore, many findings suggest the presence of structural abnormalities in networks responsible for emotional and cognitive control not only in patients with MDD and BD, but also in individuals at increased risk of developing affective disorders.

Another common symptom is insomnia. Interestingly, patients with primary insomnia also show impaired functional connectivity (FC) between the ACC, insula and limbic structures in contrast to HC. These findings indicate the presence of common connectivity impairments in both the task-positive network and the default mode network (DMN) in primary insomnia and MDD and BD presenting with insomnia. Thus, we might conclude that some of the common aberrations found in the two affective disorders may be due to overlapping symptomatology.

The study of epigenetic factors in affective disorders also shows the presence of general and specific aberrations. For example, multiple studies have shown alterations in the expression of long non-coding RNAs and microRNAs that are associated with neurodevelopmental processes, neuronal plasticity, neuroinflammation, altered neurotransmission, etc. All these processes are implicated in the etiopathogenesis of MDD and BD. Changes in microRNA expression have also been associated with treatment response to some pharmacotherapeutics, suggesting their potential as biomarkers for therapeutic monitoring. Therefore, an in-depth study of the complex etiopathophysiological mechanisms through a multimodal and multidisciplinary approach is necessary to achieve a meaningful cross-validation between the scientific-investigatory (neuroimaging, molecular, genetic, etc. data) and the clinical-deductive approach (achieving translation of scientific findings into clinical practice).

Hypotheses:

1. By analyzing the functional connectivity (FC) at the whole-brain level (seed-to-voxel) of two major regions of the salience network (ACC and AI), the accumulated literature on their impaired connectivity in mood disorders will be confirmed. We hypothesized that altered connectivity between the ACC and AI and structures from the default mode and executive networks could be a differential signature between MDD and BD.

2. Differences in the expression of long non-coding RNAs (lncRNAs), some of which have been validated in peripheral blood of MDD patients and others in post-mortem medial frontal gyrus tissue of BD patients, could be confirmed in a Bulgarian population, demonstrating concordance of these alterations between 2 different races.

3. In a task investigating cognition and emotion processing in affective disorders, changes in medial frontal gyrus activity will be observed as follows: hyperactivation of the structure in patients with BD compared to HC and hypoactivation of the medial frontal gyrus (MFG) in patients with MDD compared to HC and BD.

4. MicroRNA expression associated with sleep disturbances could help differentiate patients with MDD and BD, as insomnia is another of the overlapping symptoms in affective disorders whose molecular mechanisms remain undiscovered.

Aim and objectives

1. Aim

Exploring the differential diagnostic potential of neuroimaging and molecular markers in patients with unipolar and bipolar depression.

2. Objectives

- Assessment and analysis of resting-state functional connectivity in patients and healthy controls.
- Determining the presence or absence of differences in functional neuroimaging correlates of a cognitive task under the influence of emotional distractors in patients with unipolar and bipolar depression (using an original newly developed paradigm)
- Evaluation of the differential expression profiles of preselected lncRNAs and microRNAs associated with sleep disturbances (in collaboration with the Department of Pathophysiology) in MDD and BD compared to healthy controls.

The study contains two components:

- Application of new statistical methods to process already collected neuroimaging data.
- Implementation of a newly developed fMRI paradigm in an independent sample.

Materials and methods

1. Subjects

Subjects were adults meeting DSM-V criteria for major depressive or bipolar disorder with a current moderate or severe depressive episode, with a total Montgomery-Åsberg Depression Rating Scale (MADRS) score ≥20 and Young Mania Rating Scale (YMRS) score ≤3 when enteringthe study. The study group of depressed patients was drawn from a random sample of patients referred for participation from psychiatric institutions, including the Department of Psychiatry at St. George University Hospital, State Psychiatric Hospital Pazardzhik, Mental Health Center Plovdiv, and outpatient psychiatrists. Healthy individuals have been recruited from the community. Screening and assessment of each subject was performed by a physician with a specialty or specializing in psychiatry (D.S., S.K., A.T.).

Diagnoses are based on meeting the DSM-V diagnostic criteria and the assessment is based on: 1) admission and working diagnosis and medical history (inpatient and outpatient records); 2) interview with the patient's treating physician; 3) structured clinical interview M.I.N.I. 6.0.0.; 4) additional information from the patient's relatives if necessary.

All subjects were informed in advance of the aims, objectives and methods of the study in accordance with the Helsinki Convention on Human Rights and gave written informed consent to participate in the study. The study was approved by the Scientific Ethics Committee of the Medical University of Plovdiv (ID: P-186/22.01.2021).

1.1 Inclusion criteria:

- age over 18 and under 65;
- a DSM-V diagnosis of MDD or BD;
- current moderate or severe depressive episode (MADRS ≥20);
- absence of hypomanic/manic symptoms (YMRS) ≤3 items.
- regular intake of psychopharmacological therapy for at least 7 days prior to study inclusion (for patients)
- absence of current or past mental illness meeting DSM-V criteria (for healthy controls)
- written informed consent to participate in the study (for all participants).

1.2 Exclusion criteria:

- age under 18 and over 65;
- high suicide risk;
- Current or past diagnosis of a DSM V disorder other than MDD or BD such as addictions, organic disorders, psychotic disorder, psychoorganic disorder;

- Malignant neoplasms;
- Neurodegenerative diseases;
- Head trauma with loss of consciousness or history of previous such;
- Dementia;
- Parkinson's disease;
- Huntington's disease;
- Acute ischemic or hemorrhagic vascular accident;
- Infections;
- Pregnancy;
- Metal implants, fixators, prostheses, pacemaker, carotid and/or coronary stent, etc., incompatible with conducting MRI.

Clinical assessment, functional magnetic resonance imaging (fMRI), sampling, and analysis of neuroimaging data were performed in the Complex for Translational Neuroscience, and laboratory studies were performed in the Department of Medical Biology.

To conduct the first component of the study, available data (from two modalities: structural and resting-state fMRI) of 115 participants including healthy controls (n=45) and patients with a current depressive episode (n=70) in the context of MDD (n=35) and BD (n=35) were analyzed. After performing an image quality check, 12 individuals were excluded from the analysis due to artifacts detected from excessive head movement not repairable with fMRI processing methods. The final sample size consisted of 103 individuals, divided into groups as follows: HC = 43 individuals, MDD = 35 individuals, BD = 25 individuals.

For the purposes of the second component of the study, 96 individuals were recruited, including 50 healthy individuals and 46 patients in a depressive episode in the context of MDD or BD. A total of 18 individuals were excluded from data analysis due to established structural pathology (n=6) and due to excessive motion artifacts during the study (n=12). The final sample consisted of 78 individuals, divided into groups as follows: HC = 40 individuals, MDD = 23 individuals, BD = 15 individuals.

Due to the refusal of some subjects to give informed consent for blood sampling and insufficient funding, the sample in which differences in peripheral molecular markers (microRNAs, lncRNAs) were sought was 60 subjects, 31 healthy controls and 29 patients in a depressive episode, respectively. A technical malfunction of a storage freezer resulted in the loss of 4 blood samples and the final sample in which the expression of long non-coding RNAi was examined consisted of 56 individuals, divided into groups as follows: HC = 29 individuals, MDD = 11 and BD =16. The sample in which the expression of microRNAs was

studied included 14 HCs and the same number of patients from both groups, due to the limited availability of reagents.

2. Methods

2.1. Clinical methods for patient evaluation

Each participant was evaluated against the inclusion and exclusion criteria described above. Diagnostic refinement was based on anamnestic and clinical data as well as assessment by structured M.I.N.I interview. The latter instrument is used both to confirm a current depressive episode and to exclude comorbid psychiatric disorders, including substance use disorders, obsessive-compulsive disorder, eating disorders, neurodevelopmental disorders, etc.

The severity of the depressive episode was determined using the widely used Montgomery-Åsberg Depression Rating Scale (MADRS) for both clinical and research purposes, with a score of 20 chosen as the minimum acceptable for patients, which is equated with moderate severity, and for healthy controls less than a score of 7. Additionally, to rule out the presence of mixed episode features (in patients), each individual was assessed using the YMRS, with the score required to rule out the presence of hypomanic or manic symptomatology being less than 3 for all groups studied. To comprehensively assess the condition and the presence of anxiety symptomatology, the Hamilton Anxiety Rating Scale (HAM-A) was also administered to each participant. All patients were taking their regularly prescribed psychopharmacotherapy for at least 7 days prior to inclusion in the study.

2.2. Functional Magnetic Resonance Imaging (fMRI)

Participants were examined on a 3T MRI system (GE Discovery 750w) with 3 different MRI sequences: high-resolution structural scan (Sag 3D T1 FSPGR sequence), TR (relaxation time) 7.2 ms, TE (echo time) 2. 3 and flip angle 12°, slice thickness 1 mm, matrix 256 × 256,; two functional series, resting-state (2D EPI sequence) with eyes closed - slice thickness 3 mm, 36 slices, matrix 64 × 64, TR, 2000 ms, TE, 30 ms, flip angle 90°, 192 volumes and task-sequence with slice thickness 3 mm, matrix 64 × 64, TR 2000 ms, TE 30 ms and flip angle 90°, 352 volumes. The functional scan starts with 5 dummy time series that are automatically excluded.

During the task session, patients perform a task that activates attention and short-term memory in the presence or absence of emotional distractors. The paradigm consists of 16 active blocks, each lasting 24 seconds, and 16 off-blocks (at rest, in which the patient is instructed to concentrate on a fixation cross in the middle of the screen) lasting 20 seconds. During the active blocks, oval or square shaped pictures were presented (Fig. 1), which were monochromatic (basic blocks - no distractor) or contained a picture with a neutral (neutral blocks - neutral distractor) or negative emotional charge (negative blocks - negative distractor) (Fig. 1). The pictures were selected from the so-called International Affective

Picture System (IAPS), which is a validated image system for emotional response research. The subject's task was to follow the sequence of the figures and to press a button each time they see two or more consecutive squares (the number of responses is the same in all active blocks). An additional condition was for the participant to ignore the content of the figure.

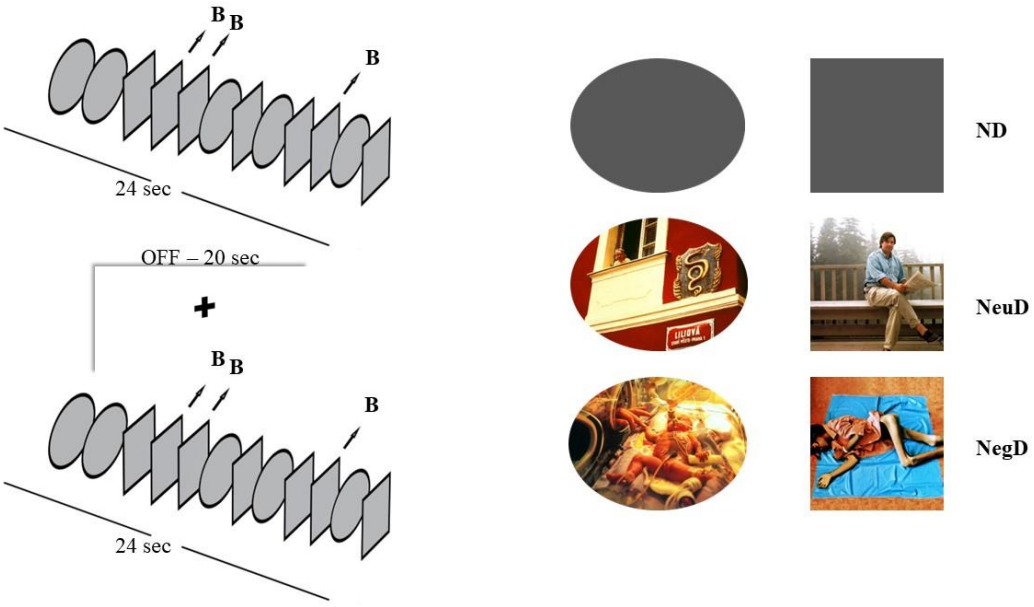

Figure 1 Schematic of the stimuli sequence in the paradigm. Abbreviations used: B – button; ND – no distractor; NeuD – neutral distractor; NegD – negative distractor

2.3 Laboratory test methods

2.3.1. Sampling

Plasma, serum, and white blood cells (WBCs) were obtained from patients and healthy controls in compliance with all standard clinical requirements. Laboratory investigations were performed in the Department of Medical Biology.

From all participants who gave informed consent, 4 mL of venous blood was collected by venipuncture into vacuum container-type tubes (BD Vacutainer®) with a red lid for serum isolation and with a purple lid for plasma and WBC isolation. After sampling, the serum isolation tubes were left at room temperature for 30 minutes and subsequently centrifuged at 3000 rpm for 10 minutes (at room temperature). The resulting serum was aliquoted (600µl) into Eppendorf type tubes and left for storage at -80ºC. Tubes with anticoagulant (purple lid) were used to obtain plasma and WBC for RNA analysis and transported on ice to the laboratory within 15 minutes after sampling. The blood was centrifuged at 3000 rpm for 10 min at 4°C, and the isolated plasma was again distributed into Eppendorf-type tubes and stored at -80ºC for subsequent analysis.

2.3.2. Preparation of WBCs and isolation of total RNA from them

WBCs were isolated from patients with MDD, BD and HC by the method of cold lysing of erythrocytes using a lysing solution containing ammonium chloride, ammonium carbonate and ethylenediaminetetraacetic acid (EDTA). The cell mass obtained after plasma separation was added to the lysing solution, then incubated at 4ºC for 10 min and centrifuged. The above cycle was repeated until complete lysis of erythrocytes. The resulting white blood cell sediment was resuspended in PBS buffer and centrifuged. After removal of the PBS buffer, 800 µl of Trizol (Invitrogen, Burlington, ON) was added to the resulting WBC pellet, followed by freezing at -80ºC or direct RNA isolation.

Trizol treatment results in lysing of the obtained WBC sludge. The samples are then separated by the addition of chloroform in a 1:5 ratio into separate fractions containing the different biological molecules. Isopropyl alcohol is added to the resulting liquid phase, whereby the RNA molecules are precipitated. The total RNA is then washed with 75% ethyl alcohol, dried and dissolved in nuclease-free water.

2.3.3. Freely circulating and exosomal plasma mRNA

Freely circulating and exosomal RNAs were obtained using the NORGEN® Midi Kit for isolation and concentration of RNA from plasma. The technique is performed with a twofold treatment with Lysis Buffer A (containing guanidine salts) and subsequent purification of RNA by two-step column chromatography. Wash Solution A was used to wash the columns during the different stages. The final elution of RNA from the Mini Spin column was in Elution solution A. The concentration of RNA in the samples was measured by spectrophotometry (using Nano Drop 2000 at 260/280 nm). The samples were stored at -80ºC.

2.3.4. Synthesis of cDNA by RT-PCR

A cDNA chain was synthesized from the resulting total RNA for subsequent determination of gene expression levels of a selected panel of long non-coding RNAs and microRNAs: let-7, miR-30c, miR-132, miR-212, miR-125b, miR-126, miR-138, miR146a, miR-182, miR-219. cDNA was obtained by reverse transcription-PCR (RT-PCR). The RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) was used and a protocol for first strand cDNA synthesis was followed. A reaction mixture containing a variable volume RNA matrix with a fixed concentration of 2 µg/µl, 0.5 µg/µl non-specific random hexamer primers and nuclease-free water was prepared. To each individual sample was added a master mix containing 5x reaction buffer - 4 µl, RiboLock™ ribonuclease inhibitor (20 U/µl) - 1 µl, 10 mM dNTP mix - 2 µl and RevertAid™ H Minus M-MuLV reverse transcriptase enzymes (200 U/µl) - 1 µl. The resulting reaction mixture was incubated for 60 min at 42ºC, after which the reaction was stopped by heating for 5 min at 70ºC. The synthesized

cDNA was diluted with nuclease-free water (1:7). The diluted samples were stored at - 20ºC.

2.3.5. Quantitative polymerase chain reaction (qRT-PCR)

RT-qPCR analysis was performed to determine gene expression levels of selected long-stranded RNAs, including TCONS_00019174, ENST00000566208, ENST00000517573, RP1-269M15.3, CTC-487M23.5, CTD-2647L4.4, RP11-453F18_B.1, RP11-273G15.2, RP11-326I11.3, RP11-434C1.1, and selected microRNAs let-7, miR-30c, miR-132, miR-212, miR-125b, miR-126, miR-138, miR146a, miR-182, miR-219. Actin, Ubiquitin, hUBC, B2M and Gapdh were used as endogenous controls. The GreenMasterMix (2x) kit (Genaxxon bioscience GmbH, Germany) used for RT-qPCR analysis contains SuperHotStart Taq DNA polymerase enzyme, PCR buffer, MgCl2, dNTP and Syber Green. The PCR reaction mixture contains: 1 µl cDNA, 2 µl forward and reverse primers for the respective long-strand RNAs or endogenous controls, 10 µl GreenMasterMix (2x) and 7 µl nuclease-free water. The final volume of the reaction mixture is 20 µl. The analysis was performed using a quantitative qPCR instrument, RotorGene Q 6000. The working program used is two-step: initial denaturation - 15 min at 95ºC, 40 cycles - denaturation - 15 sec at 95ºC, amplification - 60 sec at 58ºC, final extension - 2 min at 72ºC. Subsequent calculation of gene expression change was performed based on the 2-ΔΔCt method (Schmittgen and Livak, 2008), first by generating a ΔCt value representing the Ct of the corresponding long-chain RNA of interest minus the Ct of the averaged value of the reference genes used; generating a ΔΔCt value by subtracting the ΔCt of the patient samples from the ΔCt of the control samples; presenting the final result as the fold change in the accumulation of the corresponding long-stranded RNA transcripts in MDD, BD and HC.

2.3.6. TaqMan анализ

In order to investigate the expression levels of microRNAs involved in the regulation of YKL-40 expression, specific TaqMan analysis was performed. Commercial TaqMan primers for the corresponding microRNAs were used: let-7, miR-30c, miR-132, miR-212, miR-125b, miR-126, miR-138, miR146a, miR-182, miR-219, U6 (Thermo Fisher Scientific, USA) and TaqMan® Fast Advanced Master Mix (Thermo Fisher Scientific, USA) containing AmpliTaq™ Fast DNA Polymerase, Uracil-N glycosylase, dUTP, ROX™ Passive Reference dye and TaqMan MGB probe . The reaction mixture for the assay contains cDNA - 5 µl synthesized by reverse transcription, TaqMan primers 20x -0.47 µl for the respective microRNA, TaqMan® Fast Advanced Master Mix - 5.03 µl. Final working volume of the reaction is 10.5 µl. The analysis was performed using a RotorGene Q 6000 quantitative q-PCR instrument. The following work program was used - Uracil-N glycosylase incubation - 2 min at 50ºC,

polymerase activation - 20 sec at 95ºC, 40 cycles - denaturation - 1 sec at 95ºC, amplification - 20 sec, at 60ºC.

The analyses were performed by assoc. prof. Nikolai Mehterov, PhD

2.4. Statistical methods

Statistical analysis of participant demographic and clinical characteristics was performed using IBM SPSS 28.0 for Windows. The significance level was set at $p < 0.05$ for all tests. Student's t-test was used for continuous variables and chi-square test for categorical variables. The following methods were used: non-parametric analysis; descriptive analysis; graphical analysis; Independent-Samples Kruskal-Wallis test; Chi-square criterion; D'Agostino-Pearson test; Shapiro-Wilk test; Kolmogorov Smirnov test; Wilcoxon Signed Rank Test.

For the purpose of retrospective analysis, the CONN Toolbox running on MATLAB was used to examine functional connectivity (at rest) between the major brain regions defined according to the default atlas of the tool used, which is a combination of the FSL Harvard-Oxford atlas cortical & subcortical areas and the AAL atlas cerebellar areas. All data were preprocessed by the following steps: 1. Realignment and unwarp (spatial transformation); 2. Slice-timing correction (temporal synchronization); 3. Outlier detection; 4. Functional Direct Segmentation & Normalization; 5. Structural Segmentation & Normalization; 6. Functional Smoothing. After the preprocessing, the so-called denoising was performed, by which the BOLD signal at the whole-brain level was "homogenized". Based on the data processed in this way, a seed-to-voxel analysis was performed with selected regions of interest - anterior cingulate cortex, left and right insula - and a contrast between left and right insula signal was created in the following between-group comparisons: patients (P)>HC, MDD>HC, BD>HC, MDD>BD.

Data from the implementation of the new paradigm were processed using SPM12 running on MATLAB. At the individual level, each participant's data were preprocessed (pre-analysed) through the following steps: Slice-timing correction; Realignment; Corregistration; Normalisation; Smoothing. Each person's data were then postprocessed at the individual level and included in an ANOVA analysis to examine within- and between-group differences in activations of different brain regions under predefined contrasts between the three active conditions: neutral distractors > no distractors, no > neutral distractors, negative distractors > neutral distractors, neutral distractors > negative distractors, negative distractors > no distractors, and no distractors > negative distractors.

The molecular data were processed using Microsoft Excel 2017 and GraphPad Prism, version 8.1. The D'Agostino-Pearson, Shapiro-Wilk and Kolmogorov Smirnov statistical test for normality was used to determine the Gaussian distribution of the obtained data. The Mann-Whitney non-parametric

test was applied to compare the values between groups. Non-parametric Wilcoxon Signed Rank Test was used to conduct within-group comparison of data.

Results

1. Demographic and clinical characteristics

In the retrospective sample, there were no statistically significant differences in gender and age distribution. There was a statistically significant difference in the level of education (measured in years) between the HC and patients in both groups. No difference was found between the two patient groups in terms of clinical indicators. The results are presented in Table 1.

Table 1 Demographic and clinical characteristics of the retrospective sample

	Healthy controls **n=43**	**MDD patients** **n=35**	**BD patients** **n=25**	**Level of significance in the MDD/BD comparison**	**Level of significance in the HC/MDD and BD comparison**
Age (mean ± SD)	40,25 (±10,73)	40,97 (±10,86)	41,72 (±9,62)	p = 1.000[c]	p = 1.000[a]/ 1.000[a]
Sex (M/F)	43 (14/29)	35 (14/21)	25 (9/16)	p = 0.753[b]	p = 0.793[b]
Education (years)	15,93 (±3,26)	13,8 (±2,93)	13,88 (±2,71)	P=0.915 [c]	p = 0.012[a]/ p = 0.035[a]
MADRS	2,28 (±2,25)	30,2 (±4,14)	29,76 (±6,17)	P=0.742 [c]	p < 0.001[a]/ 0.001[a]
Age at onset	-	30.24 (11.01)	29.72 (9.19)	p = 0.850 [c]	-
Illness duration	-	130.97 (113.93)	148.6 (101.64)	p = 0.544 [c]	-
Episode duration	-	21 (36.65)	15.8 (14.99)	p = 0.508 [c]	-
Number of previous episodes	-	3.97 (3.56)	6.36 (4.72)	p = 0.035[c]	-

[a] – ANOVA; [b] - $\chi 2$ test; [c] - Independent samples t-test.

In the prospective sample, there were no statistically significant differences in the distributions of sex, age and education, although as expected there was a trend towards a statistically significant difference in the level of education between HC and BD. There was a statistically significant difference in the number

of previous episodes between the MDD and BD groups. There was also no significant difference in the percentage of correct answers in the task between the three groups. The results are presented in Table 2.

Table 2 Demographic and clinical characteristics of the prospective sample

	Healthy controls n=40	**MDD patients n=23**	**BD patients n=15**	**Level of significance in the MDD/BD comparison**	**Level of significance in the HC/MDD and BD comparison**
Age (mean ± SD)	40,73 (±10,93)	40,70 (±11,47)	46,13(±10,58)	p = 0,123[a]	p = 0,220[a]
Sex (M/F)	40 (15/25)	23 (9/14)	15 (5/10)	p = 0,131[b]	p = 0,935[b]
Education (years)	16,1 (±3,57)	14,17(±2,55)	13,80 (±2,81)	p = 0,744[a]	p = 0,051[a]
MADRS	2,51 (±1,64)	30,83 (±4,7)	29,67 (±5,07)	p = 0,749[a]	p < 0,001[a]
YMRS	0,59 (±0,79)	1,14 (±0,85)	0,93 (±0,8)	p = 0,512[a]	p < 0,036[a]
HAM-A	4,54 (±3,63)	22,05 (±8,21)	19,21 (±6,57)	p = 647[a]	p < 0,001[a]
Age at onset	-	28,68 (±10,81)	31,20 (±10,58)	p = 0,345[a]	-
Illness duration (months)	-	150,91 (±126,97)	85,87 (±109,52)	p = 0,278[a]	-
Episode duration (weeks)	-	24,32 (±34,57)	10,53 (±11,34)	p = 0,176[a]	-
Number of previous episodes	-	21 (±4,62)	14 (±7,93)	p = 0,007[a]	-
Percentage of correct answers	96,47 (±7,97)	97,21 (±4,2)	93,95 (±9,01)	p = 0,456[a]	p = 0,177[a]

[a] – Independent Samples Kruskal-Wallis test; [b] - $\chi 2$ test

Results

In the sample in which the expression of long non-coding RNAs was examined, no statistically significant difference was found in the distribution of sex and age between healthy individuals and patients, but one was found for the indicator of education. For clinical indicators, there were no differences between the two patient groups except for the number of previous episodes and, as expected, there was a statistically significant difference between HCs and patients for the MADRS and HAM-A scale scores (Table 3).

Table 3 Demographic and clinical characteristics of the sample in which lncRNAs were tested

	Healthy controls n=29	**MDD patients n=15**	**BD patients n=12**	**Level of significance in the MDD/BD comparison**	**Level of significance in the HC/MDD and BD comparison**
Age (mean ± SD)	41,66 (±11,43)	45,07 (±11,96)	46,82(±11,25)	p = 0,715[a]	p = 0,398[a]
Sex (M/F)	29 (11/18)	15 (6/9)	10 (2/10)	p = 0,187[b]	p = 0,359[b]
Education (years)	16,48 (±3,32)	13,80(±2,60)	13,09 (±2,63)	p = 0,559[a]	p = 0,004[a]
MADRS	3,00 (±1,41)	31 (±4,14)	30,36 (±6,25)	p = 0,800[a]	p < 0,001[a]
YMRS	0,71 (±0,81)	1,20 (±0,86)	1,18 (±0,87)	p = 0,937[a]	p < 0,124[a]
HAM-A	4,85 (±3,71)	22,93 (±8,18)	22,09 (±7,56)	p = 0,860[a]	p < 0,001[a]
Age at onset	-	31,47 (±12,73)	27,82 (±8,81)	p = 0,550[a]	-
Illness duration (months)	-	163,73 (±124,46)	237,82 (±106,5)	p = 0,096[a]	-
Episode duration (weeks)	-	14,53 (±17,10)	10,27 (±4,88)	p = 0,619[a]	-

Number of previous episodes	-	5,27 (±3,90)	8,09 (±2,81)	p = 0,048[a]	-

[a] – Independent Samples Kruskal-Wallis test; [b] - $\chi 2$ test

The sample in which the expression levels of selected microRNAs were examined also showed statistical homogeneity with respect to sex and age in the HC and patient groups. Again, there was a statistically significant difference in the level of education between healthy subjects and patients, as well as in the scores of the MADRS and HAM-A clinical scales. There were no differences in demographic and clinical characteristics between the MDD and BD groups, except for the indicator number of previous episodes (Table 4).

Table 4 Demographic and clinical characteristics of the sample in which microRNAs were tested

	Healthy controls n=14	**MDD patients n=15**	**BD patients n=12**	**Level of significance in the MDD/BD comparison**	**Level of significance in the HC/MDD and BD comparison**
Age (mean ± SD)	43,31 (±10,29)	45,07 (±11,96)	47,08(±11,77)	p = 0,774[a]	p = 0,635[a]
Sex (M/F)	14 (5/9)	15 (6/9)	10 (2/10)	p = 0,187[b]	p = 0,400[b]
Education (years)	16,77 (±3,96)	13,80(±2,60)	13,00 (±2,52)	p = 0,537[a]	p = 0,023[a]
MADRS	3,00 (±1,41)	31 (±4,14)	30.33 (±5,96)	p = 0,733[a]	p < 0,001[a]
YMRS	0,77 (±0,83)	1,20 (±0,86)	1,25 (±0,87)	p = 0,924[a]	p < 0,535[a]
HAM-A	4,54 (±3,78)	22,93 (±8,18)	22.08 (±7,2)	p = 0,845[a]	p < 0,001[a]
Age at onset	-	31,47 (±12,73)	27.42 (±8,51)	p = 0,550[a]	-
Illness duration (months)	-	163,73 (±124,46)	245.00 (±104.55)	p = 0,096[a]	-

Episode duration (weeks)	-	14,53 (±17,10)	10,58 (±4.78)	p = 0,619[a]	-
Number of previous episodes	-	5,27 (±3,90)	7,67 (±3.06)	p = 0,048[a]	-

[a] – Independent Samples Kruskal-Wallis test; [b] - $\chi 2$ test

2. Results of the neuroimaging data analysis of the retrospective sample

According to the literature, patients with affective disorders show aberrant connectivity in the salience network; therefore, we performed a seed-to-voxel analysis of the functional connectivity of the 3 main regions of the corresponding neural network at the whole-brain level, namely the anterior cingulate cortex, left and right anterior insula.

2.1. Functional connectivity of the anterior cingulate cortex

First, to test the robustness of the results, we conducted a within-group analysis of functional connectivity to identify expected associations between regions of the salience, frontoparietal, and default mode networks (Fig. 2)

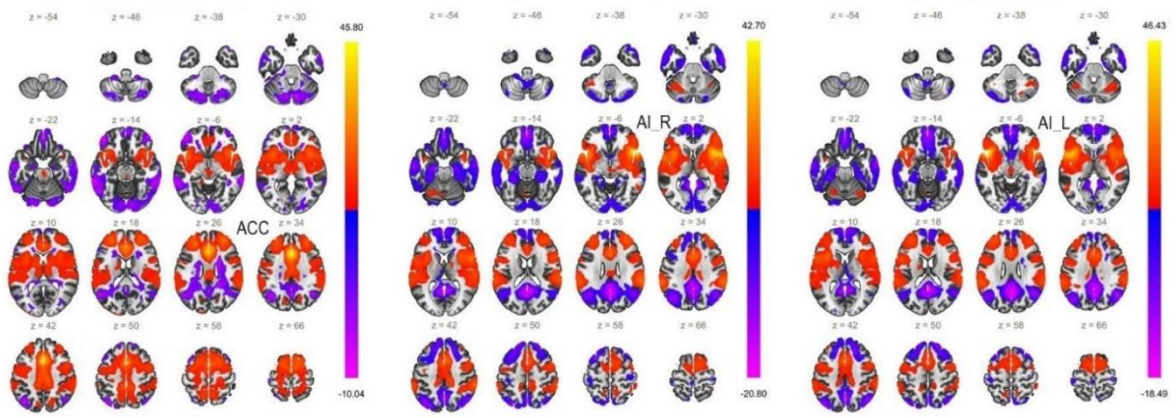

Figure 2 Functional connectivity profile of each of the selected regions in the whole sample at statistical significance threshold p<0.05 with Family-wise error (FWE) correction at cluster level.

The analysis of the functional connectivity of the anterior cingulate cortex revealed increased FC to the left and right precentral gyrus, left and right postcentral gyrus, superior parietal lobule bilaterally, and left lateral occipital cortex (inferior lobe) in patients compared with HC (Table 5, Fig. 3). At the same time, there was a reduced FC between the ACC and the parahippocampal gyrus in the group of depressive patients in contrast to HC.

Table 5 Statistically significant differences in the FC of ACC in the intergroup comparison

Intergroup contrast	**MNI coordinates x,y,z**	**Cluster size**	**Level of significance (p<0.05, FWE)**	**Regions in the cluster**
P>HC	+32 -4 +46	697	0.000	right: PostCG, PreCG, SPL
	+2 -34 +52	526	0.000	right: PostCG, SPL, PreCu, LOC
				left postCG
	-24 -48 +52	106	0.017	Left SPL
HC>P	-10 -10 -24	109	0.015	l-PHG, brain stem
BD>HC	-02 -36 +66	1393	0.000	right: PostCG, SPL
				l-PostCG, PreCu
	+24 -12 +66	120	0.009	right: PreCG, SFG
	-2 -14 +44	105	0.018	ACC, PCC, SMC
	+18 -72 +40	86	0.047	right: LOC, CC
				PreCu
HC>BD	+24 -92 -28	138	0.004	right: CerC1, CerC2
MDD>HC	+22 -56 +56	112	0.013	right: SPL, LOC
MDD>BD	-46 -70 -32	191	0.000	l-CerC1

PostCG – postcentral gyrus; PreCG – precentral gyrus; SPL – superior parietal lobule; LOC – lateral occipital cortex; PHG – parahippocampal gyrus; PreCu - precuneus; CerC1 – cerebellar crus 1; CerC2 – cerebellar crus 2; SFG – superior frontal gyrus; ACC – anterior cingulate cortex; PCC – posterior cingulate cortex; SMC – supplementary motor cortex; CC – cuneal cortex

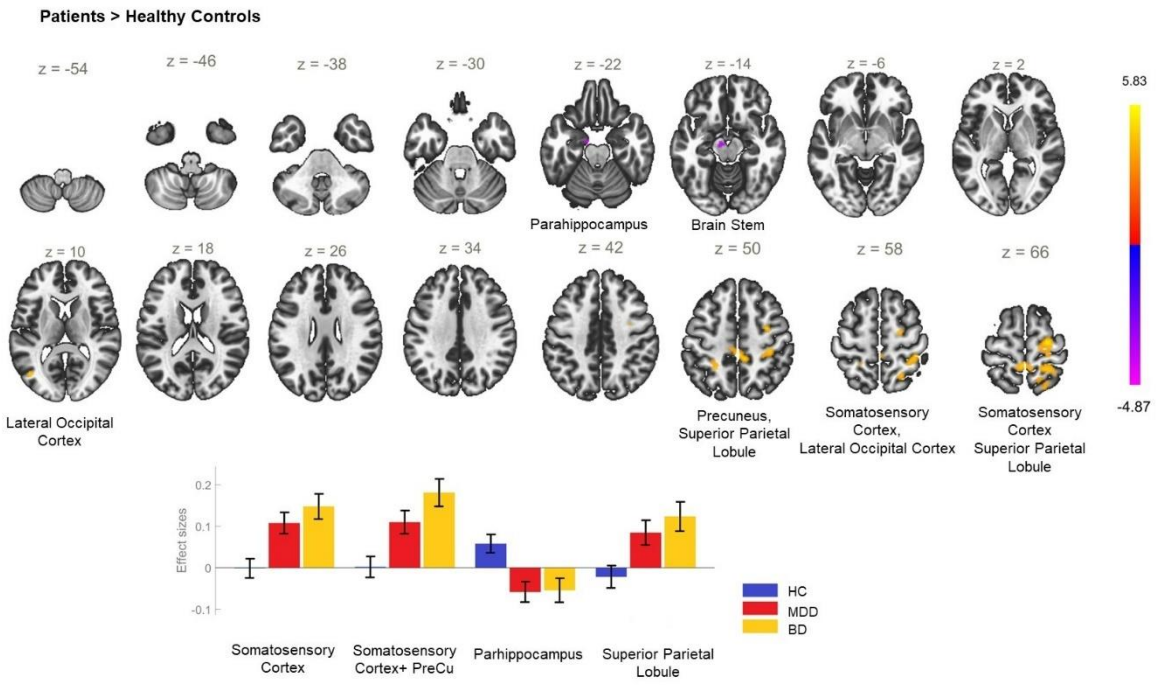

Figure 3 FC of ACC in all patients compared to HC.

In the bipolar depression group, there was increased FC between the PFC and the somatosensory cortex, superior frontal gyrus, posterior cingulate cortex, and lateral occipital cortex, and decreased FC between the PFC and the right cerebellar cruri in contrast to HC (Fig. 4).

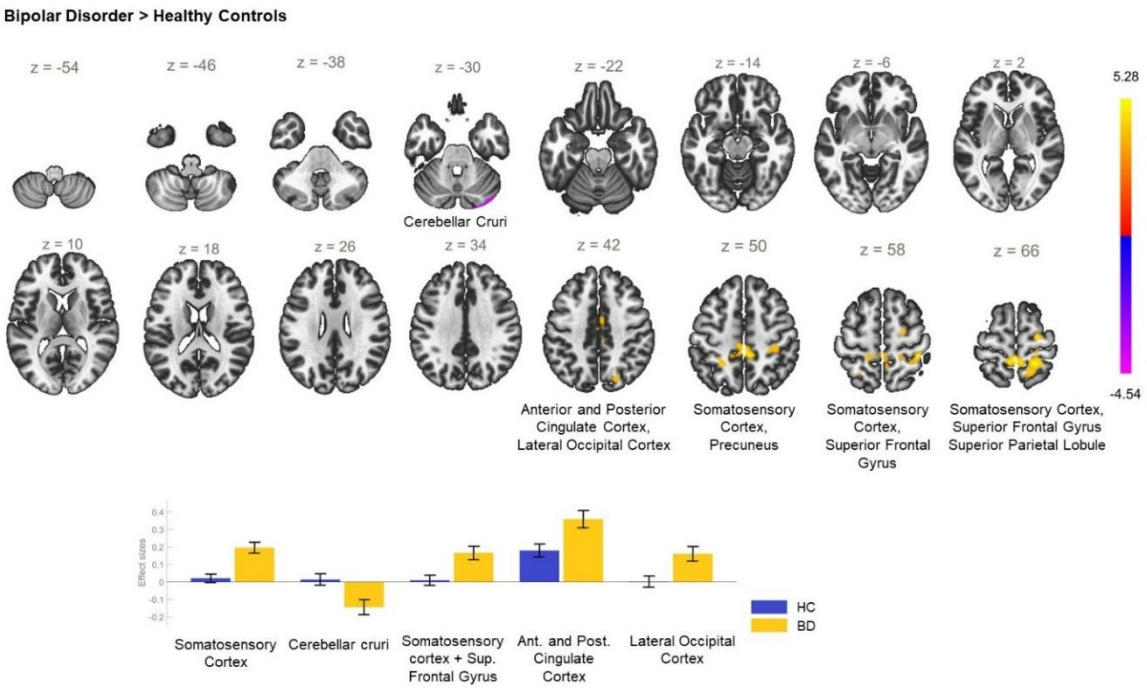

Figure 4 FC of ACC in patients with bipolar depression compared to HC.

Increased FC was observed between the ACC and the right superior parietal lobule as well as the right occipital cortex in MDD as opposed to HC (Fig.. 5).

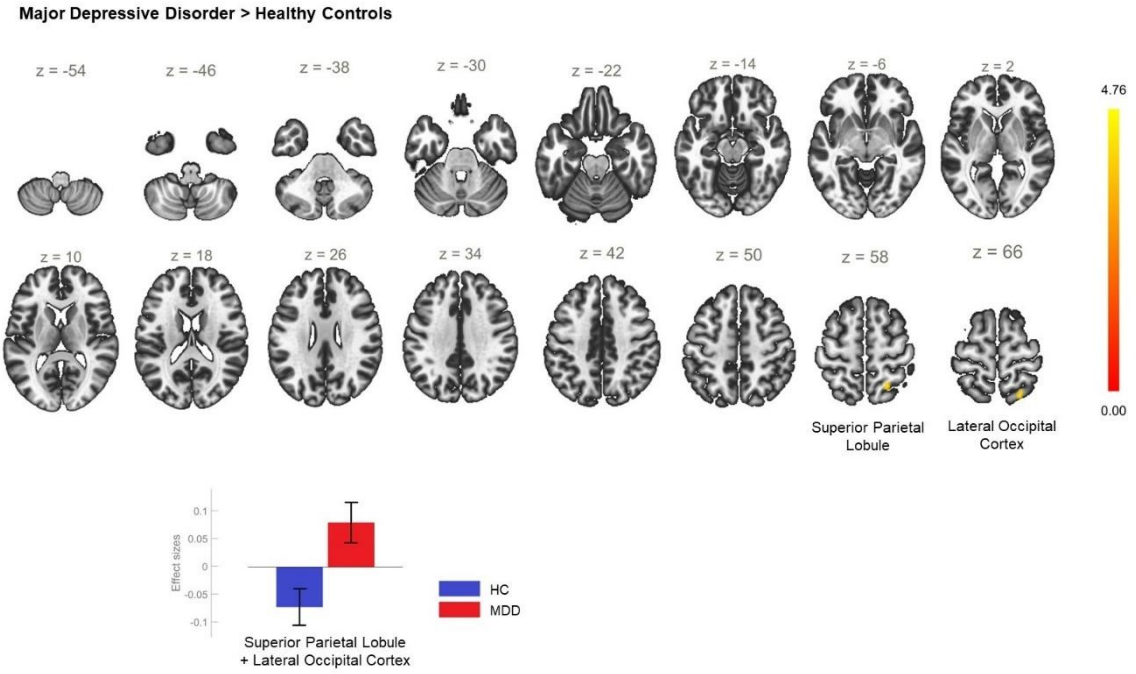

Figure 5 FC of ACC in the comparison between MDD and HC.

The direct comparison between MDD and BD showed a statistically significant hyperconnectivity of the ACC with the left cerebellar cruri in MDD that was not observed in bipolar depression (Fig. 6).

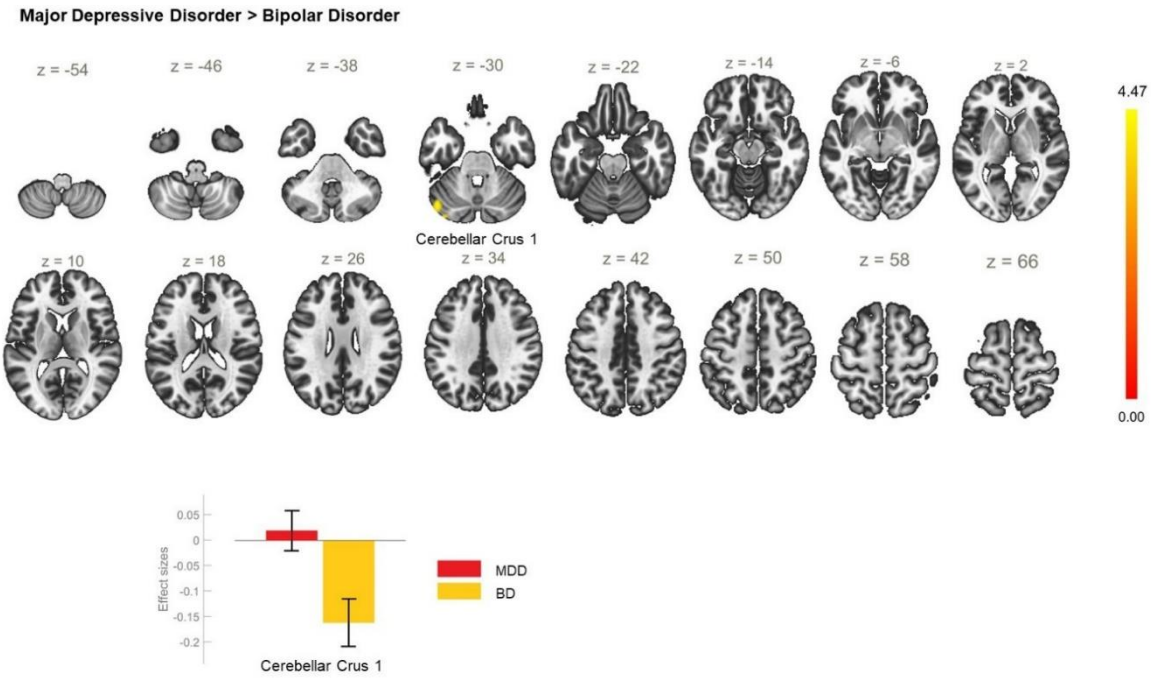

Figure 6 FC of ACC in unipolar as compared to bipolar depression.

2.2. Functional connectivity of left and right anterior insula

When comparing the FC of lAI, no statistically significant differences were found at the intergroup level. The results of the comparison of the FC of rAIs are presented in Table. 6.

Table 6 Clusters with statistically significant differences in anterior insula FC in the intergroup comparison

Intergroup contrast	MNI coordinates x,y,z	Cluster size	Level of significance (p<0.05, FWE)	Regions in the cluster
P>HC	-8 -52 +64	201	0.000	l-PostCG, PreCu
BD>HC	+54 -06 +18	108	0.017	right: PostCG, PreCG
MDD>BD	-4 +12 +58	80	0.068*	left: SFG right: SFG, SMC

Patients generally showed increased FC between the rAI and the left postcentral gyrus, the left superior parietal lobule, and the precuneus compared with healthy subjects (Fig. 7).

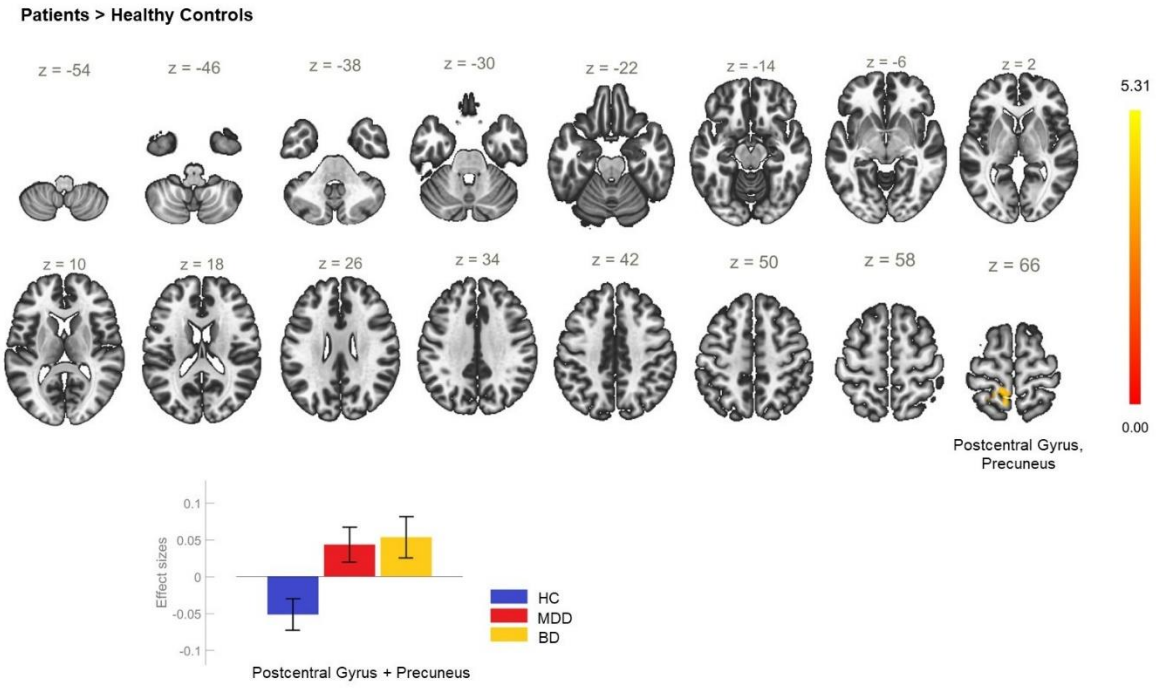

Figure 7 FC of r-AI in the comparison between patients and HC.

In the BD group, hyperconnectivity between the r-AI and right precentral gyrus and r-AI and right postcentral gyrus was observed in contrast to the group of healthy individuals (Fig. 8).

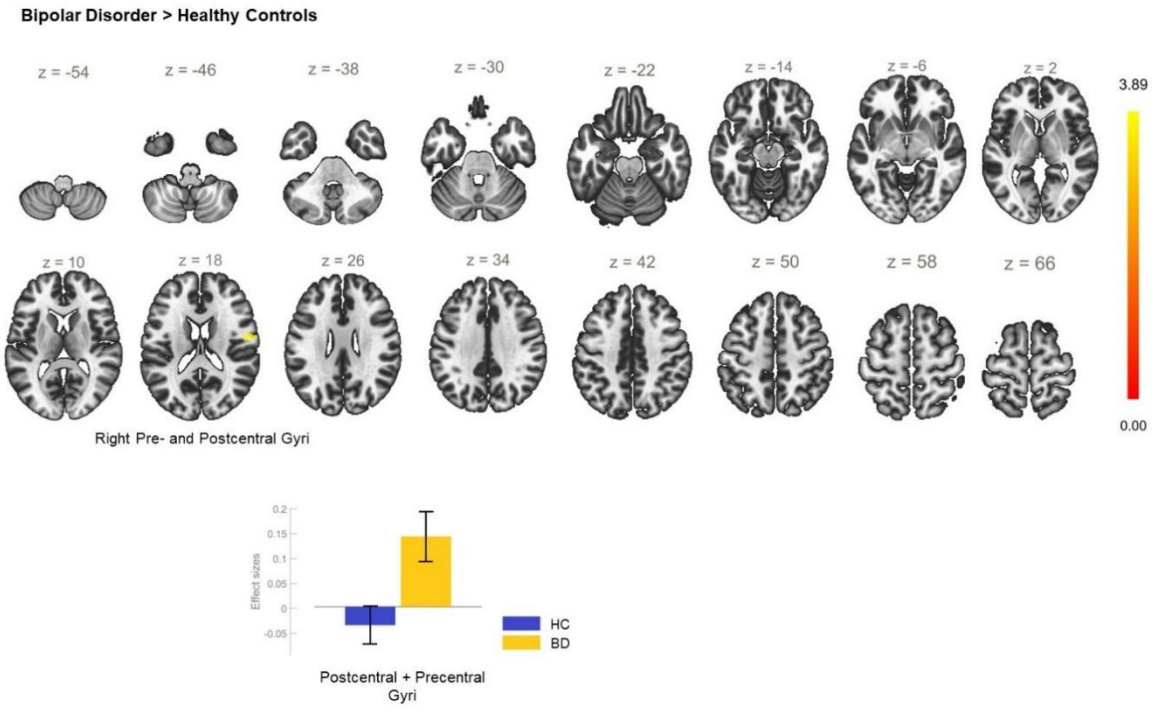

Figure 8 FC of r-AI in BD compared HC.

There were no statistically significant differences between MDD and HC , but there was a trend towards significance (p=0.068) in the comparison between

MDD and BD (Fig. 9).

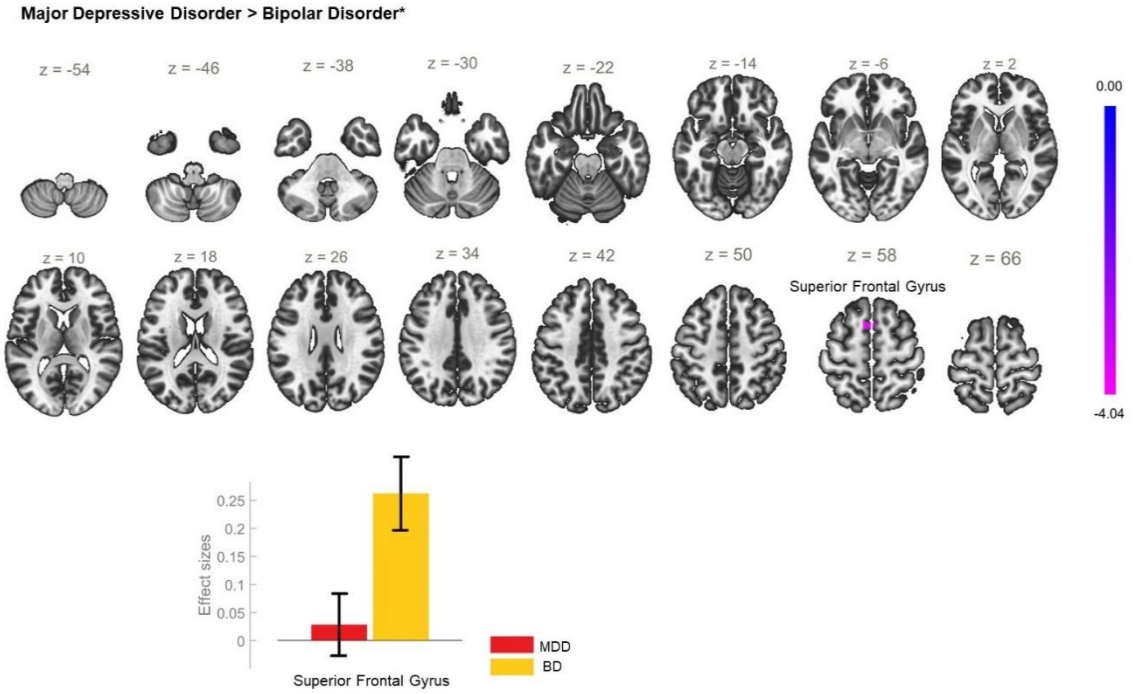

Figure 9 FC of the r-AI in MDD and BD (p=0.068).

3. Results of the neuroimaging data analysis of the prospective sample

3.1. Contrast results: neutral distractors > no distractors

At the within-group level (one-sample t-test), significant activations were found in HC with a significance level at cluster level of p<0. 05 with FWE correction in the following regions - right: occipital fusiform, lingual, middle frontal, trigeminal part of inferior frontal gyri and right external cerebellum and left: orbital (anterior, posterior, medial and lateral), middle and inferior frontal (including the opercular part), parahippocampal gyri, hippocampus and thalamus.

In the MDD group, residual activations were observed in the following structures: left: occipital pole, calcarine, posterior and lateral orbital gyrus, and medial frontal cortex and right: occipital fusiform gyrus, lingual gyrus, cuneus, posterior, medial, and lateral orbital gyri, medial frontal cortex, and anterior cingulate cortex (Fig. 10A).

In the BD group, residual activations at this contrast were found in - right: lingual gyrus, calcarine, occipital pole, occipital fusiform gyrus, thalamus, hippocampus, parahippocampus, amygdala, inferior and middle frontal gyrus, and left: medial orbital gyrus, hippocampus, parahippocampus, putamen, amygdala (Fig. 10B). The results are presented in Table 7.

Table 7 Statistically significant clusters in the contrast neutral distractors > no distractors at the within-group level

Group	MNI coordinates x, y, z	Cluster size	Level of significance p<0.05, FWE	Regions in the cluster
HC	22 -86 -10	190600	<0.001	right: OFG, LG , CerExt
	-32 30 -16	6437	<0.001	left: POG, LOG, AOG, MOG, IFG
	-52 28 30	457	<0.001	left: MFG, IFG, op.p
	-22 -28 -4	224	0.005	left: Thal, HIP, PHG
	56 34 8	209	0.007	right: IFG, tr.p, MFG
MDD	-12 -96 0	137240	<0.001	left: OP, CLC right: OFG, LG, OP, CC
	26 26 -12	427	<0.001	right: POG, MOG, AI, LOG
	-32 32 -16	268	0.002	left: POG, LOG
	8 50 -10	268	0.002	Right: MedFC, SFG, ACC left: MedFC
BD	14 -90 -6	126390	<0.001	right: LG, CLC, OP, OFG
	20 -30 -4	406	<0.001	right: Thal, HIP, PHG, AMY
	-12 8 -16	296	0.001	left: MOG, HIP, PTN, AMY, PHG
	58 32 16	221	0.005	right: IFG, tr.p, MFG

OFG – occipital fusiform gyrus; LG – lingual gyrus; CerExt – cerebellum externum; POG – posterior orbital gyrus; LOG – lateral orbital gyrus; AOG – anterior orbital gyrus; MOG – medial orbital gyrus; IFG – inferior frontal gyrus; MFG – middle frontal gyrus; IFG, op.p – opercular part of the inferior frontal gyrus; Thal - thalamus; HIP - hippocampus; PHG – parahippocampal gyrus; IFG, tr.p – triangular part of the inferior frontal gyrus; OP – occipital

pole; CLC – calcarine cortex; CC – cuneal cortex; AI – anterior insula; MedFC – medial frontal cortex; SFG – superior frontal gyrus; ACC – anterior cingulate cortex; AMY – amygdala; PTN – putamen; PreCu – precuneus;

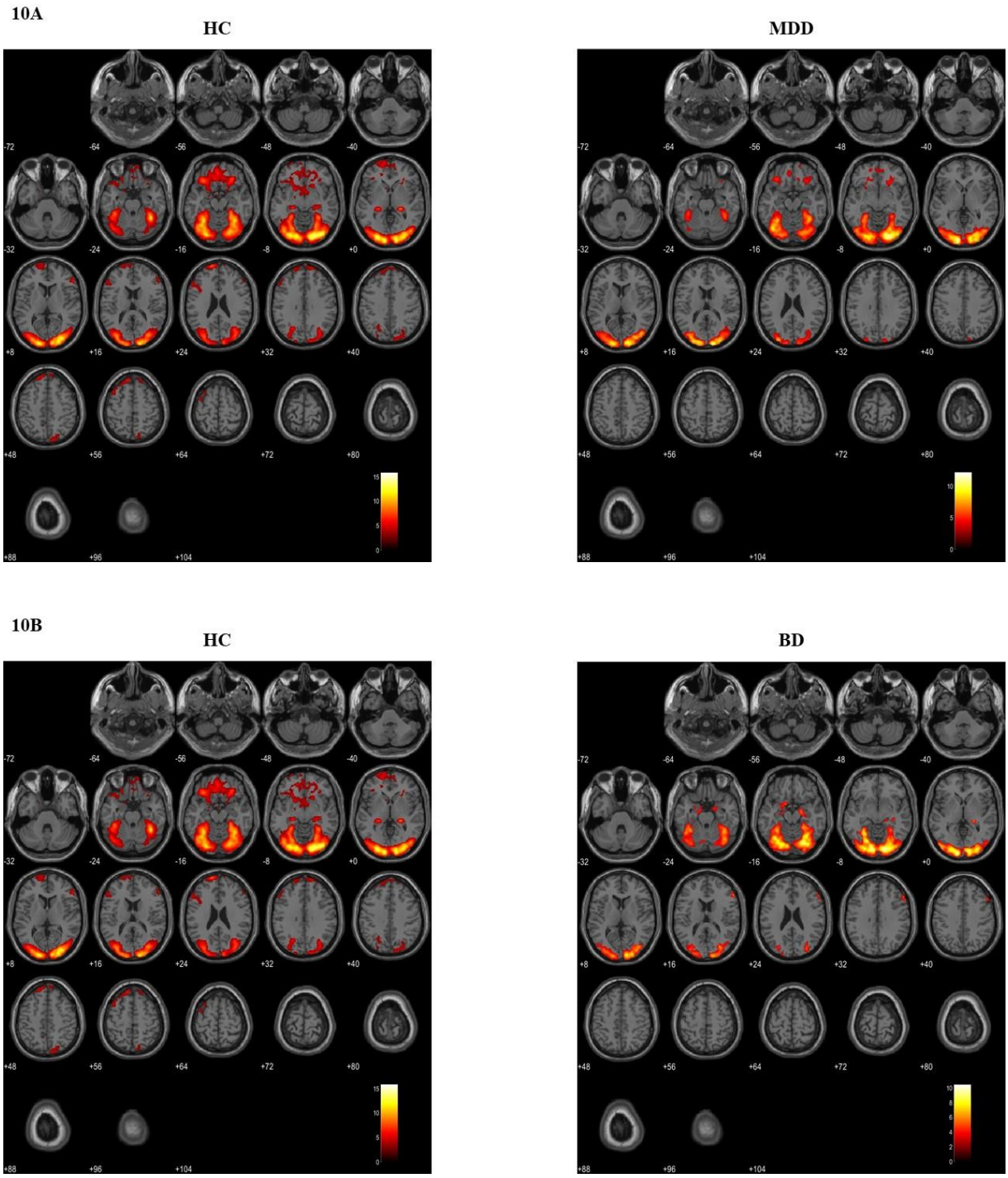

Figure 10 Residual activations in the contrast neutral distractors>no distractors at an intragroup level.

The direct comparison between HC and BD did not yield statistically significant brain activity differences in this contrast, but HC in comparison with MDD showed significant activations in several left-sided regions including: superior and middle temporal gyri, middle frontal gyrus, precuneus, posterior

cingulate cortex, superior parietal lobule, supramarginal gyrus, and angular gyrus. On the other hand, comparison between BD and MDD revealed right-sided hyperactivity in: lingual gyrus, occipital fusiform gyrus and external cerebellum in BD patients in contrast to the MDD group (Fig. 11A). At the intergroup level, the comparison between HC and all patients (P) revealed residual activations with a trend towards significance at the cluster level p<0.062 with FWE correction in HC in the left middle and left superior frontal gyri (Fig. 11B). The results thus described are presented in Table 8.

Таблица 8. Statistically significant clusters in the contrast neutral distractors > no distractors at intergroup level

Group	MNI coordinates x, y, z	Cluster size	Level of significance p<0.05, FWE	Regions in the cluster
HC > P	-32 6 44	128	0.062*	left: MFG, SFG
HC > MDD	-34 6 46	409	<0.001	l-SFG
	-10 -56 42	273	<0.002	left: PreCu, PCC, SPL
	-52 -50 18	144	0.039	left: STG, MTG, SMG, AngG
BD > MDD	36 -68 -20	252	0.003	right: CerExt, OFG, LG

MTG – middle temporal gyrus; STG – superior temporal gyrus; MTG – middle temporal gyrus; SMG – supramarginal gyrus; AngG – Angular gyrus, PCC – posterior cingulate cortex; SPL – superior parietal lobule;

In the neutral distractors>no distractors contrast, the comparison between HC and MDD showed residual activations in structures associated with cognitive functioning such as the frontal and temporal cortices, and precuneus with concomitant activation of the supramarginal gyrus and the PCC (Figure 11A). There was a trend toward significance (p = 0.062) when comparing the HC group with the group of all patients with residual activations in the middle and superior frontal gyri in HC (figure 11B).

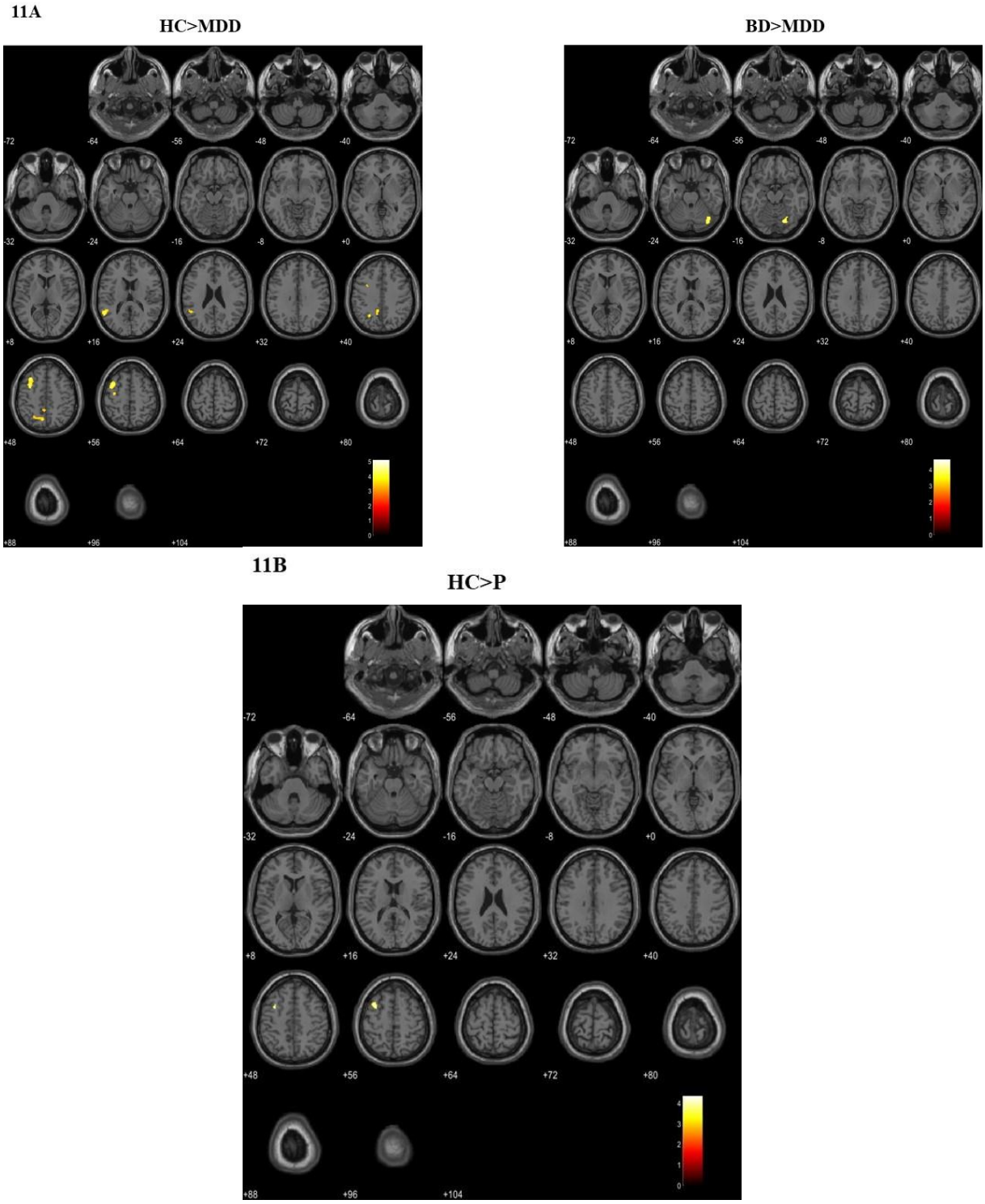

Figure 11 Residual activations in the contrast neutral distractors > ano distractors at intergroup level.

3.2. Contrast results: negative distractors>no distractors

At the intragroup level (one-sample t-test), significant activations were found in HC in the following regions (with a significance level at the cluster level of p<0. 05 with FWE correction) - right: occipital fusiform gyrus, lingual gyrus,

gyrus rectus, medial frontal cortex, precuneus, posterior cingulate cortex, middle and inferior frontal gyrus and left: middle and inferior occipital gyrus, calcarine cortex, occipital pole, lingual gyrus, anterior, posterior and lateral orbital gyrus, superior, medial, middle and inferior frontal gyrus, gyrus rectus, precuneus, posterior cingulate cortex.

MDD showed residual activations in - left: calcarine cortex, lingual gyrus, occipital pole, inferior and fusiform occipital gyri, superior frontal gyrus and frontal pole and right: lingual gyrus, occipital fusiform gyrus, occipital pole, cuneus, superior and inferior frontal gyri, thalamus, hippocampus, parahippocampus, amygdala, pallidum, putamen, posterior, lateral and medial orbital gyri and anterior insula (Fig. 12A).

In the BD group, significant activations were found in the following regions - left: calcarine cortex, lingual gyrus, occipital and temporal pole, occipital fusiform gyrus, amygdala, thalamus, hippocampus, parahippocampus, entorhinal region, fusiform gyrus, superior and medial frontal gyri, posterior orbital gyrus, gyrus rectus and anterior cingulate cortex and right: external cerebellum, occipital fusiform gyrus, lingual gyrus, calcarine cortex, occipital and temporal pole, hippocampus, parahippocampus, amygdala, entorhinal area,posterior orbital gyrus, superior, middle, medial and inferior frontal gyri, gyrus rectus, right thalamus (Fig. 12B). The above results are presented in more detail in Table 9.

Table 9 Statistically significant clusters in the contrast negative distractors > no distractors at intragroup level

Group	MNI coordinates x, y, z	Cluster size	Level of significance p<0.05, FWE	Regions in the cluster
HC	22 -86 -10	210800	<0.001	right-OFG, LG and left: IOcG, MOcG, CLC, OP, LG
	-34 32 -18	105310	<0.001	right: GR, medFC left: POG, LOG, AOG, SFG, GR, medFC
	-16 -54 10	545	<0.001	right: PreCu, PCC left: PreCu, PCC, LG
	-50 32 12	442	<0.001	left: IFG, tr,p., MFG, IFG, op.p.

	58 32 14	434	<0.001	right: IFG, tr.p., MFG, IFG, op.p.
	18 -50 8	204	0.012	right: PreCu, LG, PCC
	26 -18 76	144	0.053	right: PreCG, PostCG
MDD	-12 -94 -2	147910	<0.001	left: CLC, OP, LG, OFG, IOcG right: LG, OFG, OP, CC
	0 60 4	2539	<0.001	right: SFG, FP left: SFG, FP
	20 -30 -2	430	<0.001	right: Thal, HIP, PHG, AMY, PAL, PTN
	30 30 -8	327	0.001	right: POG, AI, IFG, op.p., LOG, MedOG, CerExt, OFG, LG, CLC, OP
BD	-10 -92 -4	152600	<0.001	left: CLC, LG, OP, OFG
	-22 -2 -20	1125	<0.001	left: AMY, EntA, Thal, HIP, PHG, FG
	22 -30 -2	977	<0.001	right: Thal, HIP, PHG, AMY, EntA, TP, POG
	-10 60 12	843	<0.001	l-SFG r-SFG
	58 32 16	639	<0.001	right: IFG, tr.p., MFG, IFG, op.p.
	-36 22 -30	233	0.006	left: TP, POG
	-2 46 -18	159	0.036	left: medFC, GR, ACC right: medFC, GR

OFG – occipital fusiform gyrus; LG – lingual gyrus; IOcG – inferior occipital gyrus; MOcG – middle occipital gyrus; CLC – calcarine cortex; OP – occipital pole; POG – posterior orbital gyrus; LOG – lateral occipital gyrus; AOG – anterior orbital gyrus; SFG – superior frontal

gyrus; GR – gyrus rectus; medFC – medial frontal cortex; PreCu – precuneus; PCC – posterior cingulate cortex; IFG, tr.p. – triangular part of the inferior frontal gyrus; MFG – middle frontal gyrus; IFG, op.p. – opercular part of the inferior frontal gyrus; PreCG – precentral gyrus; PostCG – postcentral gyrus; CC – cuneal cortex; FP – frontal pole; Thal – thalamus; HIP – hippocampus; PHG – parahippocampal gyrus; AMY – amygdala; PAL – pallidum; PTN – putamen; AI – anterior insula; MedOG – medial orbital gyrus; CerExt - cerebellum externum; EntA – entorhinal area; TP – temporal pole; ACC – anterior cingulate cortex

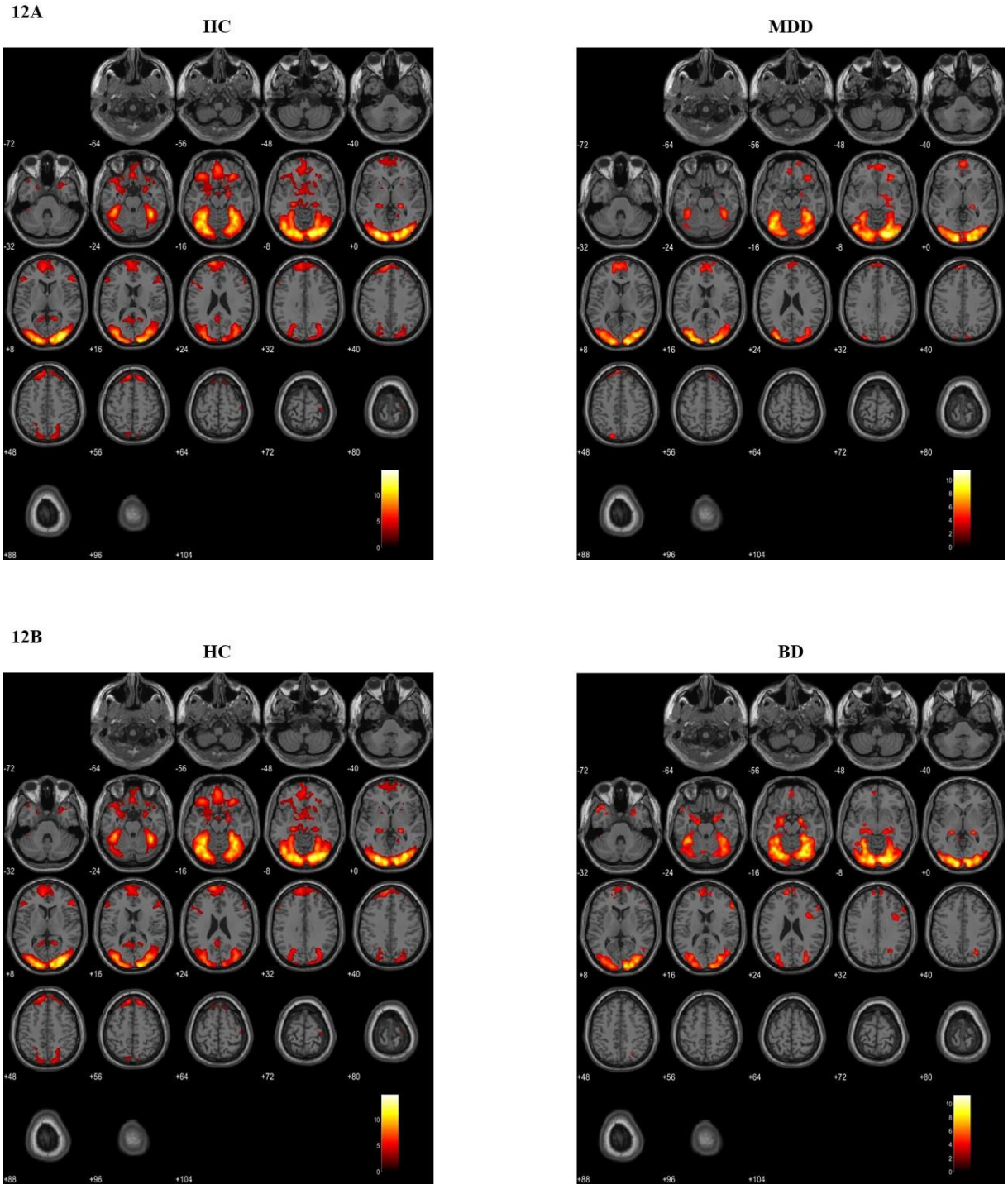

Figure 12 Residual activations in the contrast negative distractors > no distractors at intragroup level.

Results

A direct comparison between BD and MDD demonstrated the presence of statistically significant residual right-sided activations in the external cerebellum, occipital fusiform gyrus, lingual gyrus, and inferior occipital gyrus in patients with BD as opposed to MDD (Table 10, Fig. 13). The other intergroup comparisons yielded no residual activations crossing the statistical significance threshold.

Table 10 Statistically significant clusters in the contrast negative distractors > no distractors at intergroup level

Group	MNI coordinates x, y, z	Cluster size	Level of significance p<0.05, FWE	Regions in the cluster
BD>MDD	24 -72 -18	179	0.022	right: CerExt, OFG, LG, IOcG

CerExt – cerebellum externum, OFG – occipital fusiform gyrus, LG – lingual gyrus, IOcG – inferior occipital gyrus

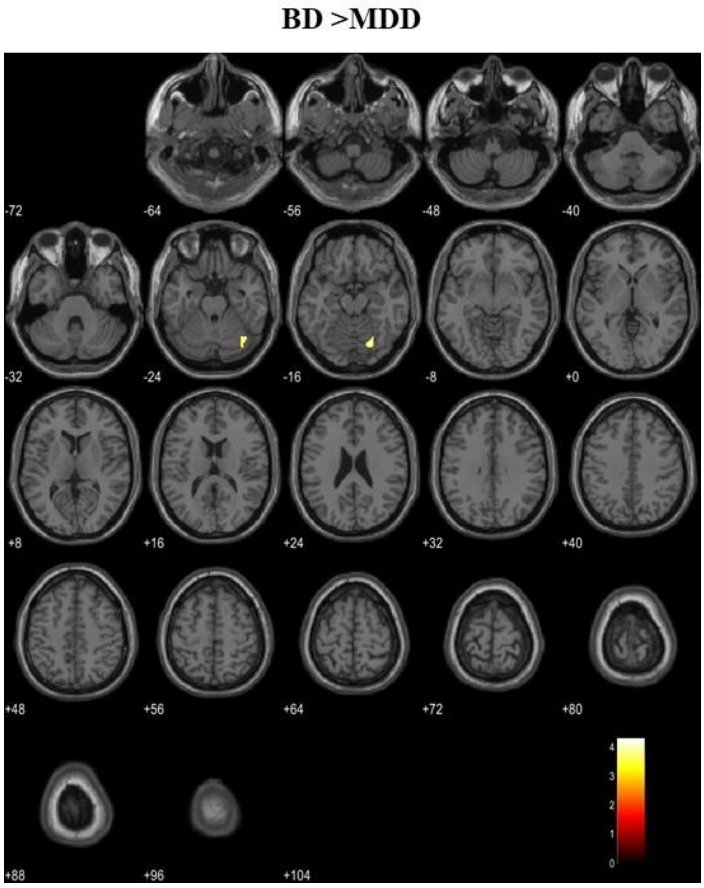

Figure 13 Residual activations in the contrast negative distractors > no distractors at intergroup level.

3.3. Contrast results: negative distractors>neutral distractors

At the intragroup level (one-sample t-test), significant activations were found in HC with a significance level at the cluster level of p<0.05 with FWE correction in the following regions - left: middle and inferior temporal gyri, middle and inferior occipital gyri, fusiform gyrus, occipital pole, superior frontal gyrus, anterior cingulate cortex, precuneus and right: inferior temporal gyrus, middle and inferior occipital gyri, occipital fusiform gyrus, superior frontal gyrus, posterior cingulate cortex and precuneus.

In MDD, statistically significant residual activations were observed in the - left: superior, middle and inferior occipital gyri, occipital fusiform gyrus, superior frontal gyrus, anterior and posterior cingulate cortex, precuneus and right: middle and inferior occipital gyri, occipital fusiform gyrus, external cerebellum, inferior temporal gyrus, superior and inferior frontal gyri, anterior cingulate cortex, frontal pole, and frontal operculum (Fig. 14A).

Significant activations in the BD group emerged in the following regions - right: middle and inferior occipital gyri, occipital fusiform gyrus, occipital pole, calcarine cortex, inferior temporal gyrus, external cerebellum, thalamus, hippocampus, parahippocampus, lingual gyrus, precuneus, posterior cingulate cortex, and left: thalamus, superior frontal gyrus, frontal and occipital pole, anterior cingulate cortex, superior, middle and inferior occipital gyri (Fig. 14B).

In this contrast, intergroup comparison yielded no statistically significant residual activations. The results are presented in Table 11.

Table 11 Statistically significant clusters in the contrast negative distractors>neutral distractors

Group	MNI coordinates x, y, z	Cluster size	Level of significance p<0.05, FWE	Regions in the cluster
HC	-44 -62 -8	2939	<0.001	left: ITG, IOcG, MTG, FG, MOcG, OP
	46 -62 -12	2118	<0.001	right: ITG, IOcG, OFG, MOcG
	-6 56 32	1056	<0.001	left: SFG, ACC right: SFG
	-4 -52 18	756	<0.001	left: PCC, PreCu right: PCC, PreCu

MDD	-40 -84 2	1261	<0.001	left: IOcG, MOcG, SOcG, OFG
	32 -82 4	679	<0.001	right: IOcG, MOcG, OFG, CerExt, ITG
	12 48 20	478	<0.001	right: SFG, ACC, FP left: SFG, ACC
	-8 -48 10	139	0.048	left: PCC, PreCu, LG
	42 28 2	137	0.051	right: FO, IFG, orb.p., IFG, tr.p.,
BD	30 -86 -4	482	<0.001	IOcG, OFG, OP, CLC, IOcG, MOcG
	42 -62 -12	459	<0.001	right: ITG, OFG, IOcG, CerExt
	-6 -14 4	290	0.001	left: thal right: thal, HIP, PHG
	-8 58 26	258	0.002	left: SFG, FP, ACC
	8 -54 2	207	0.008	right: LG, PreCu, PCC, CLC
	-22 -92 20	184	0.015	left: SOcG, MOcG, IOcG, OP

ITG – inferior temporal gyrus; IOcG – inferior occipital gyrus; MTG – middle temporal gyrus; FG – fusiform gyrus; MOcG – middle occipital gyrus; OP – occipital pole; OFG – occipital fusiform gyrus; SFG – superior frontal gyrus; ACC – anterior cingulate gyrus; PreCu – precuneus; PCC – posterior cingulate cortex; SOcG – superior occipital gyrus; CerExt – cerebellum externum; FP – frontal pole; LG – lingual gyrus; FO – frontal operculum; IFG, orb.p. – orbital part of the inferior frontal gyrus; IFG, tr.p. – triangular part of the inferior frontal gyrus; CLC – calcarine cortex; Thal – thalamus; HIP – hippocampus; PHG – parahippocampal gyrus;

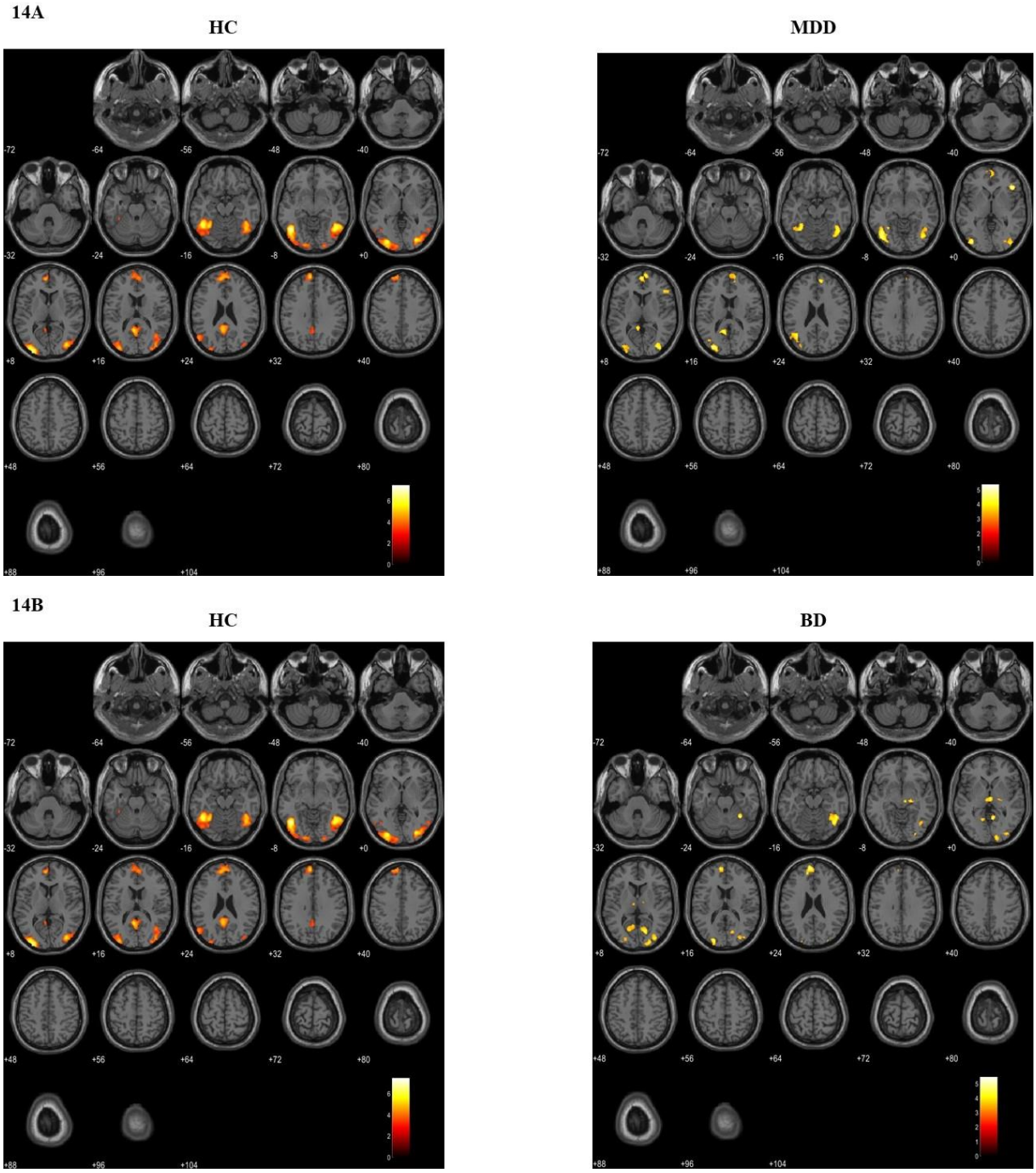

Figure 14 Residual activations in the contrast negative distractors > neutral distractors at intragroup level.

4. Results of molecular-biological analyses

At the intergroup level, no statistically significant differences were found in the expression level of the selected long non-coding RNAs in the analyzed sample of HC = 29, MDD = 11 and BD = 16. When the expression levels of selected microRNAs were examined in a sample of 14 HC, 11 MDD, and 16 BD patients, there was a statistically significant decrease in the transcriptional levels

of let-7f, miR-30c, miR-212, miR-219 (Fig. 15), miR-125b, miR-126, miR-138, miR-146a, and miR-182 (Fig. 16) in the patient group.

Patients with bipolar depression were found to have decreased expression levels of let-7f, miR-30c, miR-212, miR-219, miR-125b, miR-126, miR-138, miR-146a and miR-182 in contrast to healthy controls. MDD patients presented with lower transcriptional levels of let-7f, miR-212, miR-219, miR-126, miR-138, miR-146a and miR-182 compared to HC. There was no statistically significant difference in the expression levels of the selected microRNAs between the unipolar and bipolar depression patient groups. The levels of miR-132 also showed no significant differences at the intergroup level (Fig. 15, 16).

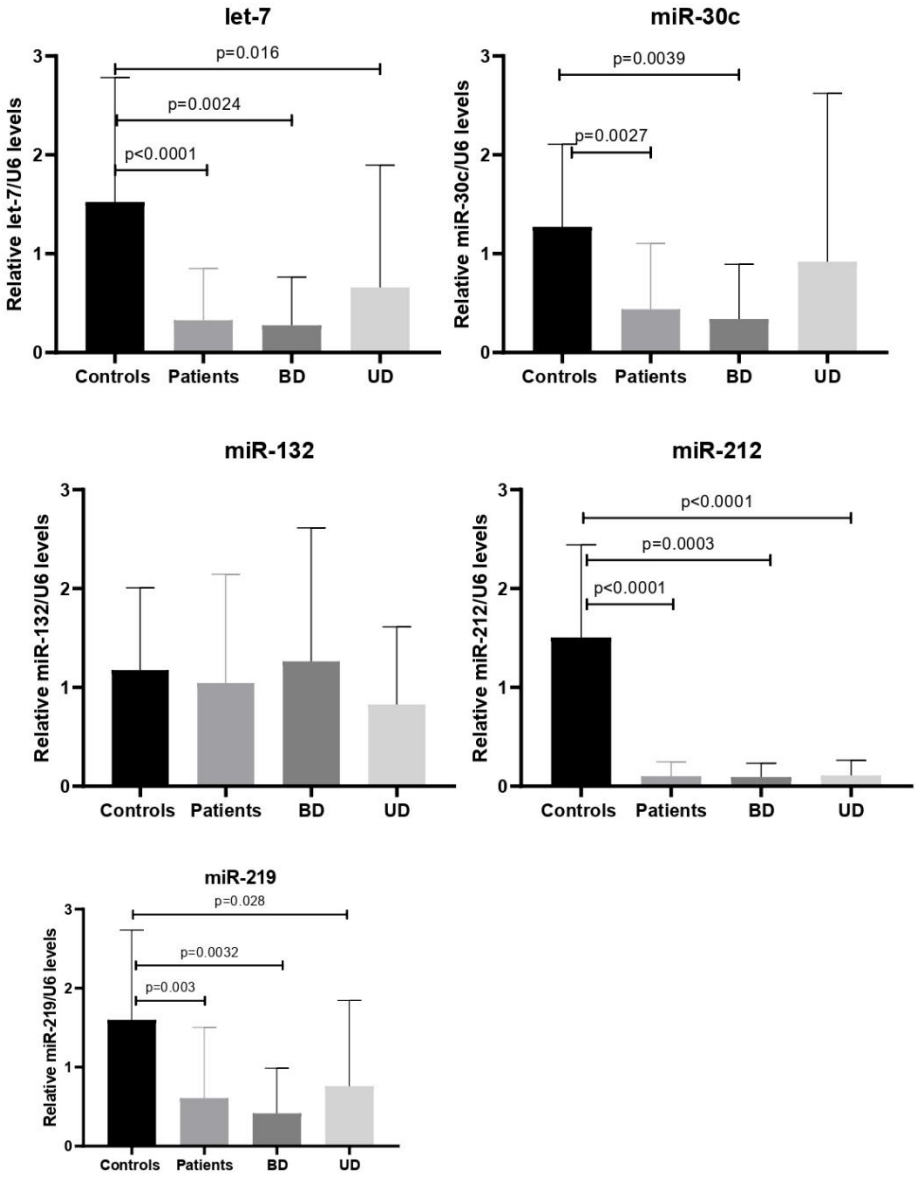

Figure 15 Intergroup differences in expression levels of selected microRNAs

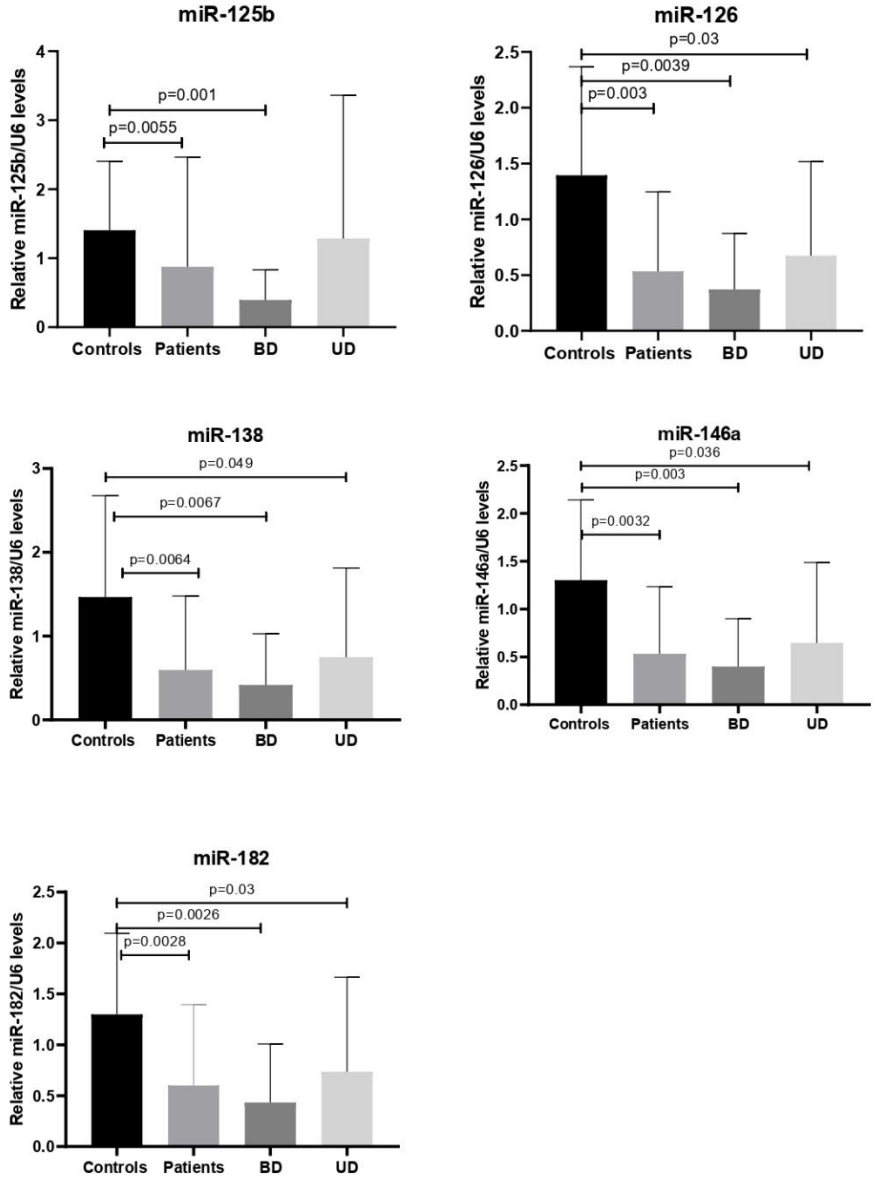

Figure 16 Intergroup differences in expression levels of selected microRNAs

Discussion

1. Discussion of the neuroimaging results of the retrospective sample

The first significant finding of this study was the increased functional connectivity between the ACC and the primary somatosensory and motor cortex, as well as the superior parietal lobule, precuneus, and right lateral occipital cortex in all patients with depressive syndrome compared to HC. These results seem to be driven by the BD patients, as the same regions were significant in the direct comparison of BD vs. HC.

It is known that the ACC has multiple functions. Of great relevance to depression is the processing of both physical and psychological pain, in which the ACC is associated with the affective component. The primary somatosensory cortex, on the other hand, is associated with the sensory-discriminative component. Increased functional connectivity between the thalamus and the somatosensory cortex (SCC) has been found in MDD compared toHC, which correlates with the severity of core symptoms of MDD, e.g., cognitive dysfunction, anhedonia, etc. Considering our findings of increased FC between ACC/SCC in the patient group, we speculate that depression may be associated with impairment in sensory-discriminative function of the SCC, leading to the phenomenological signature of psychic pain in both MDD and BD.

Another interesting finding in our study was that the FC between the ACC and the right superior parietal lobule (SPL) was increased in both MDD and BD compared to HC. The SPL has been implicated in the integration of somato-sensory and visuomotor processes and especially in the simultaneous processing of multiple visual stimuli. Therefore, the ACC-SPL hyperconnectivity may explain the common cognitive symptoms in both disorders - impaired perception, hypoprosexia and pseudodementia.

A compelling finding in this study was the reduction in FC between the ACC and the cerebellum in BD in contrast to HC and between the ACC and left cerebellar cruri in BD compared to MDD. The cerebellum is involved in the processing of psychological pain, and given the significant relationship between psychological and physical pain, and between psychological pain and suicide risk in depression, changes in its connectivity may not only be a prospective diagnostic but also a therapeutic marker. It should be noted that cerebellar dysfunctions are also relevant to psychomotor regulation, which has been theorized to be associated with increased connectivity between the ACC and primary motor cortex.

Lateralization of brain function remains one of the most intriguing areas of study, especially since it has been demonstrated in both vertebrate and invertebrate species. In our study, changes in the right AI FC demonstrated significant differences between groups in contrast to the left AI, raising the

question of the functional specificity of the right AI as a region of interest in the pathophysiology of mood disorders. Recent scientific evidence suggests an important role for the right AI in interoceptive processes. Furthermore, the r-AI is associated with the affective-perceptual form of empathy, whereas the l-AI is presumably involved in both the cognitive-appraisal and affective-perceptual forms. These findings, coupled with the present results of our study confirming the changes in the relationship between r-AI, somatosensory and motor cortex in patients with bipolar depression, suggest an impairment in interoceptive and affective integration in BD compared to HC. Furthermore, the hypoconnectivity between r-AI and right middle frontal gyrus compared with l-AI could be interpreted as an impairment of both interoception and cognitive control in unipolar depression, suggesting the possibility of different degrees of impaired connectivity in a continuum with two phenomenological substrates, namely MDD and BD.

Our study adds to the growing evidence for the involvement of the salience network in the pathophysiology of mood disorders. The observed changes may explain some of the cognitive, psychomotor, emotional, and volitional disturbances seen in the phenomenology of both MDD and BD. In addition, our findings suggest both common and distinct aberrations in unipolar and bipolar depression that can be seen as supporting the hypothesis of an existing continuum of mood disorders.

2. Discussion of the neuroimaging results of the prospective sample

The main results of the cognitive task performance with emotional distractors included the activation of structures responsible for processing both visual stimuli and basic cognitive and social-cognitive functions such as attention, memory, decision making, abstract thinking, response to social stimuli, etc. in the three groups studied: HC, MDD and BD in all contrasts studied. The comparison between HC and patients with MDD and BD in the contrast neutral distractors>no distractors, yielded a trend for hypoactivation of the left middle frontal gyrus and left superior frontal gyrus in the patient group. On the other hand, intergroup ANOVA analysis revealed hypoactivation of left middle frontal gyrus, left precuneus, left posterior cingulate cortex, left superior parietal lobule, left superior temporal gyrus, left supramarginal gyrus, and left middle temporal gyrus in the MDD as ooposed to the HC group. In addition, direct comparison of BD and MDD demonstrated hyperactivation of the right external cerebellum, occipital fusiform gyrus, lingual gyrus, and fusiform gyrus in BD. The lack of statistically significant differences between HC and BD suggests significantly milder impairments in cognitive functioning in BD compared with MDD.

In the negative distractors>no distractors contrast, statistically significant differences were found only in the comparison between BD and MDD, with increased activation of the right external cerebellum, right fusiform gyrus, right

lingual gyrus, and right inferior occipital gyrus in the BD group. The negative distractors>neutral distractors contrast did not reveal statistically significant activations. The lack of differences could be due to several reasons: insufficient number of active blocks, small sample size, and also bias due to the predominantly used negative emotion disgust.

Cognition is one of the main domains investigated to detect diagnostic markers in affective disorders. Several of the regions that stood out in the present study (superior and middle frontal gyrus, middle temporal gyrus, precuneus, superior parietal lobule) are involved in this very function. These results add to the evidence found in the hitherto available literature.

There is abundant evidence of altered activity of the precuneus, prefrontal, paracingulate, frontal opercular, posterior parietal and lateral occipital cortex, insula, amygdala, hippocampus and thalamus in tasks examining memory, attention and decision making in MDD and BD, and these changes are not always related to the state of affect. This points to a different involvement of structures mediating cognitive control in affective disorders and suggests altered connectivity and/or intercellular interactions that underlie the deficits commonly found as part of the clinical presentation of MDD and BD.

In unipolar and bipolar depression, different structural and functional disturbances have also been found in neural networks involved in emotion- and reward-processing). Differential activity of regions such as the amygdala, ACC, prefrontal cortex, and striatum is most commonly observed in the context of tasks activating cognition or emotion processing in both affective disorders. Most of the regions that emerged in the present study are also consistent with the scientific literature.

For example, aberrations in angular gyrus connectivity have been associated with anhedonia, and changes in its activity have been observed during a facial emotion recognition task in MDD versus HC. This points to a possible involvement of the angular gyrus in a network related to perception of and response to emotional stimuli and suggests a role for this structure in the pathogenesis of affective disorders.

In addition, different alterations in the connectivity between the striatum and the angular gyrus, thalamus, cerebellum, nucleus caudatus, etc. were found when comparing patients with a first and those with a recurrent depressive episode, and striatum activity was decreased in processing feedback from pleasurable stimuli (reward feedback) in patients with depression as opposed to healthy controls. The cited data also suggest the existence of a continuum of emotional and cognitive processing that includes regions that have been primarily linked to other functions, e.g., the angular gyrus, cerebellum, etc. Further exploration of this expanded connectome could provide a clearer picture of the mechanisms of affective disorders and of the heterogeneous interindividual

symptomatology characteristic not only of mood disorders but of mental illness in general.

Along with impaired emotional reactivity, another major symptom of depression is reduced volitional activity. This symptom is not limited to affective disorders and may have a separate pathophysiological mechanism. For example, there is evidence that disturbances in ventromedial prefrontal cortex activity and connectivity are associated with altered volitional activity and reward-processing not only in MDD and BD but also in schizophrenia. This suggests common mechanisms in psychiatric disorders not only at the symptomatological but also at the pathophysiological level and requires their further study as part of a more extended framework with the eventual aim of discovering pathophysiological complexes that may alter therapeutic direction. For example, transcranial magnetic stimulation is currently used for indirect stimulation of the ventromedial prefrontal cortex, and future studies could evaluate this method for the treatment of a specific symptom rather than a nosological entity.

Although until recently the function of the cerebellum was thought to be limited to motor coordination, an increasing number of studies have found a functional link between this structure and multiple cognitive and emotional processes. In addition, research suggests that aberrations in cerebellar connectivity and activity are associated with various mental illnesses, including affective disorders.

Given our own results showing a difference in cerebellar activity between MDD and BD in the contrast neutral distractors>no distractors and negative distractors>no distractors, we can conclude that the cerebellum is a structure of interest for both the pathogenesis of affective disorders and the possibility that its activity and connectivity may be a future target for diagnostic and therapeutic research.

Identifying consistent changes in brain activity and connectivity in affective disorders could help improve their diagnosis, monitoring and treatment. However, despite the many findings, there is no validated diagnostic biomarker to date, as different paradigms and processing methods are applied and there is a lack of comparability of findings across a statistically significant population sample. Therefore, more in-depth studies are needed to clarify the reasons for the currently available heterogeneous results. In addition, the methodology used in neuroimaging and data processing should also be standardised, as these have been found to be part of the reasons for the lack of sufficient reliability and validity of the results.

The altered activity shown in the present study, primarily related to regions involved in cognitive and regulatory processes, would suggest that the underlying impairments in MDD and BD are related to cognitive processing of external and internal stimuli. This is supported by the absence of residual activations in the

presence of emotional distractors, which could be interpreted as similar processing of emotions (in this case the emotion "disgust", which is a predominant emotion in the paradigm used) in healthy individuals and patients with MDD and BD.

3. Discussion of the results of the molecular-biological analysis

The significant results of the molecular-biological analysis are the low transcriptional levels of microRNAs let-7f, miR-212, miR-219, miR-126, miR-138, miR-146a, and miR-182 detected in both the patient group and the MDD and BD groups separately relative to the HC. MiR-30c and miR-125b showed reduced transcriptional levels in the patient group and the BD group compared with the HC group. MiR-132 showed no significant differential expression. No statistically significant differences were found between MDD and BD.

Most of the studied microRNAs are associated with neurodevelopmental processes, neuronal plasticity, neurodegeneration and neuroinflammation, and the changes detected in our study confirm the hypotheses about the relationship between these processes and the pathogenesis of affective disorders. Furthermore, there is evidence in the literature that most of the studied microRNAs have differential expression in MDD and BD.

For example, higher plasma transcription levels of let-7e-5p and miR-125a-5p have been found in patients with MDD and BD compared to healthy controls, and Receiver operating curve analysis of combined molecular biology and clinical data suggests that testing for these microRNAs can improve diagnostic specificity by 10%. In addition, a study by Liang et al. showed that genetic polymorphisms of the let-7 promoter, and more specifically the rs10877887 CC and rs13293512 genotypes, are associated with a higher risk of developing MDD, and stratification analysis showed that patients with the rs13293512 CC and TC genotypes have a higher risk of relapse after treatment.

Two other microRNAs were associated with the etiopathogenesis of treatment-resistant depression, namely miR-146a and miR-126. Furthermore, overexpression of miR-146a-5p in the hippocampal dentate gyrus was associated with suppression of neurogenesis and spontaneous excitatory neuronal activity in a mouse model of depression. Given the known functions of this particular brain structure, this finding could also be interpreted as a possible explanation of altered memory functions in depression.

Some of the microRNAs studied are associated with specific symptoms that occur not only in affective disorders, but also in other psychiatric and neurological disorders such as Alzheimer's disease. This suggests possible common pathogenetic mechanisms and suggests new avenues for therapeutic interventions when the intimate mechanisms that lead to the development of these phenomenological entities are studied in detail.

For example, in addition to Alzheimer's disease, a decrease in let-7 levels has also been found in patients with chronic insomnia as opposed to healthy controls. In addition, altered transcriptional levels of let-7, miR-138 and miR-125a have been found in different sleep stages, and blocking let-7b results in a change in the delta wave (electroencephalographic) during non-rapid eye movement (NREM) sleep. This suggests a possible involvement of this microRNA in the pathogenesis of insomnia, which is also one of the overlapping symptoms in depression in the context of MDD and BD.

In addition, miR-125b has been found to be important for hippocampal functioning and regulates both learning and memorization processes and anxiety. In a depressive episode in the context of both MDD and BD, transient changes in cognitive functioning are observed, suggesting a possible involvement of miR-125b in the pathogenesis of this symptom. MiR-219 has also been implicated in the pathophysiology of cognitive impairment. One of the major targets of miR-219 is the N-methyl-d-aspartate (NMDA) glutamate receptor signaling pathway, disruptions in which have been linked to cognitive deficits in affective disorders, schizophrenia, etc.

Interestingly, dysregulation of miR-125b and other microRNAs was found in patients with BD in a manic episode in contrast to HC. In the context of the results of the present study and the finding of dysregulation of miR-125b also in a depressive episode in BD and considering the lack of statistically significant difference between HC and MDD, in the future this microRNA may be a potential marker with diagnostic value for BD. In addition, these results question the hypothesis of neuroinflammation as the only common underlying etiopathogenetic mechanism in MDD and BD. This implies that the presence of neuroinflammation is not a sufficient factor for the development of depression, which is supported by the lack of improvement in clinical symptomatology with the addition of an anti-inflammatory medication to antidepressant therapy.

All of the studied microRNAs have important roles in multiple different processes, indicating that their dysregulation can lead to heterogeneous consequences and this could explain the multifaceted clinical presentation of mental illness, including MDD and BD. For example, miR-146a has a major role in the regulation of the immune response, whereas miR-126 is associated with the regulation of angiogenesis. However, increased expression of miR-146a has been found in neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease, as well as in prion disease, whose main manifestations are behavioral and cognitive impairments, suggesting a possible association with the regulation of cognitive functioning. In addition, increased expression of miR-146a and of miR-126 is associated with the early phases of brain injury in ischemia, and down-regulation of miR-126 following brain ischemia potentiates neuronal death.

Our study showed down-regulation of miR-126 and miR-146a in the BD and MDD groups relative to HC, confirming the possible involvement of both

microRNAs in the pathogenesis of MDD and BD. On the other hand, these results could imply that there are common mechanisms between affective disorders and CNS ischemia-inducing diseases, which broadens the spectrum of pathogenetic alterations beyond neuroinflammation and neurodegeneration and brings back one of the big historical questions, namely, are affective and psychiatric disorders in general divided into endogenously and exogenously induced? Or perhaps the spectrum is expanding because the alterations we find are associated only with specific symptoms rather than a nosological entity.

For some of the microRNAs, namely miR-132 and miR-212, there is evidence that they could be a marker of therapeutic response. For example, Ahmadimanesh et al. found up-regulation of miR-132 and miR-124 in patients with MDD in contrast to HC with decreased transcriptional levels of miR-16 in the patient group. Furthermore, after administration of the selective serotonin reuptake inhibitor (SSRI) sertraline, a decrease in miR-132 and miR-124 expression and an increase in miR-16 expression was observed. According to these data, the down-regulation of these two microRNAs could be a marker of therapeutic response to SSRI, but this hypothesis needs to be validated in a larger sample.

A similar potential has also been shown for miR-212. An experimental study has demonstrated the influence of electroconvulsive stimulation (an analogue of electroconvulsive therapy in humans) as an antidepressive therapy on miR-212 expression. It was found that after administration of the therapy, miR-212 transcriptional levels increased in both mouse dentate gyrus and peripheral blood while a concurrent symptomatic improvement was observed. Another study found that miR-183 and miR-212 had increased serum levels after four weeks of antidepressant treatment. Our results suggest the involvement of miR-212 but not miR-132 in the pathogenesis of depression in the context of MDD and BD.

In conclusion, we can summarize the study of microRNAs and their modulatory function on gene expression as an important step towards unraveling the etiopathogenesis of affective disorders. Furthermore, these molecular mechanisms suggest new methods for both diagnosis and monitoring of therapeutic response in MDD and BD. Our study found no statistically significant differences of the studied microRNAs between unipolar and bipolar depression, suggesting possible common mechanisms of both disorders and suggesting the investigation of a larger panel for the purpose of differentiating the two disorders.

The lack of statistically significant differences in the differential expression of long non-coding RNAs can be explained by several different arguments. First, some of the selected RNAs were isolated from post-mortem brain tissue and the absence of differential expression in peripheral blood may be due to the tissue specificity of long non-coding RNAs and the lack of association between peripheral and tissue expression levels. The lack of result for the other long non-coding RNAs could be interpreted as a rejection of the hypothesis of no interracial

differences in their differential expression, as they were selected from a study conducted in a Han Chinese population. This implies that in order to establish the role of these RNAs in affective disorders, a new study should be conducted with as broad a panel of long non-coding RNAi as possible and if feasible in therapeutically naïve patients, as psychopharmacotherapy represents a confounding factor.

Conclusions

Conclusions

1. We determined alterations in the functional connectivity of the anterior cingulate cortex and the right anterior insula in depressed patients as opposed to healthy controls that could explain some of the cognitive, psychomotor, emotional, and volitional disturbances observed in the phenomenology of both MDD and BD.
2. The increased functional connectivity between the anterior cingulate cortex and the cerebellar cruri could distinguish MDD from BD.
3. Altered activity of the external cerebellum, lingual gyrus, and occipital fusiform gyrus in a task engaging attention and short-term memory could differentiate unipolar from bipolar depression.
4. The altered activity shown in the present study, primarily related to regions involved in cognitive and regulatory processes, suggests that the underlying impairments in MDD and BD are related to the cognitive processing of external and internal stimuli.
5. The established differential expression of microRNAs directly related to the processes of neurodevelopment, neurogenesis, neurodegeneration, and neuroinflammation in patients with depression supports the hypothesis of MDD and BD as disorders with complex etiopathogenesis and provides a possible explanation for their heterogeneous clinical presentation.
6. Some of the studied miRNAs are associated with specific symptoms found not only in affective disorders, but also in other psychiatric and neurological disorders, and this suggests the possibility of providing markers for specific phenomenological entities, which also allows for new therapeutic approaches.
7. The lack of a significant difference in the expression of the microRNAs studied suggests the presence of common molecular mechanisms in MDD and BD, supporting the hypothesis of a continuum of affective disorders.
8. The lack of results from the analysis of the expression of lncRNAs can be interpreted as a rejection of the hypothesis of no interracial differences in their differential expression, since they were selected from a study conducted in a Han Chinese population.

Contributions
Theoretical and Methodological:

- A new paradigm for exploring cognitive functions with emotional distractors has been developed.

- An interdisciplinary translational approach has been applied, which has generated further data to help elucidate the etiopathogenesis of affective disorders.

Scientific and Applied

- Evidence for the role of the altered connectivity between the anterior cingulate cortex and the cerebellar cruri as a possible differentiating marker in unipolar and bipolar depression has been generated.

- We generated pilot data on the potential of examining cerebellar activity during a task engaging cognition as a possible differentiating marker for unipolar and bipolar depression.

- Pilot data have been generated for the potential establishment of molecular biological biomarkers for the diagnosis and monitoring of treatment response in affective disorders.

Presentation of the topic at national and international conferences:

Presentation of the topic at national and international conferences:

- Sixth bi-annual Conference of the European Society for Cognitive and Affective Neuroscience, Vienna (Austria) – presentation topic: „Functional Connectivity Aberrations of the Default Mode and Salience Networks Outline the Continuum Major Depressive Disorder - Bipolar Disorder – Schizophrenia“
- 31st European Congress of Psychiatry, Paris (France) – poster topic: „Altered Functional Connectivity of Salience Network in Mood Disorders“
- Symposium „Phylosophy of Mental Health: From Strategy to Practice“, Sofia (Bulgaria) – presentation topic „The continuum mental health – Major Depressive Disorder – Bipolar Disorder“
- 30th Annual Conference of the Bulgarian Psychiatric Association, Pamporovo (Bulgaria) – presentation topic „Alterations in the Functional Connectivity of Various Neuronal Networks in the Continuum of Affective Disorders“
- Conference „Science and Youth“ 2023, Plovdiv (Bulgaria) – presentation topic “Altered Activation of the Left Superior and Middle Temporal Gyri During a Cognitive Task with Emotional Distractors May Differentiate Unipolar from Bipolar Depression”

Publications

1. Todeva-Radneva, A., Paunova, R., Kandilarova, S., & St Stoyanov, D. (2020). The Value of Neuroimaging Techniques in the Translation and Transdiagnostic Validation of Psychiatric Diagnoses - Selective Review. Current topics in medicinal chemistry, 20(7), 540–553. https://doi.org/10.2174/1568026620666200131095328
2. Todeva-Radneva, A., Aryutova, K., Kandilarova, S., Paunova, R., & Stoyanov, D. (2021). The Translational Potential of Non-coding RNAs and Multimodal MRI Data Sets as Diagnostic and Differential Diagnostic Biomarkers for Mood Disorders. Current topics in medicinal chemistry, 21(11), 949–963. https://doi.org/10.2174/1568026621666210521144534
3. Todeva-Radneva A, Kandilarova S, Paunova R, Stoyanov D, Zdravkova T, Sladky R. Functional Connectivity of the Anterior Cingulate Cortex and the Right Anterior Insula Differentiates between Major Depressive Disorder, Bipolar Disorder and Healthy Controls. Biomedicines. 2023; 11(6):1608. https://doi.org/10.3390/biomedicines11061608