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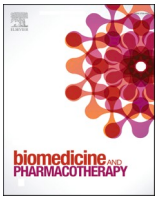

Anti-neuroinflammatory microRNA-146a-5p as a potential biomarker for neuronavigation-guided rTMS therapy success in medication resistant depression disorder

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ABSTRACT

Treatment-resistant depression (TRD) is a challenging issue to address. Repetitive transcranial magnetic stimulation (rTMS) is commonly used but shows varying efficacy, necessitating a deeper understanding of depression physiology and rTMS mechanisms. Notably, an increasing amount of recent data has displayed the connection of TRD and its clinical outcome with chronic inflammatory processes. The current study included 19 TRD patients undergoing rTMS and 11 depressed patients responding to medication as a comparison group. We assessed therapeutic efficacy using MADRS, HAM-D-17, GAD-7, and PHQ-9 tests. Inflammatory markers, neurotrophins, and associated miRNAs were measured in patients blood serum before and during treatment. A control group of 18 healthy individuals provided baseline data. The results of our study showed significantly higher levels of pro-inflammatory interleukins-6 and − 8 in TRD patients compared to drug-responders, which also related to more severe symptoms before treatment. In addition, TRD patients, both before and during treatment, exhibited higher average blood serum concentrations of pro-inflammatory interleukin-18 and lower levels of anti-neuroinflammatory miR-146a-5p compared to healthy controls. We also observed that the expression of miR-16-5p, miR-93-5p, and especially miR-146a-5p correlated with clinical changes following rTMS. Our study confirmed that TRD patients possess a higher inflammatory status, while the anti-neuroinflammatory miR-146a-5p was demonstrated to have a considerable potential for predicting their rTMS treatment success.

1. Introduction

According to the World Health Organization, it is estimated that approximately 4.4% of the world's population suffers from depression [1]. As the global population continues to grow, the incidence of depression is increasing, especially in the least developed countries. Evidently, major depression disorder significantly impacts interpersonal relationships, leads to social exclusion and contributes to suicidal deaths. Consequently, society experiences substantial human and economic losses. Unfortunately, the use of medication for the treatment of depression often fails to achieve a positive outcome. Research has demonstrated that less than one-third of patients achieve remission after 12 weeks of initial antidepressant treatment, while approximately 30% of individuals with major depressive disorder do not respond to drug treatment and are ultimately diagnosed as treatment-resistant [2].

Generally, cases of treatment-resistant depression (TRD) that do not respond to conventional drug therapies are often managed using repetitive transcranial magnetic stimulation (rTMS), with the prefrontal dorsolateral cortex (PFDLC) being the most commonly stimulated region. The clinical efficacy of this approach in treating TRD, surpassing the placebo effect, has been demonstrated in numerous studies, as summarized by Kan and colleagues [3]. However, despite the increasing application of rTMS in TRD treatment, the precise therapeutic mechanism of rTMS remains poorly understood, and clinical outcomes can vary between individual studies and patients, even when similar protocols are applied [4]. All of this prompts a deeper look at potential therapeutic markers and a more precise optimization of the rTMS procedure itself. To date, the reasons why some patients with TRD respond effectively to rTMS, while others do not, as well as physiological mechanisms of TRD formation, remain elusive.

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In this study, our objective was to investigate the physiology of TRD by analyzing biological markers that may be associated with the manifestation of pathology and therapy-induced changes. To accomplish this, we examined the dynamics of peripheral blood biomarkers (cytokines involved in inflammatory processes, neurotrophic factors and associated miRNAs) of treatment-resistant and treatment-responsive depression patients. Our aim was to identify the physiological factors that may contribute to the development of treatment resistance and to provide a more objective evaluation of the effects of rTMS therapy. It is worth noting that previous molecular studies of depression have attributed an important role to the chronic inflammatory processes, as the significance of immunological factors in the pathophysiology of TRD has been supported by several studies, as summarized by Amasi-Hartoonian et al. [5]. TRD has been associated with elevated peripheral blood concentration of interleukins IL-5, IL-6 and IL-8 [6]. In general, major depressive disorder is characterized by upregulation of pro-inflammatory cytokines and chemokines (e.g. IL-1β, IL-2, IL-6, IL-12, Monocyte Chemoattractant Protein (MCP)− 1, C-reactive protein (CRP), etc.), while anti-inflammatory cytokines (IL-4 and Transforming Growth Factor beta (TGF-β1)) have been found to be downregulated [7]. Moreover, studies have shown that anti-inflammatory drugs can alleviate depressive symptoms through modulation of inflammatory pathways [8,9].

In this study, we also examined the expression of several miRNAs known to be involved in inflammatory processes (miR-16-5p and miR-146a-5p) and also expression of miR-93-5p, which is associated with the regulation of Vascular Endothelial Growth Factor (VEGF) [10–12]. While neurotrophic factors such VEGF, BDNF (Brain Derived Neurotrophic Factor), and NGF (Nerve Growth Factor) have been shown to have neuroprotective effects and may modulate neuro-inflammatory responses, the literature on changes in their peripheral blood levels in depression is inconsistent [13–18].

The data presented strongly suggests that assessing the level of inflammation could serve as an additional prognostic marker for TRD and assist in the development of more effective treatment approaches. In this study, our aim was to test the hypothesis that inflammatory phenotype could aid in identifying TRD and predicting response to rTMS therapy.

2. Material and methods

2.1. Subjects

The age range of all subjects included in this study were between 18 and 75 years. The selection criteria for depressed patients were as follows: a medical history of at least one affective disorder, with a score of more than 18 points on the HAM-D-17 clinical test scale. For TRD patients who underwent rTMS treatment, an additional requirement was resistance to at least two different antidepressants or combinations thereof. Exclusion criteria encompassed the presence of medical conditions that may impair cognitive function, epilepsy, ongoing alcohol or drug abuse, pregnancy, and other common contraindications to rTMS treatment [19].

Nineteen patients diagnosed with treatment-resistant unipolar depressive disorder (F32.2 and F33.2), without psychotic symptoms or known infectious or oncological diseases, participated in the rTMS treatment group (8 males, 11 females, mean age 48.78 ± 16.83 years) (one subject dropped out during the study, resulting in a total of 19 patients being enrolled out from 20 overall enrolled patients). Eleven patients diagnosed with medication-responsive unipolar depressive disorder (F32.2 and F33.2), without psychotic symptoms or known infectious or oncological diseases underwent pharmacological treatment (a combination of antidepressant medications) (2 males, 9 females, mean age 54.09 ± 11.89 years) (there were 2 subjects who dropped out and 1 which was eventually diagnosed with TRD from 14 overall enrolled patients). The clinical data of the patients who participated in this study is presented in Table 1. Additionally, 18 healthy individuals (6 males, 12 females, mean age 49.22 ± 11.95 years) participated as a control group. Ethical permission from Vilnius Regional Biomedical Research Ethics Committee (approval no. 2019/11–1161–653) and informed consent were obtained.

Table 1
Clinical data of depression patients involved in this study.

Patient (code)	Diagnosis	rTMS Protocol	Medication
DT01	F33.2	iTBS	Fevarin, Quetiapine
DT02	F32.2	iTBS	Paroxetine, Trazodone
DT03	F33.2	iTBS	Escitalopram, Quetiapine, Valdoxan, Trazodone
DT04	F32.2	iTBS	Duloxetine, Mirtazapine, Quetiapine, Lorazepam
DT05	F33.2	iTBS	Mirtazapine, Paroxetine,
DT06	F33.2	cTBS	Mirtazapine, Duloxetine, Quetiapine, Trazodone
DT07	F33.2	iTBS	Quetiapine, Venlafaxine
DT08	F33.2	iTBS	Mirtazapine, Quetiapine, Lorazepam
DT09	F33.2	iTBS	Mirtazapine, Olanzapine, Bromazepam
DT10	F33.2	iTBS	Lithium, Quetiapine
DT11	F33.2	iTBS	Venlafaxine, Mirtazapine
DT12	F33.2	iTBS	Escitalopram, Quetiapine, Mirtazapine
DT13	F33.2	iTBS	Olanzapine, Paroxetine, Bromazepam
DT14	F33.2	cTBS	Venlafaxine, Mirtazapine, Quetiapine, Bromazepam
DT15	F32.2	iTBS	Escitalopram, Trazodone, Quetiapine
DT16	F33.2	iTBS	Duloxetine, Mirtazapine
DT17	F33.2	iTBS	Mirtazapine, Trazodone, Duloxetine, Quetiapine
DT18	F33.2	iTBS	Paroxetine, Trazodone
DT19	F33.2	iTBS	Mirtazapine, Duloxetine, Olanzapine
DM01	F33.2		Duloxetine, Mirtazapine, Quetiapine
DM02	F33.2		Paroxetine, Quetiapine
DM03	F33.2		Duloxetine, Trazodone, Quetiapine
DM04	F32.2		Mirtazapine, Paroxetine
DM05	F33.2		Duloxetine, Mirtazapine, Olanzapine
DM06	F33.2		Paroxetine, Quetiapine
DM07	F33.2		Venlafaxine, Mirtazapine, Pregabaline, Olanzapine
DM08	F32.2		Duloxetine, Trazodone
DM09	F33.2		Duloxetine, Trazodone
DM10	F33.2		Paroxetine, Trazodone, Quetiapine
DM11	F32.2		Duloxetine, Mirtazapine

Denote: patient code letters "DT" stand for "Depression rTMS", corresponding to rTMS group and code letters "DM" stand for "Depression Medication", corresponding to Medication group.

The definition of treatment-resistant depression followed the clinical practice of the Republican Vilnius psychiatric hospital (RVPH), which required two or more previously failed pharmaceutical treatments (average length of current episode 7.0 ± 5.9 months). During the rTMS treatment, previously ineffective medication treatment was maintained at stable levels, except for benzodiazepines, which were tapered off before the start of rTMS trial. Blood samples from rTMS patients were collected at baseline and after 10 procedures (two weeks) of rTMS treatment. For medication-treated patients, blood samples were collected at baseline before the treatment and two weeks after the start of the treatment. Blood samples from the healthy controls were collected once. All blood samples were collected at the standard time of 8 am.

2.2. rTMS application

rTMS procedures were conducted using a MagVenture Magpro X100 machine equiped with a MagVenture Cool Coil B65 liquid-cooled figure-of-eight coil (MagVenture A/S, Denmark). The stimulation intensity was determined based on to the motor threshold. For intermittent theta burst (iTBS) rTMS stimulation over the left PFDLC and continuous theta burst (cTBS) rTMS over the right PFDLC intensity was set at 80% motor

threshold. The iTBS protocol consisted of 2-second 50 Hz three-pulse bursts that were presented at 5 Hz frequency, applied in 20 trains with 8-second intervals between each train, resulting in a total of 600 impulses. The cTBS protocol consisted of 2-second 50 Hz three-pulse bursts that were presented at 5 Hz frequency, delivered in a single train lasting 40 s, amounting to a total 600 impulses. The motor threshold was defined as the lowest stimulator intensity value that elicited 50% response of visible relaxed right thumb twitch when applying single pulses over the left or right hemisphere motor hand area. The choice of stimulation site was determined by the psychiatrist based on the predominant symptoms of depression: iTBS over the left PFDLC for adynamic depression (17 patients), and cTBS over the right PFDLC for anxious depression (2 patients). The Localite TMS Navigator MR-less neuronavigation system was employed for TMS coil positioning. This system utilizes the standard MNI (MNI ICBM152 non-linear symmetric T1 Average Brain) brain model. The stimulation targets in the MNI model corresponded to the following coordinates: 1) − 41; 16; 54 for left PFDLC; 2) 40; 48; 35 for right PFDLC. Patients underwent 14–40 daily rTMS sessions, with a mean 30.32 ± 6.68 sessions, depending on their clinical response.

2.3. Clinical tests

Depressive patients underwent assessment using various clinical scales, including the Montgomery-Åsberg Depression Rating Scale (MADRS) and the Hamilton Depression Rating Scale 17 (HAM-D-17), which were administered by psychiatrist. Additionally, self-report scales, namely the Patient Health Questionnaire-9 (PHQ-9) and the Generalized Anxiety Disorder-7 (GAD-7), were utilized. The clinical tests were conducted in the afternoon, with the first assessment taking place on the day prior to the initiation of the treatment course, and the final assessment conducted on the day of the last treatment session.

2.4. Evaluation of serum cytokine and trophic factor concentration

Whole blood samples were collected into serum separator clot activator tubes (VACUETTE) and allowed to sit at room temperature for 30 min. Subsequently, the samples were centrifuged at 1000 x g for 15 min. Obtained serum was aliquoted and stored frozen at −80 °C prior to ELISA tests. Various protein concentration analysis were carried out using Human/Mouse BDNF DuoSet ELISA, Human CCL2/MCP-1 DuoSet ELISA, Human IFN-gamma DuoSet ELISA, Human IL-1 beta/IL-1F2 DuoSet ELISA, Human IL-4 DuoSet ELISA, Human IL-6 DuoSet ELISA, Human IL-8/CXCL8 DuoSet ELISA, Human Total IL-18 DuoSet ELISA, Human IL-10 DuoSet ELISA, Human beta-NGF DuoSet ELISA, Human TGF-beta 1 DuoSet ELISA, Human VEGF DuoSet ELISA (R&D Systems, Minneapolis, MN, United States) kits according to manufacturer's instructions and measured using Infinite M200 spectrophotometer (Tecan, Switzerland).

2.5. Isolation and expression analysis of microRNAs

RNA isolation from serum samples was performed using the miRNeasy Serum/Plasma kit (Qiagen, Germany) following the manufacturer's protocol. As an internal spike-in control, Caenorhabditis elegans (C. elegans) miR-39 mimic (Thermo Fisher Scientific, USA) was added during the RNA isolation process (5.6×10^8 copies to 200 μl serum samples). For the reverse transcription reaction, 10 ng of isolated RNA was used, employing the TaqMan® Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Thermo Fisher Scientific, USA). In this kit, the reverse transcription reaction is followed by miR-Amp (amplification) reaction, and the final concentration of resulting cDNA was not measured. For RT-qPCR, 5 μl of diluted (1:10) miR-Amp product was used in a 20 μl reaction. The expression levels of microRNAs were determined using TaqMan® Advanced miRNA Assays (Thermo Fisher Scientific, USA) according to manufacturer's instructions. The specific microRNAs analyzed in this study were miR-16-5p, miR-93-5p, and miR-146a-5p. To evaluate the expression of the selected microRNAs, reverse transcription quantitative PCR (RT-qPCR) was conducted using the Rotor-Gene™ 6000 thermocycler with Rotor-Gene 6000 series software (Corbett Life Science, QIAGEN, Germany). The expression levels were presented as ΔCt after normalization to the spike-in control miR-39-3p.

2.6. Statistical analysis

The statistical analysis was performed using SPSS 17.00 software. GraphPad Prism 8 was utilized for visual representation of the data. To analyze the clinical effect on the test scales before and after the treatment, paired samples t-test was employed. For assessing differences in biomarker concentration between study groups, one way ANOVA was conducted. As many biomarkers did not follow a normal distribution, concentration changes of biomarkers within the study groups during the treatment were analyzed using non-parametric Wilcoxon paired sample test. To calculate changes in clinical test scales and biomarker concentrations as a percentage between measurements, the following formula was used:

$$\text{Change\% X to Y} = (\text{MeasureY} - \text{MeasureX}) / (\text{MeasureX} / 100)\cdot$$

Spearman correlation coefficient was applied for correlation analysis.

3. Results

3.1. The overall change in clinical test scores upon rTMS and medication treatment

Significant decreases in both MADRS and HAM-D-17 clinical test scores were observed in both the drug-resistant rTMS treated depression patients' group (denoted as "rTMS" in the graphs) and the non-resistant medication-treated group (denoted as "Medication" in the graphs) (Fig. 1A-B). However, only PHQ-9 test after rTMS treatment demonstrated a significant decrease in the self-administered scales (Fig. 1C-D). When comparing the average decrease percentages of MADRS and HAM-D-17 test scores, the medication treatment group showed higher reductions (59.96% and 54.59%, respectively) compared to the rTMS treated group (40.08% and 48.86%, respectively) (Table 2). The decrease in MADRS test scores also showed a significant difference between the groups according to the t-test. Additionally, the medication group exhibited a higher average decrease in the GAD-7 self-report scale (26%) compared to the rTMS group (14.65%), although this difference was not statistically significant. Conversely, the rTMS group showed a higher average decrease in the PHQ-9 scale (20.67%) compared to the medication group (14.13%) (Table 2).

3.2. rTMS and medication effect on cytokine concentrations in blood serum

In this study we measured blood serum concentrations of several pro-inflammatory cytokines (IL-1β, IL-6, IL-8, IL-18, IFNγ and CCL2/MCP1) as well as concentrations of anti-inflammatory ones (IL-4, IL-10 and TGF-β1; although, it should be noted that IL-10 and TGF-β1 may have a dual role) (Fig. 2A-I). The concentrations of aforementioned cytokines were measured twice: before and after 10 procedures for medication-resistant depression patients treated with rTMS, and before and after two weeks of treatment for Medication group depression patients. Additionally, blood samples from healthy controls were collected once, and the concentrations of tested cytokines were also measured (in graphs denoted as "Control").

The obtained data revealed that the groups were significantly different only in terms of the pro-inflammatory cytokine IL-18 (Fig. 2F). Patients in the rTMS group, both before and during treatment, exhibited

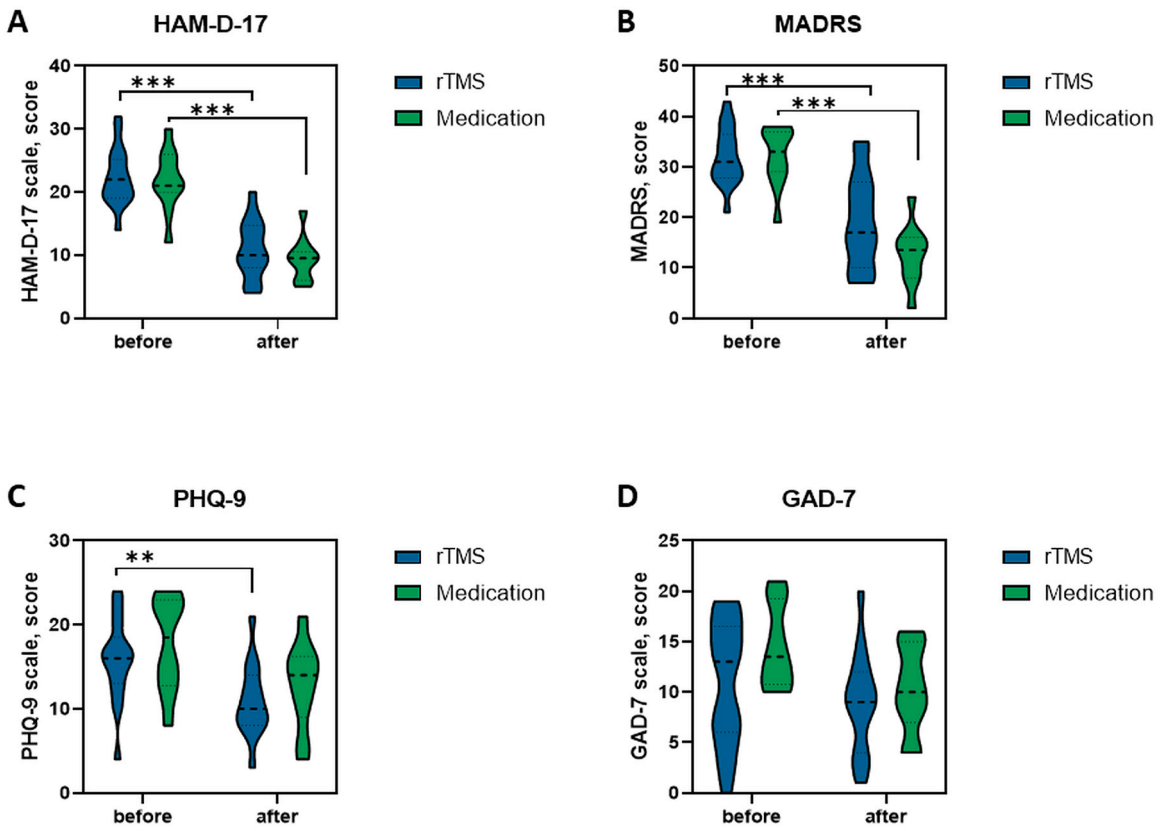

Fig. 1. Clinical changes observed in rTMS and Medication group patients. Drug treatment-resistant depression patients' treated with rTMS (denoted "rTMS"; n = 19) and depression patients' treated with medication (denoted "Medication"; n = 11) test scores in HAM-D-17 (panel A), MADRS (panel B), PHQ-9 (panel C) and GAD-7 (panel D) scales before and after two weeks during the treatment course. *Note: * * denotes significant difference with p < 0.01; * ** denotes significant difference with p < 0.0001, as evaluated using paired sample t test.*

Table 2
Descriptive statistics of percent change in clinical test scores.

		Maximum	Minimum	Mean	Std. Deviation
rTMS group	**HAM-D-17**	-84.38	-7.14	-48.86	24.37
	MADRS	-75.86	-6.90	**-40.08***	25.12
	PHQ-9	-100.00	250.00	-20.67	79.58
	GAD-7	-100.00	300.00	-14.65	98.59
Medication group	**HAM-D-17**	-76.19	-22.73	-54.59	15.12
	MADRS	-92.31	-33.33	**-59.96***	16.85
	PHQ-9	-83.33	100.00	-14.13	52.85
	GAD-7	-80.00	10.00	-26.00	29.40

* denotes significant differences between rTMS and Medication groups with p < 0.05, as evaluated using independent samples t-test.

higher average blood serum concentrations of IL-18 compared to healthy controls (406.94 and 400.72 pg/ml vs. 255.09 pg/ml; F(2, 38) = 6.728 and F(2, 38) = 6.481, respectively, with p < 0.005, as evaluated using ANOVA with Bonferroni post hoc test).

When comparing rTMS and Medication groups directly without considering the Control group, significant differences were found in concentrations of pro-inflammatory proteins IL-8 and IFNγ. The levels of these pro-inflammatory cytokines were significantly higher in the rTMS group compared to the Medication group, both before and during the treatment course (as shown in Table 3). Additionally, during the treatment course, the concentration of IL-6 was nearly 26 times higher in rTMS goup compared to Medication group (as indicated in Table 3).

Regarding differences within the groups, in the rTMS group, significant changes upon treatment were observed in TGF-β1 protein concentrations. On average, the concentration increased from 5476.26 pg/ml to 5913.47 pg/ml, with p < 0.05 (Z = −2.012), as evaluated using the Wilcoxon signed ranks test (Fig. 2G). In the Medication group, mild but significant increases in IL-1β (Fig. 2A) and IL-18 (Fig. 2F) concentrations were observed. On average, IL-1β concentration increased from 1.36 pg/ml to 1.60 pg/ml, with p < 0.05 (Z = −2.000); and IL-18 concentration increased from 332.00 pg/ml to 368.30 pg/ml, with p < 0.05 (Z = −1.988). In contrast, concentrations of IL-6 (Fig. 2C) slightly but significantly decreased in Medication group. On average, IL-6 concentration decreased from 2.45 pg/ml to 1.7 pg/ml, with p < 0.05 (Z = −2.070).

3.3. rTMS and medication effect on trophic factor and miRNA concentrations in blood serum

In addition to cytokine levels, concentrations of trophic factors BDNF, NGF, and VEGF were also measured. However, no significant differences between all three tested groups (rTMS, Medication, and Control) were identified (Fig. 3A). When specifically comparing the rTMS and Medication groups, while excluding the healthy volunteers' (Control) group, significant differences were found. The initial NGF concentrations were approximately 24-fold higher in the rTMS group compared to the Medication group. Furthermore, differences were observed in ongoing treatment BDNF concentrations, which were 1.15-fold higher in the rTMS group compared to the Medication group (a summarized in Table 3). It is important to emphasize that within each group, no significant changes were detected in BDNF, NGF, or VEGF concentrations during the treatment course.

Accordingly, no significant increases or decreases within the groups were observed in miRNA-16-5p, miRNA-93-5p, or miRNA-146a-5p

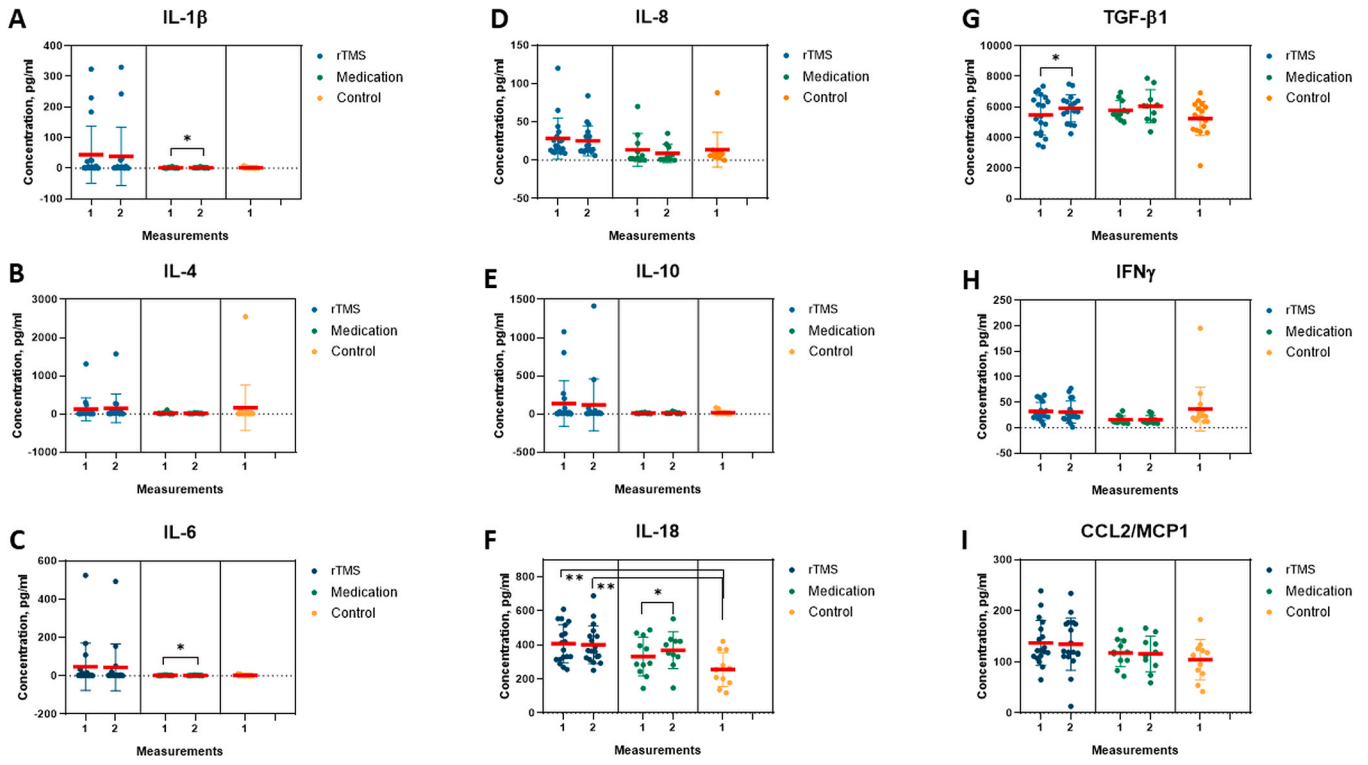

Fig. 2. Cytokine expression in rTMS (n = 19) and Medication (n = 11) group patients. Protein levels of IL-1β (panel A; Control group n = 18), IL-4 (panel B; Control group n = 18), IL-6 (panel C; Control group n = 18), IL-8 (panel D; Control group n = 11), IL-10 (panel E; Control group n = 18), IL-18 (panel F; Control group n = 11), TGF-β1 (panel G; Control group n = 17), IFNγ (panel H; Control group n = 17) and CCL2/MCP1 (panel I; Control group n = 11) in subjects' blood serum, as estimated using ELISA technique. Scatter plots with mean values and SDs formed for triplicate measurements in rTMS group (denoted "rTMS"; 1 – before treatment; 2 – after ten procedures), medication group (denoted "Medication"; 1 – before treatment; 2 – after two weeks of treatment) and for one control group measurement. *Note: within groups * denotes significant difference with $p < 0.05$, as evaluated using Wilcoxon signed ranks test; between groups * * denotes significant difference with $p < 0.005$, as evaluated using ANOVA with Bonferroni* post hoc *test.*

Table 3
Significant differences in biomarker concentrations between rTMS and Medication groups.

	Group	Mean (pg/ml)[a]	Std. Deviation (pg/ml)[a]	Significance[b]
IL-6 (after two weeks of treatment)	rTMS Medication	44.0588 1.7000	121.41894 1.15950	**0.031**
IL-8 (before treatment)	rTMS Medication	406.9444 332.0000	111.56822 113.14239	**0.012**
IL-8 (after two weeks of treatment)	rTMS Medication	400.7222 368.3000	110.96518 107.33960	**0.007**
IFNγ (before treatment)	rTMS Medication	32.2105 15.8182	17.74379 7.66574	**0.004**
IFNγ (after two weeks of treatment)	rTMS Medication	30.5000 15.6000	21.63399 8.26236	**0.031**
NGF (before treatment)	rTMS Medication	180.7368 7.6364	393.62924 8.00341	**0.014**
BDNF (after two weeks of treatment)	rTMS Medication	33812.4706 29353.3000	7139.31031 5136.68030	**0.027**
miR-93 (after two weeks of treatment)	rTMS Medication	ΔCt = 2.6351 ΔCt = 1.0634	1.57571 2.27122	**0.031**

[a] denotes that protein concentration is measured in pg/ml (Exception: miRNA quantity is evaluated in ΔCt)

[b] denotes significant differences between rTMS and Medication groups, as evaluated using Mann-Whitney U test.

levels during the treatment course. However, the ANOVA analysis of rTMS, Medication and Control groups revealed interesting differences. During the treatment course, all three miRNAs tested (miRNA-16-5p, miRNA-93-5p, and miRNA-146a-5p) showed higher ΔCt values in the rTMS group compared to the Medication group, indicating lower expression of these miRNAs in the rTMS group (Fig. 3B). Furthermore, miRNA-146a-5p levels, both initially and during the treatment time points, were significantly lower in the rTMS group compared to healthy controls group. In the rTMS group, ΔCt values were on average 0.9 and 1.3 points higher than in the Control group, illustrating 1.85–2.5 times reduced miRNA-146a-5p expression in the rTMS group compared to the healthy controls.

3.4. Correlations between cytokine, trophic factor and miRNA serum levels and clinical tests in medication-resistant and non-resistant depression

Table 4 presents significant correlations between tested biomarkers and clinical test scores in the rTMS group of medication-resistant depression patients. Negative correlations were found between initial concentration of pro-inflammatory cytokines IL-18 and IFNγ and initial scores on the GAD-7 scale. Surprisingly, this indicates that higher levels of IL-18 and IFNγ may be associated with lower subjective evaluations of anxiety. Negative correlations were also observed between the initial IL-18 concentrations and initial scores on the PHQ-9 scale. This confirms that increased levels of IL-18 may be linked to milder depression symptoms when self-evaluated. Interestingly, the ΔCt levels of miR-93-5p negatively correlated with initial PHQ-9 scores. In this case, it denotes that lower expression of miR-93-5p is linked to less pronounced self-evaluated depression symptoms.

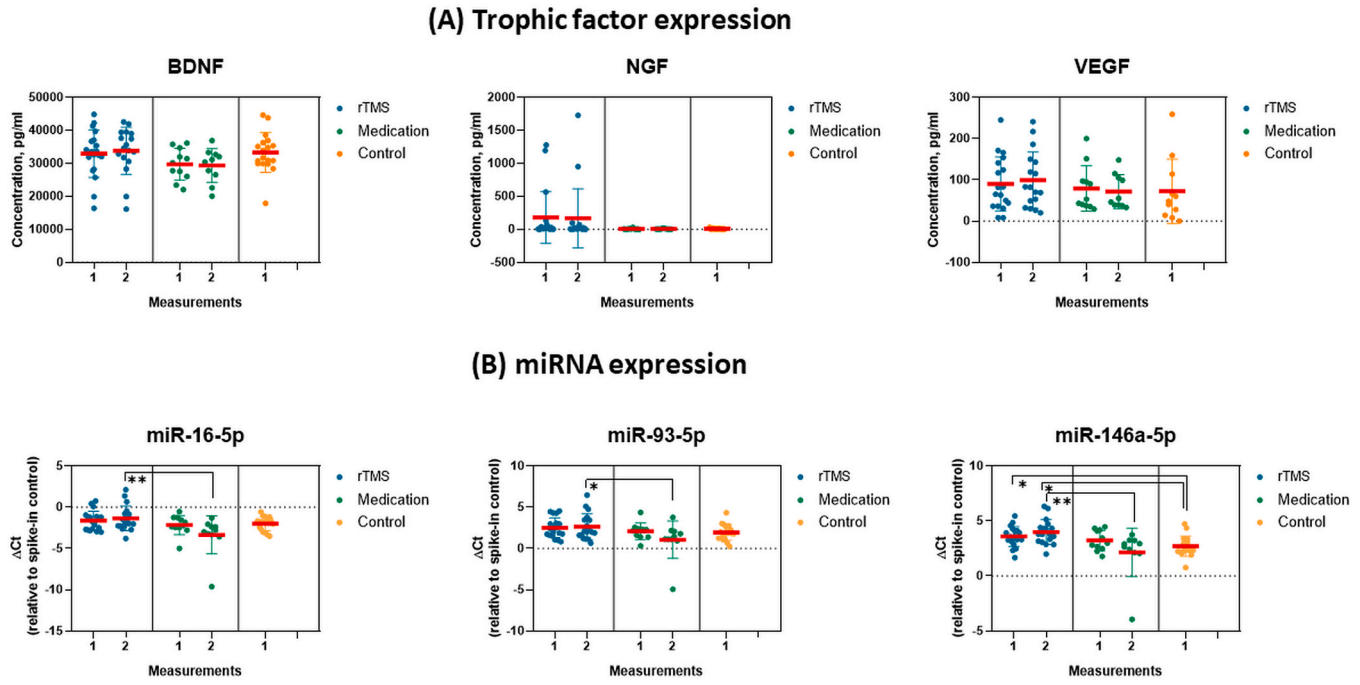

Fig. 3. Trophic factor and miRNA expression in rTMS (n = 19) and Medication (n = 11) group patients. (A) Protein levels of trophic factors BDNF (Control group n = 18), NGF (Control group n = 18) and VEGF (Control group n = 11) in subjects' blood serum, as estimated using ELISA technique. Scatter plots with mean values and SDs formed for triplicate measurements in rTMS group (denoted "rTMS"; 1 – before treatment; 2 – after ten procedures), Medication group (denoted "Medication"; 1 – before treatment; 2 – after two weeks of treatment) and for one Control group measurement. (B) RT-qPCR analysis of miR-16-5p, miR-93-5p and miR-146a-5p expression in rTMS group (1 – before treatment; 2 – after ten procedures), Medication group (1 – before treatment; 2 – after two weeks of treatment) and for one Control group (n = 18) measurement. miRNA expression levels were normalized to exogenous control and presented as mean values of ΔCt ± SD. *Note: * denotes significant difference with p < 0.05, * * denotes significant difference with p < 0.005, as evaluated using ANOVA with Bonferroni* post hoc *test.*

Table 4
Significant correlations in rTMS group.

Correlates	Correlation Coefficient	Sig. (2-tailed)
IL-6_1 **vs** GAD percent change	0.665**	0.010
IL-8_1 **vs** HAM-D_1	0.489*	0.046
IL-10_1 **vs** GAD percent change	0.608*	0.021
IL-18_1 **vs** GAD_1	-0.615*	0.011
IL-18_1 **vs** PHQ_1	-0.601*	0.014
BDNF_1 **vs** MADRS percent change	0.568*	0.027
NGF_1 **vs** GAD percent change	0.533*	0.050
IFNγ_1 **vs** GAD percent change	0.672**	0.009
IFNγ_1 **vs** GAD_1	-0.553*	0.021
IFNγ_1 **vs** HAM-D_1	0.511*	0.030
miR-16_1 **vs** HAM-D percent change	0.618*	0.011
miR-93_1 **vs** HAM-D percent change	0.612*	0.012
miR-93_1 **vs** PHQ_1	-0.531*	0.028
miR-146a_1 **vs** HAM-D percent change	0.529*	0.035
miR-146a_1 **vs** MADRS percent change	0.529*	0.043
miR-146a_1 **vs** PHQ percent change	0.674**	0.006

Denote: symbol "_1" – stands for the first measurement before rTMS procedures.
* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Regarding the percent change in clinical test scores after treatment, particular emphasis should be drawn to miR-146a-5p, as the primary ΔCt levels of miR-146-5p correlated positively with the percent change in HAM-D-17, MADRS, and PHQ-9 scores. This indicates that lower expression of miR-146a-5p was associated with a less successful rTMS effect in the treatment-resistant depression patients' group, and, conversely, higher miR-146a-5p expression was linked to a more positive treatment outcome. Additionally, basal levels of miR-16-5p and miR-93-5p were also found to correlate positively with the percent change in HAM-D scale.

In the Medication group, two significant correlations with the clinical test scores were detected. Firstly, basal levels of neurotrophin NGF correlated positively with the scores in GAD-7 scale before treatment (r = 0.661, p = 0.038). Secondly, the initial concentration of the pro-inflammatory cytokine IL-8 correlated negatively with the percent change in PHQ-9 score after treatment (r = −0.633, p = 0.049).

3.5. Correlational analysis of studied biomarkers

To gain deeper insights into the molecular mechanisms and interplay among the studied biomarkers, we examined correlations among their initial concentrations. As shown in Table 5, we found strong correlations between the pro-inflammatory cytokine CCL2/MCP1 and ΔCt values of miR-16-5p and miR-93-5p. For instance, in the Control group, the correlation between CCL2/MCP1 and miR-16-5p was highly significant, with a value of r = 0.882 (p = 0.000). Similarly, the correlation between CCL2/MCP1 and miR-93-5p was also significant (r = 0.782,

Table 5
Significant correlations between tested biomarkers.

Group	Variable No. 1	Variable No. 2	Correlation Coefficient	Sig. (2-tailed)
Combined	miR-16-5p*	IL-10	0.370	0.010
		BDNF	0.333	0.021
		CCL2/MCP1	0.715	0.000
	miR-93-5p*	CCL2/MCP1	0.655	0.000
rTMS	miR-93-5p*	IL-18	0.525	0.025
Medication	miR-16-5p*	CCL2/MCP1	0.79	0.004
	miR-93-5p*	CCL2/MCP1	0.831	0.002
	miR-16-5p*	BDNF	0.480	0.044
Control		CCL2/MCP1	0.882	0.000
	miR-93-5p*	IL-8	0.557	0.048
		CCL2/MCP1	0.782	0.004

* miRNA quantity presented in ΔCt values

$p = 0.004$). It is crucial to highlight that the correlation between CCL2/MCP1 and miRNAs weakened in the rTMS group when compared to the Control and Medication groups. In the rTMS group, the correlations of CCL2/MCP1 concentration with the ΔCt values of miR-16-5p or miR-93-5p decreased and ultimately lost statistical significance ($r = 0.458$, $p = 0.056$ and $r = 0.467$, $p = 0.051$, respectively).

Furthermore, we observed that ΔCt values of miR-93-5p exhibited positive correlations with concentrations of pro-inflammatory cytokines IL-8 and IL-18 in the Control and rTMS groups, respectively. This implies that lower expression of miR-93-5p is associated with higher levels of IL-8 and IL-18, and vice versa.

3.6. Age and gender influence on biomarkers and clinical test scores

We performed a Spearman correlation analysis to investigate the relationship between the subjects' age and the separate biomarkers tested for all study groups combined. Additionally, we correlated age with the clinical test scores and the percentage of score decrease for the patient groups. Our findings revealed mild but significant positive correlations between age and basal ΔCt values of miR-93-5p ($r = 0.409$, $p = 0.011$) as well as miR-146a-5p ($r = 0.334$, $p = 0.04$). This indicates that higher age is associated with lower expression of miR-93-5p and miR-146a-5p. For the combined patient groups, we were also able to detect a correlation between age and PHQ-9 test score decrease ($r = 0.424$, $p = 0.035$), indicating that older patients tended to subjectively rate their clinical improvement less following therapy, even though other clinical correlations failed to show any relationship with age and proved to be insignificant. However, when only the rTMS group was tested, all the previous correlations lost statistical significance (age and miR-93-5p: $r = 0.327$, $p = 0.172$; age and miR-146a-5p: $r = 0.335$, $p = 0.161$; age and PHQ-9 test score decrease: $r = 0.397$, $p = 0.143$).

We conducted an additional independent sample t-test with two distinguished gender groups, comparing biomarker concentrations for all study groups combined, and clinical test scores as well as score decrease percentage for the patient groups. We found significantly higher initial CCL2/MCP1 concentrations in male subjects (156 ± 39.33 pg/ml) compared to females (116.43 ± 30.42 pg/ml, $p = 0.014$), as well as differences in basal ΔCt values of miR-16-5p (males -1.18 ± 1.05, females -2.02 ± 0.96, $p = 0.034$) and miR-93-5p (males 2.84 ± 1.1, females 2.03 ± 0.94, $p = 0.046$). However, when comparing the genders within the rTMS group alone, all the previous differences lost statistical significance: CCL2/MPC1 (males -159.38 ± 43.86 pg/ml, females 119.2 ± 37.25 pg/ml, $p = 0.058$), ΔCt of miR-16-5p (males -1.15 ± 1.2, females -1.96 ± 1.02, $p = 0.146$), ΔCt of miR-93-5p (males 2.73 ± 1.15, females 2.36 ± 1.26, $p = 0.528$). Meanwhile, significant gender differences emerged in TGF-β1 (males 6223.75 ± 993.42 pg/ml, females 4932.64 ± 1235.5 pg/ml, $p = 0.022$) and IFNγ (males 41.88 ± 17.15 pg/ml, females 25.18 ± 15.22 pg/ml, $p = 0045$) concentrations. There were no significant gender differences concerning clinical test scores, as well as score decrease percentage, for both patient groups or the rTMS patient group alone.

Due to relatively small sample sizes, we were unable to explore the influences of age and gender on the tested biomarkers and their potential relationship with clinical symptoms and therapeutic improvement further. However, these factors must undoubtedly be subject to additional scrutiny in future studies, utilizing larger and more balanced subject groups, particularly with regards to miRNA expression.

4. Discussion

Both clinical strategies, rTMS and pharmaceutical therapy, were proven to be effective in reducing depressive symptoms. However, overall, a greater clinical improvement was observed in responders to pharmaceutical therapy when compared to TRD patients treated with rTMS. On the subjective level, TRD patients seemed to perceive a larger clinical improvement after rTMS treatment compared to regular patients after medication therapy. Nonetheless, despite the fact that we have shown differences between treatment outcomes, one has to keep in mind that these results cannot be used for a direct comparison between pharmaceutical and rTMS supplemented therapies due to the initial group differences considering TRD status. Therefore, these findings should serve an illustrator purpose only, indicating the possible hindrance effect of drug resistance towards the overall clinical efficacy of additional rTMS therapy.

As expected, TRD patients showed higher levels of pro-inflammatory cytokines IL-6, IL-8 and IFNγ compared to patients responding to pharmaceutical treatment. Such results align with the widely accepted hypothesis of inflammatory nature of TRD [8]. Additionally, the upregulation of these cytokines correlated with more severe clinical symptoms before the rTMS course and less subjectively perceived anxiety reduction after treatment. Interestingly, IL-18 was also significantly increased in rTMS patients compared to healthy controls, even though higher levels of it correlated with better self-described symptoms before the treatment.

During rTMS therapy, an increase in anti-inflammatory cytokine TGF-β1 was observed, although it did not show a significant correlation with clinical improvement. It is worth noting that TGF-β1 is known to exert neuroprotective effects and plays a major role in synaptic plasticity [20]. Additionally, rTMS is also widely recognized to elicit similar effects [21–23]. Therefore, it is plausible that TGF-β1 is one of the factors responsible for the cellular level responses to rTMS.

Surprisingly, in our study, we observed significantly higher values and greater variation in the concentration of the neurotrophin NGF in rTMS-treated TRD patients when compared to medication-responsive patients. The higher NGF concentration in the rTMS group also correlated with a lower improvement in subjectively described symptoms. Moreover, our results contradict the data of Bilgen and colleagues [24] whose study showed that peripheral blood concentrations of NGF in TRD patients were significantly lower compared to a healthy control group. In contrast, in our study, TRD patients had higher peripheral blood concentrations of NGF in comparison to healthy controls, though differences (180.74 pg/ml vs. 9.00 pg/ml) were not significant when all three study groups were compared.

Similar to our previous study [25], we observed that the concentration of mature BDNF in the peripheral blood seemed to increase during rTMS treatment, although this increase was not statistically significant. However, the increase caused significant differences to appear between TRD patients and medication responders after two weeks of treatment. Interestingly, we found that initially higher BDNF values in the rTMS group seemed to hinder possible clinical improvement. The meta-analysis performed by Meshkat and colleagues [26] demonstrated that indeed peripheral BDNF levels in TRD patients significantly increase over the treatment course (with various treatment schemes applied). In addition, no association with depressive symptoms has been found. However, in contrast to our study, authors evaluated concentrations of total BDNF (together mature and pro-BDNF forms) and thereafter suggested that the predictive value of BDNF could still be valid if separate measurements of mature BDNF or pro-BDNF are performed. Regarding the mechanism that could explain the phenomenon of high baseline BDNF concentrations potentially impeding further clinical improvement, we hypothesize that higher baseline BDNF levels may indicate a state of increased ongoing neuroplastic processes [27]. Consequently, this heightened neuroplasticity might reach a plateau, making it more challenging for rTMS to induce further neuroplastic effects [28] and reveal its full therapeutic potential. Of course, the correlation between BDNF levels and the antidepressant effect of rTMS may be much more intricate, and additional factors may play a role.

Higher ΔCt values, indicating lower expression of miR-16-5p, miR-93-5p, and miR-146a-5p during the treatment, were observed among TRD patients compared to the Medication group. Additionally, miR-146a-5p showed significant basal level group differences between TRD

patients and healthy control group. Furthermore, ΔCt values of all tested miRNAs before the treatment served as good predictors of a lesser clinical improvement across multiple symptom domains after the rTMS therapy. miR-16-5p, a key regulatory molecule in serotonergic transmission, has been previously implicated in the pathophysiology of depression, as it is downregulated in cases of MDD [29]. On the other hand, miR-93-5p, had not been previously associated with the pathophysiology of depression, and it has even been suggested as an optimal reference gene in miRNA expression studies of MDD [30]. Interestingly, in our study, when correlational analysis between tested biomarkers was performed, a significant positive correlation between ΔCt values of miR-93-5p and IL-18 was detected in the TRD group. This suggests that lower expression of miR-93-5p is associated with higher IL-18 concentrations in the TRD patients blood serum. These findings are in accordance with study performed by Juan and colleagues [31], showing that miR-93-5p may regulate the expression of the pro-inflammatory cytokine IL-18.

Nevertheless, it should be emphasized that the expression of miR-146a-5p showed the greatest predictive value, as it significantly correlated with changes in all HAM-D-17, MADRS and PHQ-9 clinical test scales. In the previous studies by other authors, miR-146a has been shown to be dysregulated in depression and to play a role in orchestrating inflammatory signaling pathways. For example, the study by Hung and colleagues [11] revealed reduced miR-146a levels in the blood cells of MDD patients and found that miR-146a expression before antidepressant treatment was inversely correlated with the severity of depression. Further research by Liu and colleagues [32] investigated the potential mechanisms by which miR-146a dysregulation may contribute to TRD. They showed that miR-146a improved depressive behaviors in depressed model mice by inhibiting microglial activation and neuroinflammatory factor expression, suggesting that dysregulation of miR-146a may contribute to the inflammation observed in TRD. Furthermore, the potential therapeutic effects of targeting miR-146a in depression was investigated, as miR-146a mimic treatment inhibited TNF-α, IL-1β, IRAK1, and TRAF6 expression in BV-2 microglial cells [32]. It is important to note that studies have confirmed miR-146a to possess not only diagnostic but also prognostic value in MDD, as it may be a promising marker to predict antidepressant response in patients [33]. Similarly, our study confirmed that miR-146a-5p can also be a promising biomarker for predicting response to rTMS therapy in TRD patients. It could be further explored as a distinguishing factor for novel, inflammatory phenotype-based depression treatment strategies in the future.

5. Conclusions

We observed higher concentrations of pro-inflammatory cytokines IL-6 and IL-8 in the blood serum of drug-resistant patients treated with rTMS, in comparison to the drug responsive group. These higher cytokine levels were also associated with more severe symptoms before treatment and more pronounced subjective symptoms of anxiety after the therapy. Furthermore, higher concentrations of NGF and IFNγ biomarkers were also found in the rTMS patient group compared to the medication group. TRD patients, both before and during treatment, also exhibited higher average blood serum concentrations of pro-inflammatory cytokine IL-18 compared to healthy controls. However, the epigenetic markers miR-16-5p, miR-93-5p, and especially miR-146a-5p exhibited the most promising prognostic potential in our study, with lower ΔCt values before rTMS therapy significantly correlating with better clinical outcomes after treatment. These findings strongly indicate that the prognostic potential of the studied miRNAs should be subject to further investigation for potential implementation into clinical practice.

Study limitations include the naturalistic basis of our research, which hindered complete matching of patient groups in terms of age, gender, medication composition, and rTMS protocol uniformity within the TRD group. Furthermore, the relatively small sample sizes limited our ability to conduct a statistically sound gender comparison, necessitating further research.

To address these limitations, future studies will require a larger and more well-rounded patient sample. This will enable us to re-evaluate the hypothesis of miRNA influence on treatment-resistant depression and gain deeper insights into the underlying biological mechanisms. Additionally, a more comprehensive patient sample will facilitate the exploration of age and gender influences on the tested biomarkers and their potential relationship with clinical symptoms and therapeutic improvement.

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CRediT authorship contribution statement

G. V. Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation; Methodology, Project administration, Supervision; Visualization, Writing – original draft. **V. V.** Conceptualization, Data curation, Formal analysis; Investigation, Methodology, Software, Writing – review & editing. **A. Z.** Investigation, Methodology, Writing – review & editing. **K. D.** Conceptualization, Data curation, Writing – review & editing. **A. G.** Conceptualization, Supervision, Writing – review & editing. **R. N.** Methodology, Resources, Writing – review & editing. All authors approved the final article.

Declaration of Competing Interest

Authors declare no conflicts of interest.

Data Availability

Data will be made available on request.

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