EUROPEAN JOURNAL OF PSYCHOTRAUMATOLOGY
2021, VOL. 12, 1987655
https://doi.org/10.1080/20008198.2021.1987655

EUROPEAN JOURNAL OF PSYCHO-TRAUMATOLOGY
THE OFFICIAL JOURNAL OF THE EUROPEAN SOCIETY FOR TRAUMATIC STRESS STUDIES

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BASIC RESEARCH ARTICLE

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Inflammation-related microRNAs are involved in stressful life events exposure and in trauma-focused psychotherapy in treatment-resistant depressed patients

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ABSTRACT

Background: About 30% of major depressive disorder (MDD) patients are classified as resistant to treatment (treatment-resistant depression, TRD). Among the factors associated with unfavourable treatment outcomes, stressful life events play a relevant role, and trauma-focused psychotherapy has been successfully proposed for the treatment of patients with a history of such events. Stressful experiences are related to enhanced inflammation and, recently, microRNAs (miRNAs) have emerged as potential mediators of the association between these experiences and psychiatric disorders. To date, no study has explored the effects of stressful life events on miRNAs in MDD patients.

Objective: The objective of the present study was to assess possible miRNA blood expression alterations in TRD patients induced by the exposure to stressful life events and to investigate the effects of trauma-focused psychotherapy on the expression profiles of the same miRNAs, as well as their possible predictivity in relation to therapy outcome.

Method: The basal levels (T0) of seven candidate miRNAs (miR-15a/miR-29a/miR-125b/miR-126/miR-146a/miR-195/let-7f) were measured in the whole blood of 41 TRD patients. A subgroup of patients (n = 21) underwent trauma-focused psychotherapy; for all of them, miRNA levels were also longitudinally assessed (T4: after 4 weeks of treatment; T8: end of treatment; T12: follow-up visit), contextually to clinical evaluations.

Results: miR-146a levels negatively correlated with recent stressful life event scores (p = .001), whereas the levels of miR-15a, miR-29a, miR-126, miR-195, and let-7f changed during the psychotherapy (best $p = 1.98*10^{-9}$). miR-29a was also identified as a response predictor, with lower baseline levels predicting non-response (p = .019) or worse improvement in mood symptoms (p = .032).

Conclusions: The study results could contribute to clarify the underlying molecular mechanisms and to identify novel biomarkers of stressful experiences and response to targeted treatments.

Los micro ARN relacionados a inflamación están involucrados en la exposición a eventos vitales estresantes y en la psicoterapia con foco en el trauma, en pacientes con depresión resistente a tratamiento

Antecedentes: Alrededor del 30% de los pacientes con un Trastorno Depresivo Mayor (TDM) son clasificados como resistentes a tratamiento (Depresión Resistente a Tratamiento, TRD por su sigla en inglés). Entre los factores asociados a resultados de tratamiento desfavorables, los eventos vitales estresantes juegan un rol relevante, y la psicoterapia con foco en el trauma ha sido propuesta con éxito para el tratamiento de los pacientes con historia de tales eventos. Las experiencias estresantes están relacionadas a un aumento de la inflamación y, recientemente, microARNs (miARNs), han surgido como potenciales mediadores de la asociación entre estas experiencias y trastornos psiquiátricos. A la fecha, ningún estudio ha explorado los efectos de los eventos vitales estresantes sobre los miARNs en pacientes con TDM.

Objetivo: El objetivo del presente estudio fue evaluar posibles alteraciones en la expresión de miARN en sangre en pacientes con TRD inducidas por la exposición a eventos vitales estresantes e investigar los efectos de la psicoterapia con foco en el trauma sobre los perfiles de expresión de los mismos miARNs, así como su posible predictividad en relación al resultado de la terapia.

ARTICLE HISTORY
Received 27 May 2021
Revised 31 August 2021
Accepted 7 September 2021

KEYWORDS
Major depressive disorder; treatment-resistant depression; stressful life events; trauma-focused psychotherapy; microRNAs; inflammation

PALABRAS CLAVE
Trastorno depresivo mayor; depresión resistente a tratamiento; eventos vitales estresantes; psicoterapia con foco en el trauma; microARNs; inflamación

关键词
重性抑郁障碍; 难治性抑郁; 应激性生活事件; 聚焦创伤心理治疗; 炎症

HIGHLIGHTS

- The peripheral levels of microRNAs implicated in inflammation are correlated to recent stressful life events in treatment-resistant depressed patients and modified by trauma-focused psychotherapy; some of them could represent predictive biomarkers of response to psychotherapy.

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Supplemental data for this article can be accessed here.

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Método: Los niveles basales (T0) de 7 miARN candidatos (miR-15a/miR-29a/miR-125b/miR-126/miR-146a/miR-195/let-7f) fueron medidos en la sangre completa de 41 pacientes con TRD. Un subgrupo de pacientes (n = 21) se sometió a psicoterapia con foco en el trauma; para todos ellos, los niveles de miARN fueron también evaluados longitudinalmente (T4: después de 4 semanas de tratamiento; T8: fin del tratamiento; T12: visita de seguimiento), contextualmente a evaluaciones clínicas.
Resultados: Los niveles de miR-146a se correlacionaron negativamente con los puntajes de eventos vitales estresantes recientes (p = .001), mientras que los niveles de miR-15a, miR-29a, miR-126, miR-195, y let-7f cambiaron durante la psicoterapia (mejor p = p = 1.98*10^{-9}). miR-29a también fue identificado como un predictor de respuesta, con menores niveles basales prediciendo falta de respuesta (p = .019) o menor mejoría en los síntomas anímicos (p = .032).
Conclusiones: Los resultados del estudio contribuyen a clarificar los mecanismos moleculares subyacentes y a identificar nuevos biomarcadores de experiencias estresantes y respuesta a tratamientos dirigidos.

难治性抑郁患者应激性生活事件暴露和的聚焦创伤心理疗法中涉及的炎症相关 microRNA

背景: 大约 30% 的重性抑郁障碍 (MDD) 患者被归类为治疗抗性 (难治性抑郁, TRD) 。在与不利治疗结果相关的因素中, 应激性生活事件起着相关作用, 已成功提出针对有此类事件病史患者的聚焦创伤心理疗法。应激性经历与炎症增强有关, 最近, microRNA (miRNA) 已成为这些经历与精神障碍之间关联的潜在中介因素。迄今为止, 还没有研究探讨应激性生活事件对 MDD 患者 miRNA 的影响。
目的: 本研究旨在评估TRD 患者中因应激性生活事件暴露引发的可能的 miRNA 血液表达改变, 并研究聚焦创伤心理疗法对相同 miRNA 表达谱的影响, 以及它们与治疗结果相关的可能的预测性。
方法: 在41 名 TRD 患者的全血中测量了 7 种候选 miRNA (miR-15a/miR-29a/miR-125b/miR-126/miR-146a/miR-195/let-7f) 的基础水平 (T0) 。21名患者亚组接受了聚焦创伤心理疗法;对于所有患者, 还根据临床评估对其 miRNA 水平进行了纵向评估 (T4:治疗 4 周后;T8:治疗结束;T12:随访)。
结果: miR-146a 水平与近期应激性生活事件得分呈负相关 (p = .001), 而 miR-15a, miR-29a, miR-126, miR-195 和 let-7f 的水平在心理治疗期间发生变化 (最佳 p = 1.98*10-9) 。 miR-29a 也被确定为反应预测因子, 较低的基线水平预测无反应 (p = .019) 或更差的情绪症状改善 (p = .032)。
结论: 研究结果有助于阐明潜在的分子机制, 并确定应激性经历和对靶向治疗反应的新生物标志物。

1. Introduction

Major depressive disorder (MDD) is the most common psychiatric disease worldwide and represents a leading cause of years lived with disability. Despite ongoing efforts to improve therapeutic options, both pharmacological and non-pharmacological, MDD remains largely undertreated (Kraus, Kadriu, Lanzenberger, Zarate, & Kasper, 2019). The first-line treatment choice is represented by pharmacological therapy; however, only about 40% and 30% of patients experience remission after the first and second treatment course, respectively, and up to one-third of them are classified as resistant to treatment (treatment-resistant depression, TRD) (Jaffe, Rive, & Denee, 2019; Rush et al., 2006). Several clinical variables are associated to an unfavourable treatment outcome, such as earlier disease onset, greater severity, presence of psychiatric comorbidity, suicidal behaviours, and stressful life events (Kautzky et al., 2017; Kraus et al., 2019). Among these factors, stressful life events, especially when experienced during childhood (early life adversity, ELA), have a particular clinical relevance since they are, in turn, associated with the other above-mentioned negative predictors (Bahk, Jang, Choi, & Lee, 2017; Dias de Mattos Souza, Lopez Molina, Azevedo da Silva, & Jansen, 2016; Nelson, Klumparendt, Doebler, & Ehring, 2017; Spinhoven, Penninx, van Hemert, de Rooij, & Elzinga, 2014; Williams, Debattista, Duchemin, Schatzberg, & Nemeroff, 2016), suggesting the existence of both a direct and indirect relationship with the TRD condition. ELA includes forms of child maltreatment such as physical, sexual and emotional abuse, and neglect, and it is defined as any act that is either actively conducted, or neglected to be conducted, by a parent or caregiver that either harms or has potential to harm a child (Brown, Fiori, & Turecki, 2019). A meta-analysis conducted on almost 200 studies highlighted that nearly half of patients suffering from MDD has a history of ELA and that maltreated children are two to three times more likely to develop MDD in adulthood (Nelson et al., 2017). Also, severe life events occurring during adulthood, such as loss of employment, chronic or life-threatening health problems, separation, financial insecurity, exposure to violence or bereavement, have been associated with the development of MDD and its greater severity (Musliner et al., 2015; Shapero, Curley, Black, & Alloy, 2019; Sheerin et al., 2018; Yacaman-Mendez, Hallgren, & Forsell, 2019), and with a worse response to pharmacological treatment (Menke et al., 2021), although with a weaker evidence and impact compared to ELA.

On these bases, trauma-focused psychotherapies, such as trauma-focused cognitive-behavioural therapy (TF-CBT) and eye movement desensitization and reprocessing (EMDR), have been proposed for the treatment of MDD and TRD patients with a history of stressful life events, showing evidence of therapeutic effectiveness (Yan et al., 2021).

From a biological perspective, MDD is associated with altered patterns of gene expression, both in the central nervous system and in peripheral tissues, such as blood. In particular, these findings provide partial support for all major theories of depression, including dysregulation in monoamine and neurotransmitter systems, altered neuroplasticity and neurogenesis, and disturbed neuroendocrine, immune and inflammatory activity (Ciobanu et al., 2016). Stressful life events, too have been associated with modifications in gene expression, which could be involved in mediating the effects of these adverse experiences on MDD risk. For example, research suggests that, through epigenetic mechanisms, ELA can induce gene expression changes affecting stress reactivity, brain function, and behaviour. It is noteworthy that, although these effects are usually long lasting, they can be reversed by pharmacological and environmental interventions (Brown et al., 2019; Turecki, Ota, Belangero, Jackowski, & Kaufman, 2014). Moreover, in peripheral blood cells of people with a history of ELA, stress-responsive transcript signatures have been described as characterized by an involvement of genes implicated in cytokine and chemokine activity, indicating an activation of the pro-inflammatory signalling (Schwaiger et al., 2016), consistently with other evidence reporting an association between early life stress and immune/inflammatory activation (Do Prado, Grassi-Oliveira, Daruy-Filho, Wieck, & Bauer, 2017). Also multiple severe recent life events have been observed to impair the expression of stress-related genes, such as the Serum/Glucocorticoid Regulated Kinase 1 (SGK1), and to enhance inflammation in response to glucocorticoid stimulation with dexamethasone (Menke et al., 2021).

In addition to those affecting coding genes, MDD- and stressful life event-related modifications have been described also in the expression of non-coding RNAs known as microRNAs (miRNAs). Since their discovery in the early 1990s, miRNAs have revolutionized the understanding of gene regulation; they act as post-transcriptional regulators of gene expression by binding to the 3′ untranslated regions of target messenger RNAs (mRNAs), altering their stability and/or inhibiting the translation (O'Carroll & Schaefer, 2013). These small, but powerful molecules have been shown to play key roles in basic neural functions and in different neurological and psychiatric disorders (Luoni & Riva, 2016; Maffioletti, Tardito, Gennarelli, & Bocchio-Chiavetto, 2014); recently, they have been proposed as possible mediators of the vulnerability to MDD conferred by stressful life events. Altered miRNA expression after experiences of early life stress have been observed in both the brain and blood of MDD animal models, although results are partially conflicting and largely unclear (Allen & Dwivedi, 2020). Findings in humans are limited to few reports. Volk and colleagues reported increased levels of miR-15a in the blood of healthy subjects exposed to childhood trauma compared to non-exposed individuals, in accordance with results obtained in cellular and animal models; this suggests a role for this miRNA in the coping mechanisms for chronic stress (Volk et al., 2016). Another study, based on a miRNomic approach, highlighted a number of dysregulated miRNAs in the blood of schizophrenic patients exposed to childhood trauma versus non-exposed ones (Cattane et al., 2019). To date, no study on MDD patients has explored the contribution of stressful life events to miRNA expression alterations.

Based on these findings, the objective of the present study was to assess possible miRNA blood expression alterations in TRD patients induced by the exposure to stressful life events, in order to clarify the molecular underpinnings of their relationship with psychopathology. Seven candidate miRNAs (miR-15a, miR-29a, miR-125b, miR-126, miR-146a, miR-195, and let-7f) were selected based on the existing literature. miR-15a was included because of its involvement in ELA (Volk et al., 2016). Since inflammation has been shown to play a relevant role in life adversities and stress (Fogelman & Canli, 2019; Schwaiger et al., 2016), other miRNAs (miR-29a, miR-125b, miR-126, miR-146a, miR-195, and let-7f) were chosen because of their implication in inflammatory mechanisms (Olivieri, Rippo, Procopio, & Fazioli, 2013).

Moreover, we aimed to investigate the effects of trauma-focused psychotherapy on the expression profiles of the same miRNAs and on those of the C-reactive protein (CRP), a widely recognized biomarker of both acute and chronic inflammation (Luan & Yao, 2018), as well as the possible predictivity of baseline levels of the same biomarkers in relation to therapy outcome.

2. Methods

2.1. Study participants and clinical assessment

Forty-one TRD patients were voluntarily enrolled in the study. The diagnostic inclusion criterion was a diagnosis of MDD according to the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV) classification system. The exclusion criteria were the following: (a) mental retardation or cognitive disorder; (b) a lifetime history of schizophrenic, schizoaffective, or bipolar disorder; (c) personality disorder, substance abuse, alcohol abuse or dependency, obsessive compulsive disorder, or post-traumatic stress disorder as the primary diagnosis; and (d) comorbidity with an eating disorder.

All patients were referred to the Psychiatric Hospital 'Villa Santa Chiara' in Verona, Italy.

TRD was defined as the failure to respond to two or more adequate trials with two or more different classes of antidepressant drugs and to an adequate trial with a tricyclic drug (TCA), corresponding to at least stage III according to the Thase and Rush staging method (Thase & Rush, 1997). All patients were assessed for exposure to ELA according to the Italian version of the Childhood Experience of Care and Abuse Questionnaire (CECA.Q) (Bifulco, Bernazzani, Moran, & Jacobs, 2005). Recent stressful life events, occurred during the 12 months before the assessment, were evaluated through the Italian versions of the Paykel Scale of stressful life events and of the Holmes-Rahe Life Stress Inventory (Baratta, Colorio, & Zimmermann-Tansella, 1985; Holmes & Rahe, 1967).

2.2. Trauma-focused psychotherapy

A subsample of the whole patient group (n = 21) was recruited in the context of a project about trauma-focused psychotherapy. Nine of them underwent TF-CBT and 12 EMDR. Each patient received 24 sessions of TF-CBT or EMDR carried out by highly experienced psychotherapists. The symptomatological assessment and blood sampling were conducted at 4 time points: baseline (T0), after 4 weeks of treatment, during the hospitalization (T4), after 8 weeks, that represented the end of the treatment and hospitalization (T8), and 4 weeks after the end of the treatment, when patients went back to the hospital for the follow-up visit (T12). The pharmacological treatment (patients receiving first-generation antipsychotics: 4.8%; second-generation antipsychotics: 33.3%; SSRIs: 38.1%; SNRIs: 38.1%; TCAs: 4.8%; NaSSAs: 33.3%; other classes of antidepressants: 38.1%; mood stabilizers: 33.3%; benzodiazepines or hypnotic drugs: 100%) was maintained the same for the whole period, with possible slight adjustments in accordance with clinical needs. Symptomatological evaluations were performed using the Montgomery-Åsberg Depression Rating Scale (MADRS) (Montgomery and Asberg, 1979) and relative dimensions according to the three-factor model (MADRSF1: mood symptoms; MADRSF2: cognitive symptoms; MADRSF3: neurovegetative symptoms) (Suzuki et al., 2005), the Beck Depression Inventory II (BDI-II) (Beck, 1993), the Beck Anxiety Inventory (BAI) (Beck & Steer, 2013), and the Pittsburgh Sleep Quality Index (PSQI) (Curcio et al., 2013); the MINI-ICF-APP was used to assess psychosocial functioning (Balestrieri et al., 2013). Patients were considered responders to the psychotherapy if the reduction in MADRS score at T12 was >50%.

The study was approved by the Local Ethics Committees (Ethics Committee for Clinical Trials of the province of Verona and Rovigo N: 234,777/11.05.16) and written informed consents were obtained after the nature of the procedures had been fully explained to and understood by the patients. The clinical trial and relative assessment have been described in detail in a previous study (Minelli et al., 2019).

2.3. Blood collection, serum separation and microRNA isolation from blood

Peripheral venous blood samples were collected in the morning between 8:00 and 9:00 a.m., after an overnight fast, in anticoagulant-free tubes for serum separation and in PAXGene Blood RNA Tubes (Qiagen) for RNA isolation. Anticoagulant-free tubes were kept at room temperature for 1 hour before serum separation by centrifugation (1.680 g for 15 minutes at 4°C). Serum samples were stored at −80°C until the time of assay. PAXGene Blood RNA Tubes were kept at room temperature for 2 hours, then frozen at −20°C for 24 hours and finally stored at a −80°C until RNA isolation was performed. Total RNA was extracted from 2.5 mL of blood with the PAXGene Blood miRNA Kit (Qiagen), designed for the simultaneous isolation of small and large RNAs. RNA quantification and quality control were carried out using spectrophotometric analysis (NanoDrop 2000, Thermo Scientific).

2.4. Determination of candidate microRNA expression levels by qRT-PCR

Reverse transcription was performed starting from 150 ng of total RNA, using miRNA-specific primers (TaqMan MicroRNA Assays, Applied Biosystems). A primer pool was created to get cDNA for hsa-miR-15a (000389), hsa-miR-29a (hsa-miR-29a-3p, 002112), hsa-miR-125b (000449), hsa-miR-126 (002228), hsa-miR-146a (000468), hsa-miR-195 (000494), hsa-let-7f (000382), and the small nucleolar RNA RNU48 (001006) as endogenous control. qRT-PCR was conducted in triplicate using the corresponding TaqMan probes (TaqMan MicroRNA Assays, Applied Biosystems) on the CFX Connect Real-Time PCR Detection System (Bio-Rad), following the manufacturer's instructions. The Ct values were normalized according to the delta Ct method on RNU48, which was stably expressed across samples.

2.5. Measurement of C-reactive protein serum concentrations

Serum levels of the C-reactive protein (CRP) were measured in duplicate through enzyme-linked immunosorbent assay (ELISA), with the C Reactive Protein Human ELISA Kit (ab99995) (AbCam), following the manufacturer's instructions. CRP content was expressed as μg of protein/mL of serum.

2.6. Statistical analysis

Meeting relative assumptions, baseline data were analysed using non-parametric tests. In particular, to detect differences between groups in demographic and clinical variables, the Mann-Whitney U test was used to compare the two groups for continuous measures, and Fisher's exact test was used for categorical measures. The Kendall rank coefficient was used to evaluate bivariate correlations. These analyses were conducted with the software IBM SPSS Statistics.

To test the variations over time in the levels of the seven candidate miRNAs and of the CRP, linear mixed models were applied, in which the dependent variable was represented by the difference between each time point measurement and the baseline measurement, and time was treated as the within-subject factor. For these analyses, data were log-transformed since they were not normally distributed and the models assumed normality. In a first step, an ANOVA analysis was performed to estimate the changes between the longitudinal and the baseline measurements over time. The pairwise contrasts between the mean outcome at different time points were computed exploiting a repeated measures ANOVA analysis. In a second step, the analyses were extended estimating two different models: in the first one we included the 'kind of psychotherapy' (EMDR versus TF-CBT) as the between-subjects factor and the interaction of this variable with the time; in the second one the between-subjects factor was represented by the 'response status' (responders versus non-responders) and also in this case the interaction between this variable and the time was included.

Relative groups (EMDR versus TF-CBT and responders versus non-responders) were compared with 95% confidence interval, computing FDR-adjusted *p*-values. These analyses were conducted with the software R version 3.6.1.

2.7. Pathway analysis

A pathway analysis was conducted to identify the biological processes in which the investigated miRNAs are involved, through the freely available online software suite mirPath v.3, dedicated to the assessment of miRNA regulatory roles and the identification of controlled pathways. To this aim, mirPath utilizes predicted interactions between miRNAs and target genes, both in coding sequences or in 3′ untranslated regions, provided by the DIANA-microT-CDS algorithm or experimentally validated miRNA interactions derived from DIANA-TarBase. A functional annotation of one or more miRNAs at the same time is subsequently conducted through the use of standard (hypergeometric distributions), unbiased empirical distributions and/or meta-analysis statistics, supporting all analyses for the Kyoto Encyclopaedia of Genes and Genomes (KEGG) molecular pathways (Vlachos et al., 2015) (http://www.microrna.gr/miRPathv3).

3. Results

3.1. Clinical assessment and outcome of psychotherapy

In the whole group of TRD patients (n = 41), the mean age was 52.1 years (standard deviation: ±8.7 years) and participants included 70.7% of women. 36.6% of patients showed psychotic symptoms, 82.9% current comorbidity in Axis I, and 65.9% current comorbidity in Axis II, whereas 19.5% had post-traumatic stress disorder (PTSD), and none had alcohol abuse, as a secondary diagnosis (the total number exceeds the number of subjects due to the presence of multiple comorbidities). Based on the CAECA.Q, 58.5% and 14.6% of patients experienced mother and father emotional abuse (antipathy), respectively, whereas 53.7% reported mother neglect and 65.9% father neglect. Moreover, 19.5% and 9.8% had a history of physical abuse by the mother and the father, respectively, and 34.1% had sexual abuse. The demographic and clinical characteristics of the subgroup of patients who underwent trauma-focused psychotherapy (n = 21) are reported in Table 1. Trauma-focused psychotherapy reduced the depressive symptomatology as measured with total MADRS scores and relative dimensions from the baseline (T0) to the follow-up visit (T12) ($p < .001$ for all of them) (Figure S1). The longitudinal analyses showed significant variations over time for all the symptomatological scores considered, with significant interactions with the response status (responders versus non-responders). Complete results are reported in Table S1. Although in our study we have included only patients with MDD as primary diagnosis, a portion of them had PTSD in comorbidity (secondary diagnosis). Since these patients could benefit more from trauma-focused psychotherapy, leading to possible confounding effects both in the clinical results and in miRNA changes, we have conducted the same analyses also by excluding the seven patients with PTSD in comorbidity (n = 14). Concerning the clinical outcomes, significant results were confirmed for all the symptomatological scores (results not shown).

Overall, in the whole group undergoing psychotherapy 16 patients out of 21 (76.2%) were classified as responders and 5 (23.8%) as non-responders. The responder and non-responder groups differed for the variations from T0 to T12 in different symptomatological scores (see Table 1).

Although no interaction between time and kind of psychotherapy (TF-CBT versus EMDR) was evidenced and both psychotherapies were effective in reducing symptomatological scores, a significant difference in total MADRS score variations from T0 to T12 was detected between the two treatment

Table 1. Sociodemographic and clinical characteristics of the group of TRD patients who underwent trauma-focused psychotherapy and of the responder and non-responder groups separately. The *p*-values refer to the comparison between responders and non-responders.

Characteristics	TRD patients (*n* = 21)	Responder TRD (*n* = 16)	Non-responder TRD (*n* = 5)	*p*-value
Age (years), mean (SD)	54.9 (8.1)	55.1 (8.2)	54.0 (8.5)	0.84 [a]
Gender (% F)	81.0	81.3	80.0	1.00 [b]
Education (years), mean (SD)	12.1 (3.4)	12.7 (3.6)	10.2 (2.2)	0.11 [a]
% of smokers	28.6	25.0	40.0	0.60 [b]
Body Mass Index (BMI), mean (SD)	27.0 (4.8)	26.5 (5.1)	28.7 (3.7)	0.13 [a]
Age of onset (years), mean (SD)	34.9 (12.3)	34.1 (10.8)	37.6 (17.7)	0.55 [a]
% of presence of psychotic symptoms	14.3	6.3	40.0	0.13 [b]
% of comorbidity with personality disorders	71.4	75.0	60.0	0.60 [b]
% of comorbidity with anxiety disorders	81.1	81.3	80.0	1.00 [b]
% of PTSD	33.3	37.5	20.0	0.62 [b]
% of psychiatric disorders among the first-degree relatives	90.5	93.8	80.0	0.43 [b]
Trauma CECA.Q: % of Mother antipathy (hostility, coldness)	76.2	68.8	100.0	0.28 [b]
Trauma CECA.Q: % of Father antipathy (hostility, coldness)	14.3	18.8	0.0	0.55 [b]
Trauma CECA.Q: % of Mother neglect	57.1	56.3	60.0	1.00 [b]
Trauma CECA.Q: % of Father neglect	81.0	75.0	100.0	0.53 [b]
Trauma CECA.Q: % of Physical abuse mother	33.3	37.5	20.0	0.62 [b]
Trauma CECA.Q: % of Physical abuse father	9.5	12.5	0.0	1.00 [b]
Trauma CECA.Q: % of Sexual abuse	42.9	43.8	40.0	1.00 [b]
N° of childhood stressful life events [c], mean (SD)	3.9 (1.9)	3.8 (1.9)	4.4 (1.9)	0.55 [a]
N° of adult stressful life events [c], mean (SD)	4.9 (1.8)	5.1 (1.6)	4.0 (2.3)	0.28 [a]
Normative value of event, Paykel score, mean (SD)	57.1 (37.8)	56.7 (35.9)	58.2 (48.1)	0.97 [a]
Normative value of event, Holmes score, mean (SD)	188.3 (116.4)	192.4 (123.9)	175.2 (99.6)	0.84 [a]
% of type of trauma-focused psychotherapy (EMDR)	57.1	68.8	20.0	0.12 [b]
Baseline MADRS score, mean (SD)	27.8 (7.0)	28.1 (7.9)	27.0 (3.6)	0.78 [a]
Baseline mood symptoms MADRS score, mean (SD)	7.5 (2.3)	7.3 (2.5)	8.2 (1.1)	0.40 [a]
Baseline cognitive symptoms MADRS score, mean (SD)	14.1 (3.3)	13.9 (3.4)	14.4 (3.2)	0.72 [a]
Baseline neurovegetative symptoms MADRS score, mean (SD)	6.3 (3.0)	6.9 (3.2)	4.4 (0.9)	0.09 [a]
Baseline BDI score, mean (SD)	35.9 (10.7)	35.3 (11.0)	37.6 (11.0)	0.60 [a]
Baseline BAI score, mean (SD)	25.9 (12.5)	26.9 (12.8)	22.8 (12.2)	0.60 [a]
Baseline PSQI score, mean (SD)	12.7 (3.7)	12.9 (4.1)	11.8 (2.2)	0.50 [a]
Baseline MINI-ICF-APP score, mean (SD)	15.6 (9.4)	13.9 (9.6)	21.0 (7.3)	**0.04** [a]
% variation T0-T12 MADRS score, mean (SD)	−67.5 (29.7)	− 82.1 (13.8)	− 21.1 (12.5)	**< 0.001** [a]
% variation T0-T12 mood symptoms MADRS score, mean (SD)	−72.8 (30.3)	− 86.0 (14.5)	− 30.6 (30.0)	**< 0.001** [a]
% variation T0-T12 cognitive symptoms MADRS score, mean (SD)	−64.8 (35.7)	− 82.0 (18.4)	− 9.6 (9.3)	**< 0.001** [a]
% variation T0-T12 neurovegetative symptoms MADRS score, mean (SD)	−66.9 (37.9)	− 77.4 (31.5)	− 33.3 (40.0)	**0.015** [a]
% variation T0-T12 BDI score, mean (SD)	−57.9 (31.4)	− 69.6 (18.0)	− 20.1 (37.4)	**0.011** [a]
% variation T0-T12 BAI score, mean (SD)	−32.8 (50.2)	− 47.5 (41.4)	+ 14.4 (50.1)	**0.032** [a]
% variation T0-T12 PSQI score, mean (SD)	−26.1 (29.6)	− 26.2 (31.8)	− 25.7 (24.1)	0.97 [a]
% variation T0-T12 MINI-ICF-APP score, mean (SD)	−45.1 (42.1)	− 60.1 (35.2)	+ 2.8 (21.0)	**0.004** [a]

Bold characters indicate significant *p*-values (<0.05).
[a]*p*-values obtained applying the Mann-Whitney U test.
[b]*p*-values obtained applying the Fisher's exact two-sided test.
[c]Stressful life events include: sexual abuse, physical abuse, emotional abuse, emotional and physical neglect, death of a close family member, divorce, marital separation, personal injury or severe illness, dismissal from work, injury or illness of a close family member, miscarriage, abortion.

groups, with EMDR inducing a more pronounced reduction (p = .049; EMDR: −79.5 ± 19.6%, TF-CBT: −51.6 ± 34.3%). For further analyses, the two treatment groups were jointly considered, since they were too small to conduct separate analyses.

3.2. Baseline miRNA levels in relation to stressful life events and other sociodemographic and clinical variables

In the entire group of patients (n = 41), baseline levels of the seven candidate miRNAs were tested for correlation with or compared according to all the sociodemographic and clinical data reported in Table 1. The results showed that miR-29a and let-7f levels positively correlated with age (p = .009, t = 0.289 and p = .003, t = 0.329, respectively). Concerning stress exposure, a negative correlation was detected between miR-146a levels and recent stressful life events; specifically, higher scores at the Holmes scale correlated with lower levels of miR-146a (p = .001, t = −0.366; data available for 33 patients) (Figure 1). Moreover, for the same miRNA a trend towards significance was also observed for a negative correlation with the Paykel scale scores (p = .061, t = −0.207). No significant result was observed in relation to ELA.

3.3. Longitudinal effects of trauma-focused psychotherapy on microRNA and C-reactive protein levels and pathway analysis

For the 21 patients who underwent trauma-focused psychotherapy, the levels of the seven candidate miRNAs and the CRP were tested for variations along the 4 time points described in subsection 2.2 (T0-T4-T8-T12).

This longitudinal analysis revealed that significant variations occurred, with similar time trends, for 5 out of the 7 miRNAs: miR-15a ($p = 2.73*10^{-5}$, F = 9.56), miR-29a (p = .007, F = 4.39), miR-126 ($p = 1.98*10^{-9}$, F = 20.64), miR-195 ($p = 5.42*10^{-6}$, F = 11.22), and let-7f ($p = 4.73*10^{-5}$, F = 9.01). A weaker significant variation

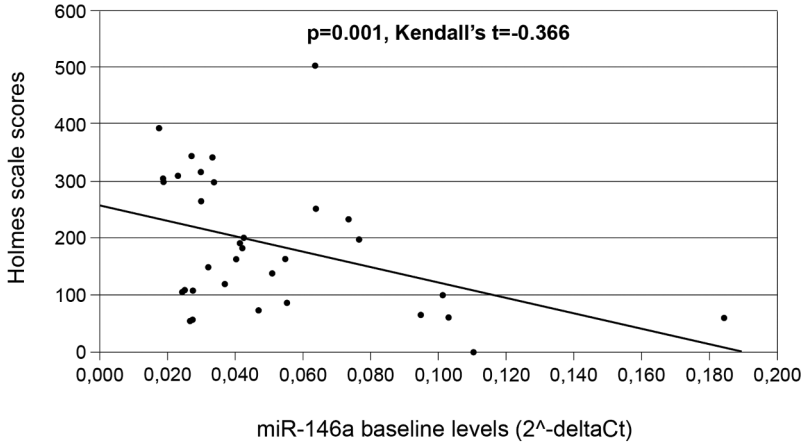

Figure 1. Correlation between recent stressful life event scores, as measured with the Holmes scale, and miR-146a baseline levels.

was observed also for the CRP (p = .035, F = 3.07). Generally, the observed trajectories were characterized by unmodified levels from T0 to T4, followed by a decrease at T8 (end of therapy) and a subsequent increase at T12 (Figure 2); p-values relative to variations between different time points (pairwise comparisons) are reported in Table 2. The same analysis was performed also by excluding the seven patients with PTSD in comorbidity. In this subsample, the results were confirmed for all the miRNAs observed as significantly changing in the whole patient sample (miR-15a: p = .001, F = 7.15; miR-29a: p = .008, F = 4.46; miR-126: $p = 1.61*10^{-5}$, F = 11.17; miR-195: p = .001, F = 7.21; let-7f: $p = 2.27*10^{-4}$, F = 8.10), suggesting that psychotherapy induces changes in the levels of these miRNAs regardless of PTSD comorbidity. On the contrary, the results were not confirmed for the CRP.

We also conducted the analyses separately in the responder (n = 16) and non-responder (n = 5) groups, observing significant results only for responders, except for the CRP (miR-15a: $p = 6.58*10^{-5}$, F = 9.18; miR-29a: p = .007, F = 4.53; miR-126: $p = 3.89*10^{-8}$, F = 18.58; miR-195: $p = 3.24*10^{-5}$, F = 9.96; let-7f: $p = 1.34*10^{-4}$, F = 8.42) (Figure S2). These findings indicate that the significant results observed in the whole group of patients receiving psychotherapy could be attributable to responders; however, it cannot be excluded that the non-significance observed in the non-responder group is due to its small sample size.

No effect of the kind of psychotherapy (TF-CBT versus EMDR) or of the response status (responders versus non-responders) was observed, and no correlation was detected between the symptomatological variations during psychotherapy and the respective variations in the levels of the candidate miRNAs and the CRP.

The pathway analysis conducted on the five miRNAs modulated during the psychotherapy showed that 59 KEGG pathways were significantly (p < .05) enriched with genes representing targets of the considered miRNAs (Table S2). These included various pathways involved in inflammation and immunity (see section 4).

3.4. 3.4 miRNA baseline levels and predictivity of psychotherapy clinical outcome

In the group of patients who underwent psychotherapy (n = 21), the baseline levels (T0) of the candidate miRNAs and the CRP were compared in responders versus non-responders. Baseline levels of miR-29a were observed to be lower in non-responders, thus being predictive of non-response to psychotherapy (p = .019, fold change = −2.33; responders: 0.007 ± 0.001, non-responders: 0.003 ± 0.005). When testing miRNA baseline levels for correlation with symptomatological variations from T0 to T12, although no correlation was highlighted for total MADRS scores (p = .085, t = −0.272), significant results were observed for miR-29a in relation to changes in MADRS mood symptoms (p = .032, t = −0.349) and, with a trend, in cognitive (p = .053, t = −0.309) and neurovegetative symptoms (p = .065, t = −0.302). Finally, a correlation was detected also with T0-T12 changes in BDI scores (p = .046, t = −0.314). All the above-mentioned significant correlations were negative, suggesting that lower baseline levels of miR-29a were predictive of a lower decrease in symptomatology, in line with the results which indicated an association of lower baseline levels with non-response.

4. Discussion

Concerning the exploration of the role of miRNAs in stressful life events, the results of this work indicated that miR-146a levels were lower in patients with higher recent stressful life event scores. miR-146a has been involved in MDD and antidepressant treatment, although with conflicting findings. Indeed, one study highlighted an up-regulation and one a down-regulation of this miRNA in the prefrontal cortex of suicide committers compared to psychiatrically healthy controls (Lopez et al., 2017; Smalheiser et al., 2012), whereas Hung and colleagues observed reduced miR-146a levels in the blood of MDD patients (Hung, Wu, Tsai, Huang, & Kang, 2019). Similarly, both an increasing (Hung et al., 2019) and a decreasing (Enatescu et al., 2016; Lopez et al., 2017) effect

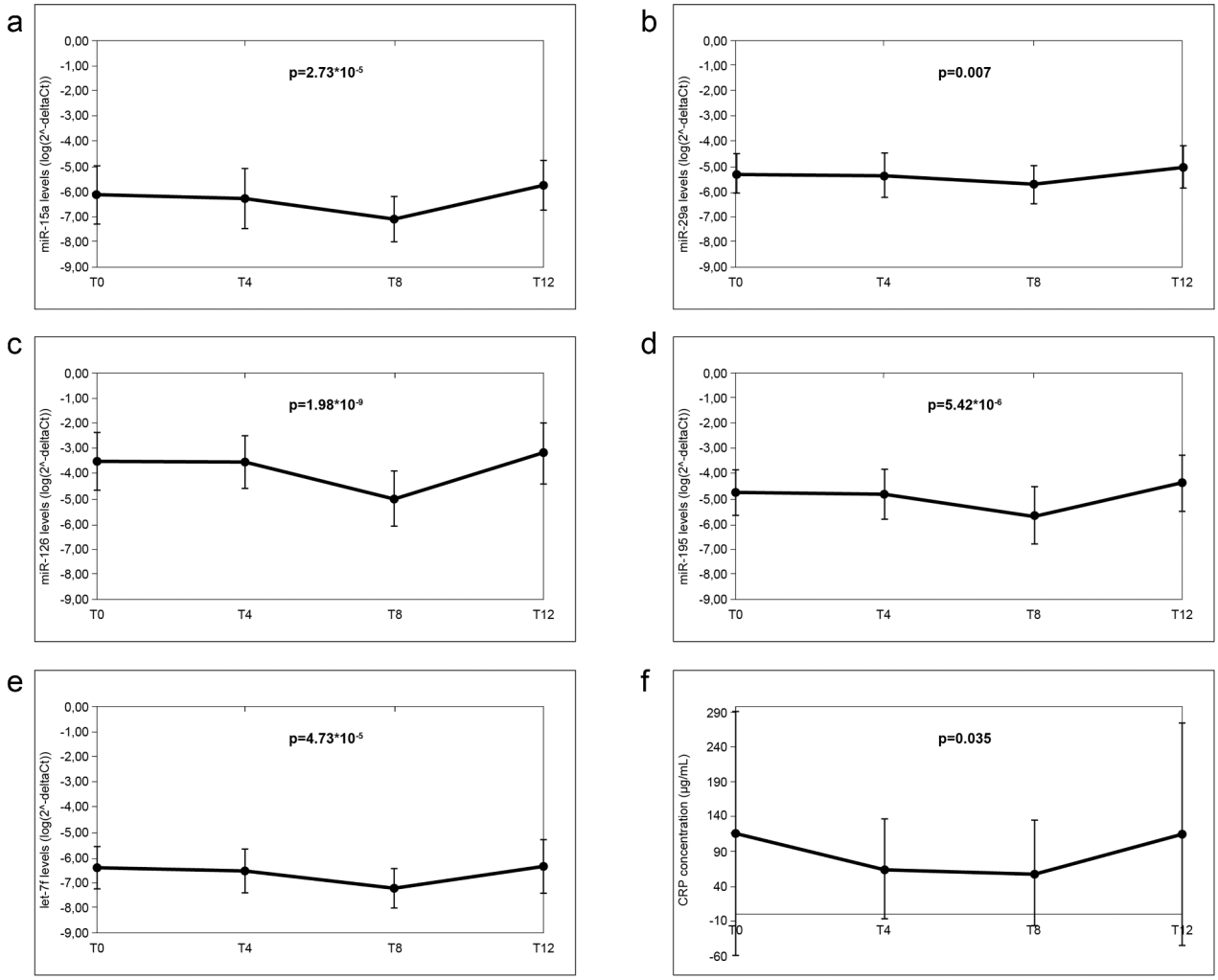

Figure 2. Longitudinal trajectories (T0-T4-T8-T12) of the C-reactive protein and miRNAs during trauma-focused psychotherapy. A: miR-15a; B: miR-29a; C: miR-126; D: miR-195; E: let-7f; F: CRP. *p*-values refer to overall changes over time.

Table 2. Pairwise comparisons for the CRP and the miRNAs showing significant overall changes over time during trauma-focused psychotherapy (adjusted *p*-values). Bold characters indicate significant *p*-values.

	T0-T4	T0-T8	T0-T12	T4-T8	T4-T12	T8-T12
miR-15a	*p* = .615	***p* = 5.92*10^{-4}**	*p* = .172	***p* = .003**	*p* = .074	***p* = 1.30*10^{-6}**
miR-29a	*p* = .779	*p* = .099	*p* = .168	*p* = .118	p = .118	***p* = .002**
miR-126	*p* = .803	***p* = 8.65*10^{-9}**	*p* = .239	***p* = 2.56*10^{-8}**	*p* = .188	***p* = 3.09*10^{-12}**
miR-195	*p* = .830	***p* = 2.19*10^{-4}**	*p* = .123	***p* = 3.52*10^{-4}**	*p* = .097	***p* = 1.28*10^{-7}**
let-7 f	*p* = .492	***p* = 4.12*10^{-5}**	*p* = .822	***p* = 8.50*10^{-4}**	*p* = .441	***p* = 2.87*10^{-5}**
CRP	*p* = .266	***p* = .046**	*p* = .825	*p* = .339	*p* = .232	***p* = .046**

of antidepressant treatment has been described in the blood of MDD patients. However, the literature is almost completely consistent in reporting an anti-inflammatory activity of this miRNA. For example, the over-expression of miR-146a has been shown to significantly decrease the levels of pro-inflammatory cytokines in a cellular model of inflammation (Lv et al., 2017) and to suppress hippocampal neuroinflammation in an animal model of cognitive dysfunction (Chen et al., 2019). Overall, the literature data concerning the role of miR-146a in MDD are complex and should be carefully interpreted in relation to the results of the present study, whereas the described anti-inflammatory activity of this miRNA, whose levels have been observed to be reduced in relation to recent stressful life event scores in this work, suggests that enhanced levels of inflammation could be present in MDD patients who experienced these events. This is in line with the well-documented inflammatory effects induced by stressful and traumatic experiences (Hori & Kim, 2019). Regarding ELA, no significant effect was highlighted in relation to the expression of the considered candidate miRNAs.

In the present study, trauma-focused psychotherapy was shown to induce a significant improvement of depressive symptomatology in TRD patients, with a high percentage of response (nearly 76%). Although both EMDR and TF-CBT were effective, the therapeutical effects of EMDR were better maintained at the

follow-up. These results are in line with a previous randomized controlled trial conducted by our research group (Minelli et al., 2019).

The expression of miR-15a, miR-29a, miR-126, miR-195 and let-7f significantly changed during the psychotherapy, also when excluding patients with PTSD in comorbidity. All these miRNAs showed similar trajectories, characterized by unmodified levels from the beginning to week 4 of psychotherapy, a decrease at the end of it (8 weeks), and an increase at the follow-up visit occurring after other 4 weeks. Since both pro- and anti-inflammatory roles were described for these miRNAs (Olivieri et al., 2013; Shang et al., 2019; Ye et al., 2016), we also measured the levels of the CRP as a recognized biomarker of inflammatory activation. Also the CRP was modified during the psychotherapy, in a similar way to miRNAs, even if with weaker statistical significance and a flattened trend. Thus, it could be cautiously hypothesized that a reduction of inflammation occurs during the psychotherapy, with a subsequent reactivation at the follow-up which is not, nonetheless, paralleled by any symptomatological exacerbation. However, the role of the above-mentioned miRNAs is not clear, since their observed variations are not always in line with their most documented function (pro- or anti-inflammatory). Far from over-simplistic explanations, these miRNAs are likely to be involved in various complex and dynamic regulatory networks, as indicated by the pathway analysis which evidenced that their target genes participate in various biological functions directly or indirectly associated with inflammation (TNF signalling (Zelová & Hošek, 2013), fatty acid metabolism (Innes & Calder, 2018), extracellular matrix–cell interaction (Sorokin, 2010)), but also in more typically immunological functions (Hippo signalling (Taha, Janse van Rensburg, & Yang, 2018), mTOR signalling (Suto & Karonitsch, 2020)), as well as in stress response (steroid metabolism (Sze & Brunton, 2020) and depression mechanisms (circadian rhythm (Varinthra & Liu, 2019) and neurotrophin signalling (Levy et al., 2018)).

Finally, through the present work, miR-29a was identified as a biomarker of response to psychotherapy; in particular, lower baseline levels were predictive of non-response or worse symptomatological improvement, as measured both by objective (MADRS) and subjective (BDI) scales. However, the results concerning the discrimination between responders and non-responders should be considered in the light of the limitation represented by the smallness of the non-responder group. miR-29a has been implicated both in stress and inflammation, with heterogeneous results. An excess of glucocorticoids has been shown to reduce miR-29a expression (Ko et al., 2013; Komori, 2016), whereas an upregulation of this miRNA has been reported in stressed animals (Sillivan, Jones, Jamieson, Rumbaugh, & Miller, 2019). Both pro- and anti-inflammatory activities of miR-29a have been described, even if the first one is more widely documented (Fabbri et al., 2012; Ranganathan et al., 2017; Tang et al., 2017).

Overall, the results here presented indicate for the first time an involvement of inflammation-related miRNAs in stressful life events and in trauma-focused psychotherapy in MDD patients, confirming the previously described link between inflammatory alterations and trauma or stress. A limitation of the study is represented by the small sample size and in particular by the scarce number of non-responder patients, which did not allow to obtain reliable results when separately considering the patients according to response or non-response to psychotherapy. Also, although the pharmacological treatment was maintained the same during the psychotherapy, the different kinds of antidepressant and other psychiatric drugs used could have differentially affected miRNA expression changes. These effects have not been evaluated in the present study due to the smallness of treatment-based subgroups. Thus, further investigations in larger samples are needed to replicate and extend these findings. Moreover, functional experiments will help to clarify the biological role of the identified miRNAs. Finally, beyond the approaches based on candidate miRNAs, miRNomic studies without *a priori* hypotheses will be extremely useful to identify new miRNAs involved in stressful experiences and response to treatments. Research in this field could have an important translational relevance, helping to find novel biomarkers of trauma and response to targeted therapies, as well as to disclose the underlying molecular mechanisms.

Acknowledgments

We would like to express our sincere gratitude to all the volunteers who participated in the study. We thank all the staff of the Psychiatric Hospital "Villa Santa Chira". We also want to thank Marcello Vitali for his kind help with artworks.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was partly supported by the EMDR Europe Research Foundation under Grant [2018-02] and by the Italian Ministry of Health under Grant [Ricerca Corrente 2020]. The post-doc position of Dr. Elisabetta Maffioletti was partly funded by the Psychiatric Hospital 'Villa Santa Chiara', Verona, Italy.

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Data availability statement

The data supporting the results and analyses presented in this paper cannot be made publicly available for privacy and ethical reasons.

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