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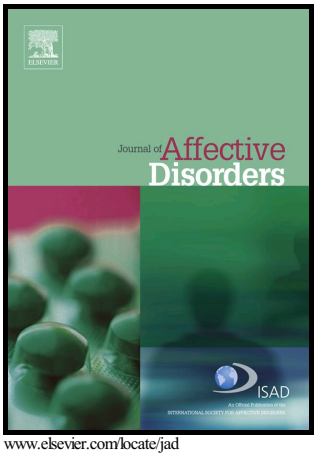

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Peripheral whole blood microRNA alterations in major depression and bipolar disorder

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Abstract

Major depression (MD) and bipolar disorder (BD) are severe and potentially life-threating mood disorders whose etiology is to date not completely understood. MicroRNAs (miRNAs) are small non-coding RNAs that regulate protein synthesis post-transcriptionally by base-pairing to target gene mRNAs. Growing evidence indicated that miRNAs might play a key role in the pathogenesis of neuropsychiatric disorders and in the action of psychotropic drugs. On these bases, in this study

[1] These authors contributed equally to this work

we evaluated the expression levels of ~~the entire miRNome~~ 1733 mature miRNAs annotated in miRBase v.17, through a microarray technique, in the blood of 20 MD and 20 BD patients and 20 healthy controls, in order to identify putative miRNA signatures associated with mood disorders. We found that 5 miRNAs (hsa-let-7a-5p, hsa-let-7d-5p, hsa-let-7f-5p, hsa-miR-24-3p and hsa-miR-425-3p) were specifically altered in MD patients and 5 (hsa-miR-140-3p, hsa-miR-30d-5p, hsa-miR-330-5p, hsa-miR-378a-5p and hsa-miR-21-3p) in BD patients, whereas 2 miRNAs (hsa-miR-330-3p and hsa-miR-345-5p) were dysregulated in both the diseases. The bioinformatic prediction of the genes targeted by the altered miRNAs revealed the possible involvement of neural pathways relevant for psychiatric disorders. In conclusion, the observed results indicate a dysregulation of miRNA blood expression in mood disorders and could indicate new aveneus for a better understanding of their pathogenetic mechanisms. The identified alterations may represent potential peripheral biomarkers to be complemented with other clinical and biological features for the improvement of diagnostic accuracy.

Keywords: microRNA, major depression, bipolar disorder, blood, biomarker, let-7

Introduction

Major Depression (MD) and Bipolar Disorder (BD) are mood disorders recognized by the World Health Organization as major causes of disability worldwide, as they frequently prevent affected individuals from leading independent lives and hold features of chronicity and potential life threat.

Several hypotheses, focused on alterations in monoamine neurotransmitters and their related signalling or, more recently, on the involvement of other biological pathways, particularly those regulating neurogenesis and neuroplasticity mechanisms (Pittenger and Duman, 2008), neuroimmune function (Müller and Schwarz, 2007) and glutamatergic neurotrasmission (Popoli et

al., 2011) have been proposed to explain the pathogenesis of these disorders. However, the etiology and pathophysiology of MD and BD are still not completely understood, thus limiting the hypothesis-driven discovery of novel therapeutic targets.

One of the major issues that still need to be addressed in mood disorders concerns the possibility to dissect MD from BD, since these pathologies show overlapping symptoms and can be misdiagnosed (Hirschfeld, 2013). Moreover, a correct differential diagnosis of MD or BD is crucial for an appropriate treatment from the onset and, in turn, for successful treatment outcomes. As a consequence, the identification of biomarkers, that can reflect MD- and BD-specific pathophysiologic processes, may be helpful and also provide novel biological targets for the development of personalized treatments (Culpepper, 2014).

MicroRNAs (miRNAs) are evolutionary conserved, small non-coding RNAs (20-22 nucleotides in length) that play an important role in the post-transcriptional regulation of gene expression. MiRNAs may act by inducing target gene mRNA deadenylation and degradation or by repressing translation, thus inhibiting protein synthesis. Each single miRNA can target hundreds of different mRNAs, and a single mRNA can be targeted by several miRNAs, allowing a coordinate and fine-tuned regulation of protein expression (O'Carroll and Schaefer, 2013). This also explains why changes in miRNAs are associated with several human pathologies (Pasquinelli, 2012), including complex disorders as cancer (Farazi et al., 2013) and cardiovascular diseases (Papoutsidakis et al., 2013).

Recent and growing evidence indicated miRNAs as key players in different processes occurring in the central nervous system (CNS). Almost 50% of all the miRNAs so far identified are expressed in the human brain, and their putative target genes are involved in the regulation of basic neural processes, such as neurogenesis and neuroplasticity (Olde Loohuis et al., 2012). These findings led to the investigation of the potential involvement of miRNAs both in the pathogenesis and in the pharmacotherapy of mental disorders (Maffioletti et al., 2014; Tardito et al., 2013); Considerable

evidence showed alterations in miRNAs expression in post-mortem brains of patients suffering from schizophrenia and BD, as well as in depressed suicide committers (Beveridge et al., 2010; Kim et al., 2010; Moreau et al., 2011; Miller et al., 2012; Smalheiser et al., 2012; Smalheiser et al., 2014). Concerning the effects of psychotropic drugs on miRNA expression, mood stabilizers were described to alter miRNAs levels in the rat hippocampus (Zhou et al., 2009) and in lymphoblastoid cell lines from BD patients (Chen et al., 2009). A role for miR-16 was suggested in the mechanism of action of the antidepressant fluoxetine (Baudry et al., 2010) and this drug, together with desipramine, was also reported to induce miRNAs modulation in rat hippocampus (Tardito et al., 2015). Finally, ketamine (an NMDA receptor antagonist with antidepressant effect) and electroconvulsive shock therapy were described to reverse changes in miRNAs expression induced by early life stress in rat hippocampus (O'Connor et al., 2012).

Besides their presence in cells, miRNAs were also observed in a highly stable cell-free form in body fluids (Cortez et al., 2011) and were detected in several peripheral biological matrices, including whole blood, plasma, serum, cerebrospinal fluid (CSF) and saliva, among the others (Cogswell et al., 2008; Mitchell et al., 2008; Park et al., 2009). The correlation observed between miRNAs expression levels in periphery and some pathological tissues suggests that the evaluation of their concentrations could provide useful biomarkers for several diseases (Laterza et al., 2009; Skog et al., 2008). A handful of studies has shown miRNA level modifications in peripheral tissues (in particular, blood and its derivatives) from MD patients, both compared to healthy controls and following pharmacotherapy, whereas only one study is available for BD. These findings are summarized in Table 1.

~~The first study on peripheral miRNAs expression in drug-free MD patients was conducted by our research group. We evaluated miRNA level changes in the whole blood of depressed patients after 12 weeks of antidepressant treatment with escitalopram, reporting a significant modulation for 30 miRNAs, potentially implicated in several biological pathways associated with brain functions~~

(Bocchio-Chiavetto et al., 2013). Other studies conducted on peripheral blood mononuclear cells from MD patients, compared to healthy controls, reported a differential expression of 14 (Belzeaux et al., 2012) and 5 miRNAs (Fan et al., 2014). Moreover, after an effective 8-weeks treatment with different classes of antidepressant drugs, a modulation was detected for 8 miRNAs (Belzeaux et al., 2012). Furthermore, an increase in the levels of 2 miRNAs, targeting the neurotrophin brain-derived neurotrophic factor (*BDNF*), was observed in the serum of MD patients (Li et al., 2013). More recently, an intriguing study evidenced a decrease in both post-mortem brains and plasma from MD patients in the levels of miR-1202, a miRNA regulating glutamate receptor (*GRM4*) expression. Moreover, miR-1202 plasma levels were modulated by antidepressant treatment and correlated with clinical response (Lopez et al., 2014). Finally, a decrease in miR-135, a miRNA regulating serotonergic neuron activity, was found in both post-mortem brains and blood of MD patients (Issler et al., 2014). Concerning BD, only one study was conducted to date in peripheral tissues, reporting a decrease in plasma miR-134 levels in BD drug-free patients during manic phase compared to healthy controls; consistently, the miRNA content increased after a 4-weeks treatment with different combinations of antipsychotics and/or mood stabilizers (Rong et al., 2011). However, no studies have been conducted to date comparing miRNA peripheral levels in MD and BD patients in order to identify potential markers useful for the differential diagnosis.

Table 1: studies which have shown miRNA level modifications in peripheral tissues from MD and BD patients, both compared to healthy controls or after pharmacological treatment. PBMCs, peripheral blood mononuclear cells.

Experimental setting	**Main finding**	**Reference**
MD patients vs. healthy controls (PBMCs)	Alteration of 14 miRNAs	Belzeaux et al., 2012
MD patients before/after AD treatment	Modulation of 8 miRNAs	
MD patients before/after AD treatment (blood)	Modulation of 30 miRNAs potentially implicated in biological pathways associated	Bocchio-Chiavetto et al., 2013

	with brain functions	
MD patients vs. healthy controls (serum)	Higher levels of 2 miRNAs targeting the brain-derived neurotrophic factor (BDNF)	Li et al., 2013
MD patients vs. healthy controls (PBMCs)	Alteration of 5 miRNAs potentially implicated in biological pathways associated with brain functions	Fan et al., 2014
MD patients vs. healthy controls (blood)	Lower levels of miR-135, which regulates the activity of serotonergic neurons	Issler et al., 2014
MD patients before/after AD treatment (plasma)	Increase in the levels of miR-1202, whcih targets the metabotropic glutamate receptor-4 (GRM4)	Lopez et al., 2014
MD patients vs. healthy controls (blood)	Lower levels of miR-320a and higher levels of miR-451, miR-17-5p and miR-223-3p	Camkurt et al., 2015
BD patients (in manic phase) vs. healthy controls (plasma)	Lower levels of miR-134	Rong et al., 2011
BD patients before/after treatment with mood stabilizers	Increase in the levels of miR-134	

Based on these lines of evidence, the aim of the present study was to investigate, by using a hypothesis-free approach, putative alterations in blood miRNA profiles related to the pathogenesis of MD and BD, in order to identify possible shared or specific signatures associated with these mood disorders.

Materials and methods

Study participants

Twenty patients with MD and 20 with BD (10 type I and 10 type II) were recruited. They had to fulfill the following inclusion criteria: (a) principal diagnosis of MD or BD type I or II, according to the DSM-IV-TR criteria; (b) current major depressive episode; (c) a minimum total score of 14 on the 17-item Hamilton Rating Scale for Depression (HAM-D_{17}) to select patients with at least moderate depression, according to the American Psychiatric Association Task Force for the Handbook of Psychiatric Measures (2000); (d) at least 18 years of age. The following exclusion criteria were considered: (a) current or previous diagnosis of organic mental disorder, schizophrenia

or other psychotic disorder; (b) current alcohol and/or substance-related disorders; (c) current eating disorders; (d) uncontrolled or serious medical condition; (e) pregnancy or post-partum period (f) body mass index (BMI) ≥ 30; (g) current treatment with antidepressant or mood stabilizer drugs. All the diagnoses were confirmed by means of the Structured Clinical Interview for DSM Axis I Disorders (SCID-I). Socio-demographic and clinical characteristics of the recruited patients were obtained through the administration of a semistructured interview: age at onset, duration of illness, psychiatric comorbidity and family history of psychiatric disorders were ascertained either from clinical charts and by direct questioning the study participants. In addition, the following clinical rating scales were administrated: the Hamilton Rating Scale for Anxiety (HAM-A) and the Clinical Global Impression (CGI).

A control group of 20 unrelated volunteers (15 females, 5 males, age 45.05 ± 10.79) was also enrolled. None of these subjects presented a positive personal and familial anamnesis for psychiatric DSM-IV-TR disorders, according to the clinical interview and confirmed by the Mini-International Neuropsychiatric Interview (MINI), was affected by any medical diseases or was in pharmacological treatment (including oral contraceptives) or has a BMI ≥ 30.

The study was performed in accordance with the ethical standards laid down in the Declaration of Helsinki. Written informed consent was obtained from each subject after a complete description of the study, which was approved by the local ethics committees.

~~There is a prevalence of female gender in the MD subgroup (17/20, 85%) compared to the BD subgroup (12/20, 60.0%), although without a significant difference (p > 0.05). BD patients show an earlier age at onset of the disorder than MD patients (24 vs 40.7 years; chi-square = 0.051; p < 0.001) and a longer duration of illness (19.55 vs 8.70; chi-square = 0 .733; p < 0.01). The severity of the depressive symptomatology at the study inclusion was similar in the two subgroups, as revealed by the scores of the assessment scales, and no other significant difference in demographic and clinical variables between MD and BD subgroups was present.~~

Blood collection and storage

Peripheral venous blood samples were collected from all the patients and controls in PAXGene tubes in the morning, after an overnight fast. The tubes were kept at room temperature for 2 hours, then frozen at -20 °C for 24 hours and finally moved to a -80 °C freezer, according to the manufacturer's instructions. The tubes were finally sent for analysis in dry ice.

MicroRNA isolation and expression analysis by microarray

Total RNA was extracted from 2.5 mL of blood with the PAXGene Blood miRNA Kit (Qiagen, CA, USA), designed for the simultaneous isolation of small and large RNAs; RNA concentration and quality were assessed through a NanoDrop spectrophotometer (Thermo Scientific, MA, USA).

A volume corresponding to 500 ng of total RNA from each blood sample was processed with the FlashTag Biotin HSR RNA Labeling kit (Affymetrix, Santa Clara, CA, USA) and subsequently hybridized onto the GeneChip miRNA 3.0 Arrays (Affymetrix, Santa Clara, CA, USA), which cover all the 1733 mature miRNAs annotated in miRBase (online miRNA database, http://www.mirbase.org) version 17 (April 2011). Washing/staining and scanning procedures were respectively conducted on the Fluidics station 450 and the GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA, USA) following the manufacturer's instructions.

~~Raw data were imported and analyzed with the software Partek Genomic Suite 6.6 (Partek, St. Louis, MO, USA). Principal component analysis (PCA) was carried out to identify outliers and the statistical analysis for the evaluation of differences in miRNA levels was performed by Analysis of variance (ANOVA) test, with 3 comparisons: BD patients vs controls, MD patients vs controls and BD patients vs MD patients. As cut-offs for the identification of the differentially expressed miRNAs, fold changes (FCs) < -1.2 or > 1.2 and FDR < 0.05 were considered.~~

Real-time PCR validation

Among the miRNAs found as significantly modulated from the microarrays data, we selected for validation in Real Time PCR (RT-PCR) those that were in common between MD and BD patients and those that had already been implicated in psychiatric disorders or in antidepressant treatment by other studies or had been involved in brain functions relevant for mood disorders. In this selection we did not consider the more recently annotated miRNAs, as still scarcely studied.

For the selected miRNAs, RT-PCR was conducted using the TaqMan MicroRNA Assays (Applied Biosystems, CA, USA), following the manufacturer's instructions; the reactions were run on the StepOnePlus instrument (Applied Biosystems). ~~The Ct values were normalized according to the deltaCt (dCt) method on the endogenous controls RNU44 and RNU48. The normalization stability score of these small nucleolar RNAs was confirmed by the analysis with the geNorm software.~~

~~The differential expression analysis was performed according to the deltadelta (ddCt) method by applying a t-test, whereas putative correlations of miRNA expression levels with subject's gender and age were assessed with the Pearson's test.~~

Statistical analysis

Data were expressed as mean ± standard deviation. Possible differences in socio-demographic and clinical variables between the groups were evaluated by chi-square test for categorical variables and by t-test for quantitative ones.

Raw microarray data were imported and analyzed with the software Partek Genomic Suite 6.6 (Partek, St. Louis, MO, USA). Principal-component analysis (PCA) was carried out to identify outliers and the statistical analysis for the evaluation of differences in miRNA levels was performed by Analysis of variance (ANOVA) test, with 3 comparisons: BD patients vs controls, MD patients vs controls and BD patients vs MD patients. As cut-offs for the identification of the differentially

expressed miRNAs, fold changes (FCs) < -1.2 or > 1.2 and FDR (False Discovery Rate) corrected-p values < 0.05 were considered.

Concerning Real-time PCR validation, the Ct values were normalized according to the deltaCt (dCt) method on the endogenous controls RNU44 and RNU48. The normalization stability score of these small nucleolar RNAs was confirmed by the analysis with the geNorm software. Normal distribution of the data was evaluated through the Shapiro-Wilk test and the differential expression analysis was performed according to the deltadelta (ddCt) method by applying a t-test, whereas putative correlations of miRNA expression levels with subject's gender and age were assessed with the Pearson's test.

Target gene prediction and pathway analysis

A gene set enrichment analysis was conducted on the putatively regulated target genes of the differentially expressed miRNAs. The goal was to identify the biological processes that might be affected by the miRNAs differentially expressed in MD and BD patients as compared to controls. To this purpose, the first step was the identification of the miRNA targets. Since the experimentally validated miRNAs-mRNAs interactions represent a relatively small subset with respect to potential interactions, a number of computational prediction tools have been developed to generate sets of candidate target genes for a given miRNA. However, the different tools often lead to a significant variability in target genes lists due to the differences in the used prediction algorithms. This is mainly because the different tools are based on the evaluation of distinct properties (e.g., thermodynamics and evolutionary conservations) and thus they provide complementary information. In order to have a more reliable prediction, we exploited ComiR, a combinatorial miRNA target prediction tool integrating the information retrieved from some of the most well-known target prediction algorithms, including Miranda, PITA, TargetScan and mirSVR. Specifically, this prediction tool computes each algorithmic score and generates as output the

combined probability of a gene to be regulated by an individual miRNA or sets of miRNAs using a support vector machine method (Coronnello and Benos, 2013). Using ComiR, we computed the probabilities of interaction between the miRNAs differently regulated and all the annotated 3'UTR sequences retrieved from ENSEMBL database.

We performed the analysis considering the different sets of differentially expressed miRNAs, namely: miRNAs associated with MD, miRNAs associated with BD and miRNAs commonly regulated between MD and BD. In order to classify whether or not a given gene had to be considered as a "good" miRNA target, we arbitrarily chose a medium-stringent threshold in the interaction probability computed by ComiR (i.e., 0.9), to include in the analysis only interactions that are more likely to occur. We performed the enrichment analysis using the tool David (Huang et al., 2009), with respect to the KEGG pathways potentially affected by miRNA expression variations.

Results

Socio-demographic and clinical characteristics of study participants

There was a prevalence of female gender in the MD subgroup (17/20, 85%) compared to the BD subgroup (12/20, 60.0%), although without a significant difference ($p > 0.05$). BD patients showed an earlier age at onset of the disorder than MD patients (24 vs 40.7 years; $p < 0.001$) and a longer duration of illness (19.55 vs 8.70; $p < 0.01$). The severity of the depressive symptomatology at the study inclusion was similar in the two subgroups, as revealed by the scores of the assessment scales, and no other significant difference in demographic and clinical variables between MD and BD subgroups was present (Table 2). All the patients were drug-naïve or drug-free from antidepressant and mood stabilizer drugs.

Table 2: demographic and clinical characteristics of Major Depression (MD) patients, Bipolar Disorder (BD) patients and healthy controls (CTRL)

	MD (n = 20)	BD (n = 20)	CTRL (n = 20)	Statistical analysis	
				p[1]	p[2]
Age (years, mean ± S.D.)	47.70 ± 11.91	43.55 ± 15.25	45.05 ± 10.79	NS	NS
Sex (n (%))					
- Females	17 (85%)	12 (60%)	15 (75%)	NS	NS
- Males	3 (15%)	8 (40%)	5 (25%)		
Age at disorder onset (years, mean ± S.D.)	40.70 ± 13.52	24.00 ± 9.78		<0.001	
Length of illness (years, mean ± S.D.)	8.70 ± 14.91	19.55 ± 13.34		<0.01	
Lifetime psychiatric comorbidity (n (%))					
- Anxiety disorders	1 (5%)	1 (5%)		NS	
- Eating Disorders (past)	0 (0%)	1 (5%)			
- Personality disorders	4 (20%)	3 (15%)			
Assessment scales scores (mean ± S.D.)					
- HAM-D_{17}	23.60 ± 3.74	22.45 ± 5.26		NS	
- HAM-A	14.40 ± 4.05	12.55 ± 4.48			
- CGI-s	4.05 ± 0.60	4.15 ± 0.36			

[1]Comparison between MD and BD patients.

[2]comparison between patients and CTRL. NS = not significant difference.

MicroRNA expression analysis

The miRNome analysis identified sets of miRNAs altered in MD and BD patients as compared to controls and a subset of miRNAs up-regulated in both patient groups. In particular, as shown in the Venn Diagram (Figure 1), 7 miRNAs were differentially expressed in MD patients with respect to controls: hsa-let-7a-5p, hsa-let-7d-5p, has-let-7f-5p, hsa-miR-1915-3p (down-regulated) and hsa-miR-199a-5p, hsa-miR-24-3p, hsa-miR-425-3p (up-regulated). Thirteen miRNAs were altered in

BD patients as compared to controls: hsa-miR-720, hsa-miR-140-3p, hsa-miR-1973, hsa-miR-30d-5p, hsa-miR-3158-3p, hsa-miR-330-5p, hsa-miR-378a-5p, hsa-miR-4521, hsa-miR-21-3p (up-regulated) and hsa-miR-1915-5p, hsa-miR-1972, hsa-miR-4793-3p, hsa-miR-4440 (down-regulated). Finally, 3 miRNAs were similarly up-regulated in MD and BD patients (hsa-miR-29c-5p, hsa-miR-330-3p, hsa-miR-345-5p).

<Figure 1 here>

No significant difference was observed comparing miRNA blood levels in MD vs BD patients. Significant data are reported in Table 3, whereas the results about all the 1733 mature miRNAs analyzed are shown in Supplementary Table 1.

Table 3: A, significant microarray results comparing miRNA blood levels in MD patients vs controls (CTRL); B, significant microarray results comparing miRNA blood levels in BD patients vs controls

A		
Transcript ID	**FDR corrected p-value**	**FC MD vs CTRL**
hsa-let-7d-5p	0.037	-1.43
hsa-miR-1915-3p	0.039	-1.65
hsa-miR-29c-5p	0.040	1.68
hsa-let-7f-5p	0.040	-1.61
hsa-miR-330-3p	0.041	1.48
hsa-miR-425-3p	0.042	1.34
hsa-miR-24-3p	0.043	1.27
hsa-let-7a-5p	0.044	-1.31
hsa-miR-199a-5p	0.045	2.16
hsa-miR-345-5p	0.045	1.48
B		
Transcript ID	**FDR corrected p-value**	**FC BD vs CTRL**

hsa-miR-720-5p	0.007	1.88
hsa-miR-3158-3p	0.007	1.63
hsa-miR-4521-5p	0.007	1.69
hsa-miR-345-5p	0.010	1.49
hsa-miR-1972-5p	0.011	-3.05
hsa-miR-4440-5p	0.018	-2.21
hsa-miR-1973-5p	0.018	1.29
hsa-miR-4793-3p	0.027	-2.74
hsa-miR-140-3p	0.027	1.33
hsa-miR-30d-5p	0.028	1.34
hsa-miR-330-3p	0.030	1.53
hsa-miR-330-5p	0.030	1.45
hsa-miR-1915-5p	0.039	-1.59
hsa-miR-378a-5p	0.042	1.54
hsa-miR-21-3p	0.043	1.38
hsa-miR-29c-5p	0.045	1.79

Considering microarray expression analysis and literature information (see Materials and Methods), we selected for RT-PCR validation a total number of 13 miRNAs: 5 from the comparison between MD patients and controls (hsa-let-7a-5p, hsa-let-7d-5p, hsa-let-7f-5p, hsa-miR-24-3p, hsa-miR-425-3p), 5 from the comparison between BD patients and controls (hsa-miR-140-3p, hsa-miR-30d-5p, hsa-miR-330-5p, hsa-miR-378a-5p; hsa-miR-21-3p), and the 3 commonly up-regulated in MD and BD patients vs controls (hsa-miR-29c-5p, hsa-miR-330-3p, hsa-miR-345-5p). RT-PCR results confirmed all the significant differences (Table 4 and Figure 2) except for hsa-miR-29c-5p, which didn't reach the statistical significance although the FC was in the same direction of the microarray analysis. Moreover, significant differences were also observed between MD and BD patients for all the analyzed miRNAs. Overall, a highly significant correlation ($r = 0.93$, $p < 0.001$) between the miRNA FCs obtained from microarray and RT-PCR analysis was found.

Table 4: deltadeltaCts (ddCt, mean ± standard deviation (SD)), fold changes (FC) and p-values, for the different comparisons, of miRNAs differentially expressed in MD patients compared to healthy

controls (CTRL), in BD patients compared to healthy controls and commonly altered in both the diseases. FC between 0 and 1 are reported as -1/FC; NS = not significant p-value

		MD vs CTRL (1) ddCt (mean ± SD) FC	BD vs CTRL (2) ddCt (mean ± SD) FC	MD vs BD (3) ddCt (mean ± SD) FC	Comparison p-value
miRNAs specifically altered in MD	hsa-let-7a-5p	0.60 ± 0.14 -1.51	-	0.51 ± 0.14 -1.42	(1) 4.43E-09 (2) NS (3) 8.11E-10
	hsa-let-7d-5p	1.25 ± 0.19 -2.38	-	1.14 ± 0.19 -2.21	(1) 1.30E-20 (2) NS (3) 9.52E-22
	hsa-let-7f-5p	2.05 ± 0.39 -4.13	-	2.04 ± 0.39 -4.10	(1) 1.43E-22 (2) NS (3) 1.01E-22
	hsa-miR-24-3p	-0.58 ± 0.17 1.50	-	-0.69 ± 0.17 1.61	(1) 7.50E-09 (2) NS (3) 2.16E-10
	hsa-miR-425-3p	-0.64 ± 0.11 1.56	-	-0.65 ± 0.11 1.57	(1) 2.12E-14 (2) NS (3) 3.77E-13
miRNAs specifically altered in BD	hsa-miR-140-3p	-	-0.65 ± 0.17 1.57	0.63 ± 0.22 -1.55	(1) NS (2) 1.06E-08 (3) 1.86E-12
	hsa-miR-21-3p	-	-0.74 ± 0.15 1.67	0.70 ± 0.26 -1.63	(1) NS (2) 2.71E-11 (3) 1.24E-12
	hsa-miR-30d-5p	-	-0.41 ± 0.22 1.33	0.44 ± 0.23 -1.36	(1) NS (2) 6.28E-06 (3) 2.21E-07
	hsa-miR-330-5p	-	-0.55 ± 0.15 1.46	0.57 ± 0.21 -1.49	(1) NS (2) 1.25E-10 (3) 5.43E-12
	hsa-miR-378a-5p	-	-0.42 ± 0.20 1.33	0.38 ± 0.25 -1.30	(1) NS (2) 2.67E-06 (3) 4.65E-06
	hsa-miR-330-3p	-0.38 ± 0.24 1.30	-0.74 ± 0.16 1.67	0.36 ± 0.24 -1.29	(1) 8.58E-05 (2) 4.88E-12 (3) 2.03E-006
miRNAs altered in MD and BD	hsa-miR-345-5p	-0.45 ± 0.18 1.37	-0.65 ± 0.18 1.57	0.2 ± 0.18 -1.15	(1) 1.09E-07 (2) 1.28E-11 (3) 1.03E-003

<Figure 2 here>

Target gene prediction and pathway analysis

With the aim to identify biological processes affected by the differentially expressed miRNAs, we first predicted the potentially regulated mRNAs and then we performed an enrichment analysis to infer putative biological pathways involved in miRNA regulation. The target gene prediction analysis was carried out considering the interaction score between 21684 ENSG gene IDs and all the miRNAs confirmed by RT-PCR as differentially expressed in MD and BD patients. We detected 229 mRNAs regulated by MD-specific miRNAs, 486 mRNAs regulated by BD-specific miRNAs, and 227 mRNAs which represent targets of the miRNAs commonly regulated in MD and BD (Supplementary Table 2). Notably, some sets of miRNAs, and in particular the 3 belonging to the let-7 family, acted mostly on the same gene targets. Indeed, the hierarchical cluster analysis clearly identified a coordinated regulated action exerted by hsa-let-7a-5p, hsa-let-7d-5p and hsa-let-7f-5p. The resulted dendrogram obtained using Ward's clustering method in R environment is reported in Figure 3.

<Figure 3 here>

The subsequent enrichment analysis of the identified target genes with respect to the KEGG pathway database showed a significant over-representation of several pathways putatively altered in MD and BD disorders (Table 3). In particular we found that, although some miRNAs were specifically modulated in MD or BD as compared to controls, some of their target genes belong to pathways that are significantly modulated in both the diseases, including *Wnt signaling pathway,*

mTOR signaling pathway, ErbB signaling pathway and Insulin signaling pathway. Conversely, other pathways seemed to be more disease-specific, as significant only in MD, like *Jak-STAT signaling pathway and Ubiquitin mediated proteolysis*, or only in BD patients, like *Long-term potentiation*, *Phosphatidylinositol signaling system*, *Neurotrophin signaling pathway* and *Gap junction.*

Table 5: KEGG pathways significantly enriched in the miRNA target gene lists respectively associated with miRNAs differentially expressed in MD and BD. Only pathways with p-value < 0.05 in at least one set are shown. NS = not significant p-value

KEGG pathway	p-value MD	p-value BD
Wnt signaling pathway	0.007	0.032
mTOR signaling pathway	0.020	0.004
Colorectal cancer	0.020	0.011
Endocytosis	0.021	0.018
ErbB signaling pathway	0.023	0.004
Insulin signaling pathway	0.040	0.006
Pathways in cancer	0.040	0.030
Jak-STAT signaling pathway	0.025	NS
Ubiquitin mediated proteolysis	0.042	NS
Long-term potentiation	NS	7.50E-04
Non-small cell lung cancer	NS	8.99E-04
Phosphatidylinositol signaling system	NS	0.006
Glioma	NS	0.010
Neurotrophin signaling pathway	NS	0.010
Melanogenesis	NS	0.025
Aldosterone-regulated sodium reabsorption	NS	0.031
Oocyte meiosis	NS	0.042
Vascular smooth muscle contraction	NS	0.045
Gap junction	NS	0.045

Discussion

In this study, by assessing the whole miRNome expression in the blood of MD and BD patients (all drug-naïve or drug-free from antidepressants and mood stabilizers), we observed a dysregulation in

a number of miRNA transcripts, some specific for MD or BD, whereas others common to both the diseases. Moreover, the RT-PCR results also evidenced significant differences, in terms of miRNA levels, between MD and BD patients.

Regarding MD, RT-PCR validation assays confirmed a significant increase of miR-24-3p and miR-425-3p levels and a decrease for let-7a-5p, let-7d-5p and let-7f-5p expression. These miRNAs were previously implicated in psychiatric diseases, as well as in neuronal molecular mechanisms and behavioural functions. In particular, miR-24-3p was suggested to be a main hypothalamic regulator of oxytocin (Choi et al., 2013), the neuropeptide that regulates several social behaviours such as stress modulation, aggressive behaviour and social recognition (Chini et al., 2014). Interestingly, miR-24-3p was found to be down-regulated in rat hippocampus following chronic treatment with two mood stabilizers, lithium and valproate (Zhou et al, 2009). Moreover, miR-425-3p, here up-regulated in MD patients, was found increased also in a similar study conducted in the peripheral blood of MD patients (Belzeaux et al., 2012). Particularly noteworthy are the data on let-7 family miRNAs. Indeed, in this study we showed a down-regulation of let-7d-5p and let-7f-5p in MD patients and interestingly, in our previous work (Bocchio-Chiavetto et al., 2013), we found them increased in the peripheral blood of MD patients after a 12-weeks treatment with escitalopram, suggesting that these miRNAs may be involved in both the pathogenesis of MD and in the effects of antidepressant drugs. On the other hand, other components of the let-7 family (namely, let-7b and let-7c) were found to be common targets of mood stabilizers in rat hippocampus (Zhou et al., 2009). Let-7 miRNAs belong to the most highly expressed miRNAs in the human brain (Anacker and Beery, 2013) and are supposed to exert a powerful influence on gene expression in the CNS. In particular, let-7 miRNAs exert a pivotal action on neuronal differentiation and maturation during neurodevelopment (Shao et al., 2010) and also on neurogenesis and neuronal plasticity functions in the adult brain.

Our data are consistent, since they showed a down-regulation in MD patients of 3 miRNAs belonging to the let-7 family that are able to regulate almost the same target genes (Figure 3). Moreover, these 3 miRNAs are coded in the same genetic cluster on chromosome 9 (hsa-let-7a-5p, chr9 94175957-94176036; hsa-let-7d-5p, chr9 94176347-94176433; hsa-let-7f-5p, chr9 94178834-94178920), suggesting a possible impaired transcriptional co-regulation.

Concerning the BD-specific miRNAs, we found an increased blood content of miR-30d-5p, miR-140-3p, miR-330-5p, miR-21-3p and miR-378a-5p. The blood expression of miR-30d-5p and miR-140-3p was increased also in MD patients after AD treatment in our previous study (Bocchio-Chiavetto et al., 2013). With regard to miR-330-5p, it was predicted to regulate many genes involved in neuronal plasticity and neurodevelopment (Cohen et al., 2014). However, in contrast with our data, a decrease of miR-330-5p miRNA was observed in post-mortem brains of BD patients (Moreau et al., 2011) and miR-21-3p levels were found reduced in MD fibroblast cultures (Garbett et al., 2014). Concerning this discrepancy, a tissue-specific miRNA regulation may likely occur, also considering a possible inverse relationship between intracellular and extracellular miRNA content. Moreover, the pharmacological long-term treatment could affect miRNA expression in post-mortem brain samples. Finally, the findings on miR-378a-5p might be of interest, considering that this miRNA is mainly involved in lipid and metabolism homeostasis, that are probably compromised in BD patients, which indeed show an increased vulnerability to develop metabolic syndrome (McElroy and Keck, 2014).

The 2 miRNAs found significantly altered in both the diagnostic groups, miR-330-3p and miR-345-5p, are predicted to regulate several target genes with a putative role in the shared pathogenetic mechanisms between MD and BD, for example the 5-hydroxytryptamine receptor 2C (*HTR2C*), monoamine oxidase A (*MAOA*), dopamine receptor D1 (*DRD1*), calcium/calmodulin-dependent

protein kinase 2 (*CAMKK2*), neurotrophic tyrosine kinase receptor, type 3 (*NTRK3*), clock homolog (*CLOCK*), cAMP responsive element binding protein 1 (*CREB1*), gamma-aminobutyric acid A receptor, alpha 2 (*GABRA2*), cannabinoid receptor 1 (*CNR1*), 5,10-methylenetetrahydrofolate reductase NADPH (*MTHFR*). Furthermore, the parallel dysregulation of these miRNAs in both the disorders suggests their involvement in depressive symptoms manifestation, since both MD and BD patients enrolled for this study are in a depressive state.

Finally, considering RT-PCR results, all the analyzed miRNAs (including MD-specific, BD-specific and commonly altered ones) showed a differential expression when directly comparing MD vs. BD patients. In particular, the levels of the 2 commonly altered miRNAs, higher both in MD and BD patients compared to healthy controls, were also significantly higher in BD vs. MD patients, with MD showing intermediate levels between controls and BD patients.

Overall, the bioinformatic analysis indicated that most of the genes potentially affected by the altered miRNAs are involved in mechanisms associated with neuroplasticity regulation and intracellular signal transduction, further supporting a role for these miRNAs in mood disorders etiology.

However, the reported miRNA alterations have been observed in peripheral blood and it's currently not clear to what extent peripheral miRNA modifications could reflect alterations occurring in the CNS. The alterations observed in the periphery might directly reflect brain modifications, since miRNAs can pass through membranes in free form or in microvesicles (Laterza et al., 2009; Skog et al., 2008), but it is also possible that changes in blood miRNA expression are due to the alteration/normalization of systems that cause molecular and cellular changes within the brain and

peripheral organs as a result of neuroendocrine or neuroimmune responses (Anacker et al., 2011; Janssen et al., 2010). Future studies investigating miRNA levels in exosomes, which act as cell-to-cell communicators and can derive from the CNS (Sheinerman and Umansky, 2013), may be useful to clarify the observed modifications. We are also aware that the sample size of this study is small and further confirmation in larger samples is needed. Finally, most of the enrolled patients were drug-free, but not drug-naïve from psychotropic drugs, so we can't exclude an influence of previous therapies on the observed results.

In conclusion, here we report a peripheral blood dysregulation in the expression levels of a panel of miRNAs specific for MD or BD patients, together with common alterations, which could potentially influence several pathways relevant for brain functions. The identification of the genes and biological pathways controlled by these miRNAs could provide new information for clarifying the pathogenesis of these diseases. Moreover, ~~although further studies in larger samples are needed,~~ the described miRNA alterations may provide potential biomarkers, which could be integrated with clinical and other biological information to enhance the diagnosis and treatment of mood disorders.

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Figure 1: Venn diagram showing the miRNAs specifically modulated in MD and BD patients compared to healthy controls, and those commonly altered in both the diseases.

Figure 2: expression levels of the miRNAs specifically modulated in MD patients compared to healthy controls (A), in BD patients compared to healthy controls (B) and commonly altered in both the diseases (C). The results of the following comparisons are shown: patients (MD or BD) vs. healthy controls and MD vs. BD patients, with * = p-value < 0.05. CTRL = healthy controls.

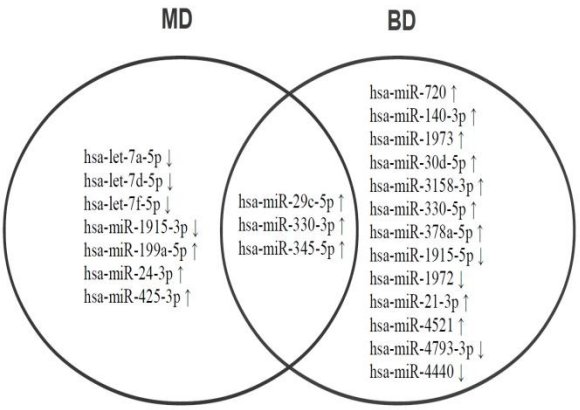
Figure 3: dendrogram showing the hierarchical cluster analysis results of miRNA-target genes interaction.

Highlights

- 5 miRNAs are specifically altered in the blood of MD patients.
- 5 miRNAs are specifically altered in the blood of BD patients in a depressive phase.
- 2 miRNAs are commonly altered in both the mood disorders.
- The dysregulated miRNAs potentially affect the expression of brain-relevant genes.

Supplementary Table 1: microarray results for all the 1733 mature miRNA analyzed, with corresponding p-values and fold changes (FC). The reported p-values are FDR corrected.

Supplementary Table 2: list of the predicted target genes of the 12 miRNAs validated by RT-PCR. Only the genes which are targeted by at least one miRNA are included.


MD
BD
hsa-let-7a-5p ↓
hsa-let-7d-5p ↓
hsa-let-7f-5p ↓
hsa-miR-1915-3p ↓
hsa-miR-199a-5p ↑
hsa-miR-24-3p ↑
hsa-miR-425-3p ↑
hsa-miR-29c-5p ↑
hsa-miR-330-3p ↑
hsa-miR-345-5p ↑
hsa-miR-720 ↑
hsa-miR-140-3p ↑
hsa-miR-1973 ↑
hsa-miR-30d-5p ↑
hsa-miR-3158-3p ↑
hsa-miR-330-5p ↑
hsa-miR-378a-5p ↑
hsa-miR-1915-5p ↓
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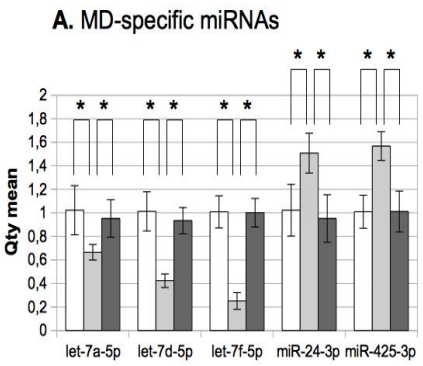
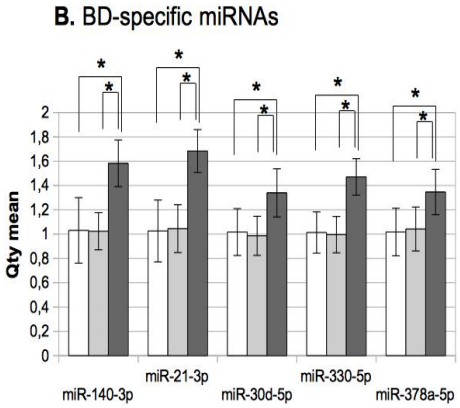
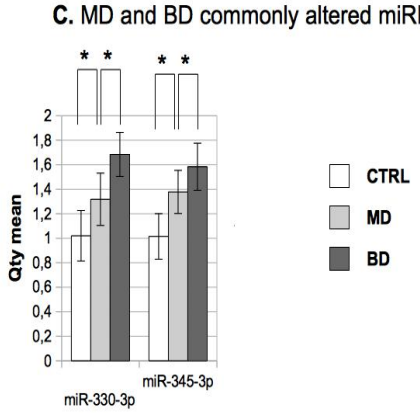
hsa-miR-21-3p ↑
hsa-miR-4521 ↑
hsa-miR-4793-3p ↓
hsa-miR-4440 ↓


A. MD-specific miRNAs
Qty mean
let-7a-5p
let-7d-5p
let-7f-5p
miR-24-3p
miR-425-3p


B. BD-specific miRNAs
Qty mean
miR-140-3p
miR-21-3p
miR-30d-5p
miR-330-5p
miR-378a-5p


C. MD and BD commonly altered miRNAs
Qty mean
miR-330-3p
miR-345-3p
CTRL
MD
BD