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# Responder and nonresponder patients exhibit different peripheral transcriptional signatures during major depressive episode

R Belzeaux<sup>1,2,3</sup>, A Bergon<sup>2,4,5</sup>, V Jeanjean<sup>1,2</sup>, B Lloriod<sup>4,5</sup>, C Formisano-Tréziny<sup>6,7</sup>, L Verrier<sup>2</sup>, A Loundou<sup>8,9</sup>, K Baumstarck-Barrau<sup>8,9</sup>, L Boyer<sup>9,10</sup>, V Gall<sup>4,5</sup>, J Gabert<sup>6,7,11</sup>, C Nguyen<sup>4,5</sup>, J-M Azorin<sup>2,3</sup>, J Naudin<sup>2</sup> and EC Ibrahim<sup>1</sup>

To date, it remains impossible to guarantee that short-term treatment given to a patient suffering from a major depressive episode (MDE) will improve long-term efficacy. Objective biological measurements and biomarkers that could help in predicting the clinical evolution of MDE are still warranted. To better understand the reason nearly half of MDE patients respond poorly to current antidepressive treatments, we examined the gene expression profile of peripheral blood samples collected from 16 severe MDE patients and 13 matched controls. Using a naturalistic and longitudinal design, we ascertained mRNA and microRNA (miRNA) expression at baseline, 2 and 8 weeks later. On a genome-wide scale, we detected transcripts with roles in various biological processes as significantly dysregulated between MDE patients and controls, notably those involved in nucleotide binding and chromatin assembly. We also established putative interactions between dysregulated mRNAs and miRNAs that may contribute to MDE physiopathology. We selected a set of mRNA candidates for quantitative reverse transcriptase PCR (RT-qPCR) to validate that the transcriptional signatures observed in responders is different from nonresponders. Furthermore, we identified a combination of four mRNAs (*PPT1*, *TNF*, *IL1B* and *HIST1H1E*) that could be predictive of treatment response. Altogether, these results highlight the importance of studies investigating the tight relationship between peripheral transcriptional changes and the dynamic clinical progression of MDE patients to provide biomarkers of MDE evolution and prognosis.

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## Introduction

Responses to current treatments of a major depressive episode (MDE) are often inconsistent and unpredictable. More than one-third of patients will not respond to the first antidepressant prescribed. Less than half of treated patients demonstrate complete remission.<sup>1,2</sup> Practice guidelines recommend waiting from 2 to 8 weeks with the same treatment to evaluate initial clinical response<sup>3–5</sup> (that is, a 50% reduction in depressive symptoms as measured by depression rating scales such as the Hamilton rating scale for depression (HDRS) or the Montgomery–Asberg depression rating scale (MADRS)).<sup>6</sup> Moreover, studies suggest that clinical response generally occurs after about a month,<sup>7</sup> and could occur after 10 weeks of treatment initiation.<sup>8</sup> On the other hand, waiting for such a long time without efficacy is associated with extended suffering, disability and risk of suicide.<sup>9</sup> Without clinical parameters clearly predictive of treatment outcome, it is therefore challenging to make a valid prognosis for MDE. In addition, there has not been any established biological marker at present that can predict treatment

response for the individual patient before the initiation or during the early course of antidepressant treatment.<sup>10</sup>

Epidemiological studies suggest that environmental factors, especially exposure to stressful life events, have a significant role in triggering major depression.<sup>11</sup> It has been proposed that the combination of certain environmental factors with genetic predispositions would result in an epigenetic and persistent dysregulation of cerebral gene programs, leading to phenotypic manifestations of psychiatric disorders, including depression.<sup>12</sup> To study such gene program alterations in a central nervous system disorder, we and others underlined the potential interest of using a peripheral tissue sample, such as blood, as these tissues share numerous biological pathways with the central nervous system.<sup>13–15</sup> Indeed, significant gene expression similarities were found between blood and brain,<sup>16,17</sup> including numerous candidate genes in mood disorders.<sup>18,19</sup> Moreover, compared with post-mortem tissues or cell lines cultured with long-term serial passage, freshly collected blood cells offer the possibility to more precisely correlate the stage of clinical

<sup>1</sup>Aix Marseille Université, CNRS, CRN2M UMR 7286, Marseille, France; <sup>2</sup>APHM, Hôpital Sainte Marguerite, Pôle de Psychiatrie Universitaire Solaris, Marseille, France; <sup>3</sup>FondaMental, Fondation de Recherche et de Soins en Santé Mentale, Paris, France; <sup>4</sup>INSERM, TAGC UMR\_S 1090, Marseille, France; <sup>5</sup>Aix Marseille Université, TAGC UMR\_S 1090, Marseille, France; <sup>6</sup>INSERM, UNIS UMR\_S 1072, Marseille, France; <sup>7</sup>Aix Marseille Université, UNIS UMR\_S 1072, Marseille, France; <sup>8</sup>Aix Marseille Université, Faculté de Médecine Timone, Unité d'aide méthodologique, Marseille, France; <sup>9</sup>Department of Public Health, APHM, Hôpital La Timone, Marseille, France; <sup>10</sup>Aix Marseille Université, Research Unit EA 3279, Marseille, France and <sup>11</sup>APHM, Hôpital Nord, Laboratoire de Biochimie-Biologie Moléculaire, Marseille, France

Correspondence: Dr EC Ibrahim, Aix Marseille Université, CNRS, CRN2M UMR 7286, 51 Bd Pierre Dramard, 13344 Marseille Cedex 15, France.

E-mail: el-cherif.ibrahim@univ-amu.fr

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evolution and treatment with biological observations. Several publications have shown transcriptional variations in peripheral blood that discriminate MDE patients from controls<sup>14,20–25</sup> and also patients during several weeks of treatment.<sup>13,19,26–31</sup>

Of note, most gene expression studies investigating MDE have been conducted with a candidate gene design that is biased toward pre-existing theory. On the other hand, pangenomic studies offer insights from novel molecular signatures and possible underlying biological mechanisms, but have the drawback of multiple testing and PCR validation. In addition, when validating potential biomarkers with or without hypothesis-driven investigations of transcriptional signatures, the physiological and stochastic variability of gene expression of samples within the same patient, between different patients, and more importantly within and between healthy controls, remain largely underestimated, raising the possibility of biases and false-positive gene candidates. Thus, although some studies suggest that the majority of gene expression is stable within individuals and have low dependence on physiological parameters,<sup>32</sup> repetitive measurements of gene expression in both patients and controls may help discriminate real variations triggered by disease evolution and treatment pharmacodynamics from other causes of gene expression variability.

We and others have shown that genes dysregulated during MDE belong to various molecular families.<sup>13,33</sup> One possible explanation for this convergent regulation of structurally distant markers might be due to common master regulator(s) yet to be characterized. MicroRNAs (miRNAs) are a class of non-coding RNAs that regulate gene expression by binding to their target mRNAs. Recently, it has been hypothesized that miRNAs have a role in brain disorders and emerging evidence suggests that they regulate neuropathology associated processes, such as brain development, dendritic spine morphology and neurite outgrowth.<sup>34</sup> The estimated number of human miRNAs exceeds 1500 (miRBase release 18) with >60% of all human mRNA predicted to contain conserved miRNA targets. Thus, miRNAs represent candidates for psychiatric diseases-associated genetic and epigenetic factors and constitute promising targets for novel drugs.<sup>35</sup> So far, although several studies investigated altered levels of miRNAs in schizophrenia, only one work comprehensively examined miRNA expression in the prefrontal cortex of depressed suicide subjects.<sup>36</sup>

In this study, we undertook a longitudinal follow-up of severe MDE patients and their matched controls, recruited in naturalistic conditions, to further characterize transcriptional biomarkers of MDE evolution. After conducting genome-wide screens and quantitative reverse transcriptase PCR (RT-qPCR) validation, we evaluated whether specific RNA signatures (both mRNA and miRNA) from blood collected at 0, 2 and 8 weeks after study inclusion can distinguish MDE patients from controls and may be predictive of the treatment response.

## Materials and methods

**Design setting and subjects.** The study design was a naturalistic, prospective, longitudinal and comparative study with assessments of MDE patients and healthy controls at

baseline (week 0), 2 and 8 weeks after study inclusion. Sixteen patients who met the Diagnostic and Statistical Manual of Mental Disorders, fourth edition, text revision (DSM-IV-TR) criteria for major depressive disorder participated to the study.<sup>37</sup> Inclusion criteria were: (i) treated or untreated MDE (ii) 17-item HDRS score  $\geq 20$  corresponding to severe or very severe MDE.<sup>38</sup>

At the end of the 8-week clinical follow-up, patients were classified as responders and nonresponders according to the consensual definition of clinical response corresponding to a minimal reduction of 50% of the HDRS score from the initial evaluation.<sup>6</sup>

For the control group, age- and sex-matched subjects were evaluated to exclude any psychiatric disorder history using the French version of standardized interview validated for health control subjects (SCID-NP).

**Blood mRNA extraction.** Peripheral blood mononuclear cells (PBMCs) were isolated from the blood by Ficoll density centrifugation. Total RNAs were extracted from the PBMCs with the mirVana miRNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. RNA concentration was determined using a nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

**Preparation of samples and microarray assay.** Sample amplification, labeling and hybridization onto Agilent whole human genome oligo microarrays containing 50 599 different oligonucleotide probes (SurePrint G3 Human GE 8 × 60 K, Agilent Technologies) essentially followed the one-color microarray-based gene expression analysis (low RNA input linear amplification PLUS kit) recommended by Agilent Technologies (details in Supplementary Information). The scanned images were analyzed with Agilent feature extraction software 10.5.1.1 to obtain background subtracted and spatially detrended processed signal intensities. All data were normalized by quantile normalization, and only 39 784 oligonucleotide probes with signal intensities detectable (that is, above background according to the 'glsWellAboveBg' Agilent feature extraction value) in  $\geq 70\%$  of samples (from either MDE patients or controls and at either baseline or 8 weeks later) were subsequently analyzed. The microarray data are available from the gene expression omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) under the series accession number GSE38206.

**Gene and chromosome band enrichment analyses.** Lists of significant genes were uploaded on DAVID (database for annotation, visualization and integrated discovery) for identifying statistically relevant signaling pathways,<sup>39</sup> with high classification stringency,  $P$ -value ( $p$ ) < 0.05 and false discovery rate (FDR)  $\leq 5\%$ . To determine the chromosomal cytoband enrichment in our lists of significant genes, we used the ToppFun algorithm and restricted the search to  $P < 0.05$ .<sup>40</sup>

**miRNA quantification.** Four hundred nanogram of total RNA were reverse transcribed using the TaqMan MicroRNA RT kit (Applied Biosystems, Foster City, CA, USA) in combination

with the stem-loop Megaplex primer pools (A and B v3.0, Applied Biosystems) without preamplification and according to manufacturer's recommendation. For each RT pool, the equivalent of 320 ng of total RNA converted into cDNA was mixed with TaqMan Universal PCR Master Mix II No AmpErase UNG (Applied Biosystems). Hundred microliter of mix (A or B) was loaded into each port of the corresponding 384 wells Human miRNA TaqMan low density array (that is, A or B) and run for 40 cycles on a ABI PRISM 7900 HT according to manufacturer's protocol with SDS v2.4 software. Normalized expression level of each miRNA was quantified as  $2^{-\Delta\Delta Ct}$  with the DataAssist software (Applied Biosystems, v3.0), relatively to the normalized expression level of the same miRNA in a calibrator sample (details in Supplementary Information).

**Target prediction.** For each list of differentially expressed miRNAs the Ingenuity pathway analysis software (Ingenuity systems), which relies on three popular algorithms (TargetSan, TarBase and miRecords), was queried to identify targets within lists of differentially expressed genes in our microarray analysis. Only highly predicted and experimentally validated targets were considered for further analysis.

**Real-time RT-PCR for candidate gene validation.** RNA was reverse transcribed with the High Capacity cDNA archive kit (Applied Biosystems). The resulting cDNA was combined with a TaqMan universal PCR Master Mix (Applied Biosystems) and 48 PCR reactions were simultaneously performed in triplicate on an ABI PRISM 7900HT thermocycler using tLDA technology according to manufacturer recommendations (Applied Biosystems). For each tested candidate gene, primer sets and probes were selected using the web portal of the manufacturer (Applied Biosystems). The expression level of each candidate gene was calculated as  $2^{-\Delta\Delta Ct}$  with the DataAssist software (Applied Biosystems, v3.0), in which each candidate gene is quantified relative to the expression of one reference gene, or a combination of two reference genes exhibiting proximal level of expression compared with the target gene, and also with a calibrated sample (details in Supplementary Information).

### Statistical analysis

**Demographic and clinical data.** Demographic and clinical variables were compared between patients and matched-controls for cohort A and between responder and non-responder patients with a Fisher exact test for qualitative variables and a *t*-test for quantitative variables.

**Microarray data.** Differential gene expression were obtained using the MultiExperiment Viewer 4 (MeV4, TM4 software suite) and SAM (significant analysis of the microarrays program) were measured with a FDR threshold set at 5%.<sup>41,42</sup> Student's *t*-tests were also applied to determine *P*-values. The data were analyzed using either a two-class unpaired (for patients versus controls comparison) or two-class paired (for internal comparisons within patients or within controls) response type. Details are provided in Supplementary Information and include the Supplementary Figure 1 for the calculation of the number of samples required.

**miRNA study.** To select differentially expressed miRNAs (at 0 and 8-week visits), non-parametric unpaired Mann–Whitney tests were used to compare the fold change (FC) between patients and matched-controls with a threshold *P*-value of 0.05. Non-parametric paired Wilcoxon tests were used to compare the FC between the two evaluations within the MDE group with a threshold *P*-value of 0.05.

**Candidate gene validation.** For exploratory purposes and to compare microarray data with RT-qPCR data, parametric unpaired *t*-tests were used to compare the FC between patients and matched controls at first evaluation and 8 weeks later within either the whole cohort (FC<sub>all</sub>), the initial sub-cohort A (FC<sub>A</sub>), or the subsequent sub-cohort B (FC<sub>B</sub>).

To explore differences between responders and non-responders, we calculated and compared marginal estimated mean of each group in a mixed linear model.

To explore the association between mRNA expression of best candidates and clinical evolution, we calculated Spearman's correlations between HDRS evolution (0 to 8- and 2 to 8-week intervals) and FC at inclusion or 2 weeks later, respectively. A discriminant function analysis was used with the goal of establishing a predictive score to classify patients in the responder versus nonresponder groups. Sensitivity, specificity, positive/negative predictive value and the confidence intervals of either each selected mRNA candidates or a combination of the best classifiers were computed for the combination of selected variables. Receiver-operating characteristic (ROC) curves analysis was used to determine the area under the curve.

## Results

**Demographic and clinical variables.** We recruited a group of 16 severe MDE patients (MDE group) and 13 healthy sex- and age-matched controls (control group). A summary of clinical and therapeutic data for each patient is reported in Table 1. Patients and controls were all of Caucasian origin and the analysis of socio-demographic parameters (Table 1 and Supplementary Tables 1 and 2) showed no significant difference among patients and controls (Supplementary Table 3).

MDE patients demonstrated different patterns of clinical evolution. Ten patients showed a clinical response (responders), whereas six patients did not experience a clinical response at 8 weeks of follow-up (nonresponders). To increase the chances of identifying transcriptional variations between MDE and control subjects, we decided to use only samples obtained from responder MDE patients and their matched controls for genome-wide screening. For simplification, we separated our study cohort into sub-cohort A (including nine MDE responders and nine matched-controls) and sub-cohort B, corresponding to the 11 other individuals (seven MDE patients (one responder and six nonresponder) and four controls) whose samples were not screened by microarray. In addition, we did not find any significant differences between responders and nonresponders based on variables reported on Table 1 and the Supplementary Table 1, such as HDRS score at inclusion, episode duration before inclusion, total number of MDE, history of suicide

**Table 1** Major depression sub-cohorts and the paired control cohorts

Cohort	Pairs	Major depression group										Control group	
		Sex	Age at first visit	Episode number	Inclusion		2 weeks		8 weeks		Sex	Age at first visit	
					HDRS	Medications	HDRS	Medications	HDRS	Medications			
A	1	F	61	2	40	Venl, Ola, Arip, Bzd	36	Mirt, Arip, Bzd	14	ECT, Mirt, Arip, Hydrox	F	59	
	2	F	58	4	27	Dul, Mirt, Li	24	Dul, Mirt, Li	2	Dul, Li	F	56	
	3	F	46	1	25	N	18	Esc	3	Esc	F	48	
	4	M	42	3	26	Dul, Mirt, Bzd	13	Dul, Mirt, Bzd	8	Dul, Mirt, Bzd	M	37	
	5	F	63	5	28	Dul, Mirt, Bzd	9	Dul, Mirt, Li, Arip, Bzd	7	Dul, Mirt, Li, Arip, Bzd	F	65	
	6	M	57	1	22	Miln, Mirt	9	Miln, Mirt	8	Miln, Mirt, Flx	M	44	
	7	F	70	4	26	Clom, Flx, Venl, Amlp, Bzd	16	Venl, Amlp, Bzd	4	Venl, Amlp, Bzd	F	64	
	8	M	59	4	28	Venl, Mirt, Arip	23	Venl, Mirt	7	ECT, Venl, Mirt	M	55	
	9	M	59	3	29	Flv, Mirt, Bzd	17	Dul, Li, Bzd	14	Dul, Clom, Li, Bzd	M	56	
	Mean	5F/4M	57.2	3	27.9		18.3		7.44		5F/4M	53.8	
	s.e.m.		2.83	0.47	1.66		2.83		1.43			3.07	
B	10	M	56	1	30	Ami	16	Ami, Bzd	16	Venl, Ami, Li, Bzd	M	53	
	11	M	74	2	37	Prx, Ola, Bzd	29	Prx, Ola, Bzd, Hydro	32	Sert, Mirt, Li, Ola, Bzd	M	72	
	12	F	47	4	21	Dul	9	Dul	8	Dul	F	53	
	13	F	51	1	28	Sert, Bzd	25	Sert, Bzd	—	—	F	60	
	14	M	65	5	21	Venl, Ola	17	Moclo, Ola	20	Moclo, Ola	—	—	
	15	M	41	2	24	Venl, Bzd	8	Venl, Bzd	20	Venl	—	—	
	16	M	35	2	22	Venl, Mirt, Esc, Ola	23	ECT, Esc, Venl, Ola	25	ECT, Esc, Venl, Mirt	—	—	
	Mean	2F/5M	52.7	2.43	26.1		18.1		20.0		2F/2M	59.5	
	s.e.m.		5.12	0.57	2.24		3.01		3.36			4.48	
Total	Mean	7F/9M	55.3	2.75	27.1		18.3		12.5		7F/6M	55.5	
	s.e.m.		2.71	0.36	1.33		2.00		2.24			2.55	

Abbreviations: Amlp, amisulpride; Ami, amitriptyline; Arip, aripiprazole; Bzd, benzodiazepine; Clom, clomipramine; Dul, duloxetine; ECT, electro-convulsive-therapy; Esc, escitalopram; Flv, Fluvoxamine; Flx, fluoxetine; HDRS, Hamilton rating scale for depression; Hydrox, hydroxyzine; Ipro, iproniazide; Li, lithium; Miln, milnacipram; Mirt, mirtazapine; Moclo, moclobémide; N, no; Ola, olanzapine; Prx, paroxetine; Sert, Sertraline; Venl, venlafaxine.

attempts, and familial history of MDE or other severe psychiatric disorders (Supplementary Table 4).

**Microarray analysis of altered mRNA expression.** The 36 samples obtained after collecting PBMC total RNAs from responder patients and matched controls of cohort A were used to characterize MDE genome-wide microarray transcriptional signatures. After conducting multiple SAM analysis with FDR set at 5%, we identified lists of transcripts differentially expressed between MDE patients at week 0 (HDRS  $\geq 20$ ) and controls, as well as between MDE patients at week 8 (with minimal reduction of  $\geq 50\%$  of HDRS) and controls. To challenge the statistical significance of the microarray results, we also performed *t*-test analysis, using an arbitrary chosen cutoff  $\geq 1.50$  FC in expression and *P*-value of  $\leq 0.001$ . About 200 transcripts were dysregulated among MDE patients at baseline compared with controls (Supplementary Tables 5 and 6), while nearly 130 were altered in MDE patients at 8 weeks (Supplementary Tables 7 and 8), with a core of 70 transcripts remaining dysregulated in the same way at both inclusion and 8 weeks later in MDE compared with controls (Supplementary Tables 9 and 10). We found similar numbers of overexpressed and under-expressed transcripts when comparing MDE patients to controls. The strong decrease in the number of dysregulated transcripts in MDE patients at 8 week is suggestive of a

state-dependent signature of MDE in relation to clinical improvement. Thus, we also performed paired statistical analysis to identify transcripts whose level of expression would change with either treatment response or clinical evolution in patients (baseline versus 8-week samples) but did not identify such a signature at a pangenomic level at an FDR of 5%.

Nevertheless, because we tested each control twice in the microarray analysis, we also determined whether certain candidate transcripts showing variations between patients and controls may be false positives. Indeed, a SAM analysis of paired control samples at baseline and at 8 weeks showed that false positive transcripts were not present in our lists of dysregulated transcripts between MDE and control subjects with FDR set at 5%. Furthermore, we determined that variations for 53 transcripts reached statistical significance using a *t*-test ( $FC \geq 1.50$ ;  $P < 0.001$ ; Supplementary Tables 11 and 12). Importantly, among transcripts exhibiting variations compared with controls, we were able to find a few previously identified candidate genes for MDE physiopathology, such as *NPY* and *GRIK5*, highlighting the importance of multiple measurements in control samples to exclude transcriptional variations not specifically associated to disease pathophysiology.

As presented in Table 2 and Supplementary Table 13, using gene ontology analysis of dysregulated MDE transcripts, the

**Table 2** Gene ontology analysis of dysregulated genes (FDR ≤ 5%)

Category	ID	Term	Genes	Count	%	P-value
<i>MDE<sub>0w</sub> vs C (FC ≥ 1.50)</i>						
Biological process	GO:0008219	Cell death	<i>API5, FIG4, GSPT1, ARHGEF6, APAF1, ELMO2, HIPK3, PPT1, PDCC6IP, PKM2, SGPP1, TPP1, KRAS</i>	13	12.4	1.6E-3
Biological process	GO:0007033	Vacuole organization	<i>FIG4, HEXB, PPT1, TPP1</i>	4	3.8	1.9E-3
Cellular component	GO:0016023	Cytoplasmic membrane-bounded vesicle	<i>AGFG1, DENND1A, CHIC2, HEXB, HGF, MAPKAP1, PPT1, PDCC6IP, SNAP23, TPP1</i>	10	9.5	3.3E-3
Molecular function	GO:0000166	Nucleotide binding	<i>ACTBL2, ACSL4, APAF1, ARL8B, ATP2A2, CMPK1, CTBP1, CELF2, GNAQ, GSPT1, HNRNPF, HIPK3, KRAS, MAT2A, MAPK6, MYH9, PKM2, POTEE, POTEF, POTEKP, PTBP1, RAB18, RAP2C, UBE2E2, UBE2H</i>	25	23.8	4.2E-3
<i>MDE<sub>0w</sub> vs C (FC ≤ -1.50)</i>						
Cellular component	GO:0000786	Nucleosome	<i>HIST1H1A, HIST1H1D, HIST1H1E, HIST1H3F/HIST2H3A/HIST1H3D, HIST1H4E/HIST1H4A/HIST1H4K/HIST1H4L</i>	5	8.9	8.6E-6
Cellular component	GO:0044427	Chromosomal part	<i>CENPP, HIST1H1A, HIST1H1D, HIST1H1E, HIST1H3F/HIST2H3A/HIST1H3D, HIST1H4E/HIST1H4A/HIST1H4K/HIST1H4L</i>	6	10.7	1.1E-3
Biological process	GO:0006325	Chromatin organization	<i>HIST1H1A, HIST1H1D, HIST1H1E, HIST1H3F/HIST2H3A/HIST1H3D, HIST1H4E/HIST1H4A/HIST1H4K/HIST1H4L, TLK2</i>	6	10.7	1.1E-3
<i>MDE<sub>8w</sub> (responders) vs C (FC ≥ 1.50)</i>						
Biological process	GO:0007040	Lysosome organization	<i>HEXB, PPT1, TPP1</i>	3	5.5	2.6E-3
<i>MDE<sub>8w</sub> (responders) vs C (FC ≤ -1.50)</i>						
Biological process	GO:0006323	DNA packaging	<i>ASH1L, HIST1H1C, HIST1H1D, HIST1H1E, HIST1H3F/HIST1H3D, HIST1H4J/HIST1H4K/HIST1H4L/HIST1H4E/HIST1H4A</i>	6	12.5	7.4E-6
Biological process	GO:0006334	Nucleosome assembly	<i>HIST1H1C, HIST1H1D, HIST1H1E, HIST1H3F/HIST1H3D, HIST1H4J/HIST1H4K/HIST1H4L/HIST1H4E/HIST1H4A</i>	5	10.4	4.4E-5
Biological process	GO:0051276	Chromosome organization	<i>ASH1L, HIST1H1C, HIST1H1D, HIST1H1E, HIST1H3F/HIST1H3D, HIST1H4J/HIST1H4K/HIST1H4L/HIST1H4E/HIST1H4A, SMC1A, TLK2</i>	8	16.7	1.1E-4
Biological process	GO:0006325	Chromatin organization	<i>ASH1L, HIST1H1C, HIST1H1D, HIST1H1E, HIST1H3F/HIST1H3D, HIST1H4J/HIST1H4K/HIST1H4L/HIST1H4E/HIST1H4A, TLK2</i>	7	14.6	2.2E-4
Biological process	GO:0034622	Cellular macromolecular complex assembly	<i>HIST1H1C, HIST1H1D, HIST1H1E, HIST1H3F/HIST1H3D, HIST1H4J/HIST1H4K/HIST1H4L/HIST1H4E/HIST1H4A, RPL24</i>	6	12.5	8.3E-4
Biological process	GO:0048562	Embryonic organ morphogenesis	<i>CLIC5, EDN1, HOXA3, HOXA7</i>	4	8.3	3.7E-3

Abbreviations: FC, fold change; FDR, false discovery rate; GO, gene ontology; PPT1, palmitoyl-protein thioesterase 1; RT-qPCR, quantitative reverse transcriptase PCR. Bold entries refer to candidate transcripts tested for RT-qPCR validation.

most significant biological processes associated with MDE signatures were related to the nucleosome and chromatin. This result suggests a trait-dependent signature because it was observed for both baseline and 8-week samples. For overexpressed transcripts, processes identified included cell death and vacuole or lysosome organization. In addition, the chromosomal localization of dysregulated transcripts revealed significant enrichment of certain cytobands (FDR ≤ 1%), such as the 6p22 region containing histone genes, and the 2q21, 9p21 and 22q13 cytobands (Supplementary Table 14).

**Differentially expressed miRNAs in MDE.** In addition to microarray screening, the same RNA samples were used to search for miRNAs that could be differentially expressed

between MDE and control subjects. Out of the 762 miRNAs assayed in parallel by multiplex RT-qPCR, approximately one-third revealed mean expression values above the background level for detectability (243 miRNAs were amplified with a Ct < 33) and were further analyzed (Supplementary Table 15). We compared miRNAs expression in MDE and controls and found significantly up- and also downregulated miRNAs (FC > 1.20 or < -1.20; P < 0.05), as shown in Table 3. Comparison of miRNA expression between MDE patients and controls at baseline and at 8 weeks showed a similar number of dysregulated RNAs (14 miRNAs, with nine miRNAs upregulated and five downregulated). Only two miRNAs showed stable overexpression in MDE patients during the 8-week follow-up compared with controls, miR-941 and miR-589. We also identified miRNAs

**Table 3** Dysregulated miRNAs

miRNA	Cytoband	FC	P-value	miRNA	Cytoband	FC	P-value
<i>MDE<sub>ow</sub> vs C</i>							
hsa-miR-589	7p22.1	4.05	2.99E-3	hsa-miR-517b	19q13.42	-2.60	1.01E-2
hsa-miR-579	5p13.3	2.49	4.34E-2	hsa-miR-636	17q25.1	-2.22	5.67E-3
hsa-miR-941	20q13.33	2.10	1.33E-2	hsa-miR-1243	4p25	-2.01	2.79E-2
hsa-miR-133a	18q11.2	2.04	3.50E-2	hsa-miR-381	14q32.31	-1.57	3.50E-2
hsa-miR-494	14q32.31	1.75	2.20E-2	hsa-miR-200c	12p13.31	-1.47	3.50E-2
hsa-miR-107	10q23.31	1.68	2.20E-2				
hsa-miR-148a	7p15.2	1.48	2.79E-2				
hsa-miR-652	Xq23	1.35	4.35E-2				
hsa-miR-425-3p	3p21.31	1.21	2.79E-2				
<i>MDE<sub>8w</sub> vs C</i>							
hsa-miR-941	20q13.33	3.49	1.55E-2	hsa-miR-376a-5p	14q32.31	-10.84	2.05E-2
hsa-miR-589	7p22.1	3.00	2.50E-2	hsa-miR-1267	13q33.3	-4.38	4.39E-3
hsa-miR-331-5p	12q22	2.31	2.79E-2	hsa-miR-100-3p	11q24.1	-2.29	4.34E-2
hsa-miR-342-5p	14q32.2	1.77	2.79E-2	hsa-miR-571	4p16.3	-1.78	1.17E-2
hsa-let-7b	22q13.31	1.67	4.35E-2	hsa-miR-454	17q22	-1.25	1.72E-2
hsa-miR-345	14q32.2	1.50	7.62E-3				
hsa-miR-33a-3p	22q13.2	1.49	1.60E-2				
hsa-miR-363	Xq26.2	1.29	2.05E-2				
hsa-miR-331-3p	12q22	1.23	1.33E-2				
<i>MDE<sub>ow,8w</sub> vs C</i>							
hsa-miR-941	20q13.33	3.33	8.69E-4				
hsa-miR-589	7p22.1	3.12	2.28E-4				
<i>MDE<sub>ow</sub> vs MDE<sub>8w</sub></i>							
hsa-miR-20b-3p	Xq26.2	3.77	2.34E-2	hsa-miR-331-5p	12q22	-4.42	1.95E-2
hsa-miR-433	14q32.2	2.34	3.91E-3				
hsa-miR-409-3p	14q32.31	2.32	2.34E-2				
hsa-miR-410	14q32.31	2.00	3.91E-2				
hsa-miR-485-3p	14q32.31	1.86	2.73E-2				
hsa-miR-133a	18q11.2	1.43	3.91E-3				
hsa-miR-145	5q32	1.25	3.91E-2				

Abbreviations : FC, fold change; miRNA, microRNA.

exhibiting significant variations of expression among patients with clinical improvement (seven upregulated and one downregulated). Thus, our results confirm the potential utility of miRNA signatures as markers of MDE evolution.

**MiRNA target prediction.** We next identified and analyzed predicted targets of miRNAs differentially expressed in MDE using the Ingenuity pathway analysis software. We performed an inverse correlation analysis at the probe level between the expression of a specific miRNA and the expression levels of all its predicted mRNA targets. Only differentially expressed targets from our own microarray screen were conserved. As shown in Supplementary Table 16, fourteen dysregulated miRNAs have putative mRNA targets that are differentially expressed in MDE, suggesting that a common RNA regulatory network functions in MDE.

**qPCR validation of microarray results.** To validate our microarray findings (Table 4), we selected fourteen mRNAs that are significantly dysregulated in MDE responders based on their function highlighted by the gene ontology analysis (Table 2), and because they have also been described in previous studies of MDE.<sup>13,43,44</sup> Table 4 reports the values obtained after RT-qPCR of sub-cohort A (MDE responders only), of sub-cohort B (mostly MDE nonresponders) and of the entire cohort, in comparison with the FC obtained from

the microarray screen. Importantly, these comparisons showed that the microarray and RT-qPCR data from cohort A are the most closely matched, with significant alterations of expression in MDE validated for *IL1B*, *IRF2*, *PPT1* (palmitoyl-protein thioesterase 1), *SORT1* and *TNF*. For many genes, the results obtained in cohort B were quite divergent from those obtained with cohort A, especially at baseline, including the histone genes, *IL1B* and *TNF*, suggesting that the transcriptional signature of MDE is closely related to the responder/nonresponder status of the patients.

To analyze the transcriptional profile of responder versus nonresponder patients in more detail, we had also collected RT-qPCR samples 2 weeks after study inclusion. We applied a mixed linear statistical model to compare the two groups of patients at the three visits with correction for age and sex (Table 3). Results of the analysis demonstrated that an mRNA signature is closely associated with the degree of clinical response. Responders underexpress *ACTBL2*, histone genes and *POTEKP*, and conversely overexpress *IL1B*, *TNF*, *PPT1* and *TPP*. As we noted that significantly more mirtazapine treatment was prescribed to responders at the second visit (Table 1, Fisher Exact Test  $P=0.026$ ), we included mirtazapine treatment as a co-variable, adjusted by age and sex, in the mixed linear model. For all the genes except *HIST1H1E*, the statistical analyses remained significant (Supplementary Table 18). In addition, we evaluated other potential confounding factors with the same method, that is, tobacco



patients, which raises the question of whether depression may be partly an inflammatory disorder.<sup>63</sup>

Our analysis revealed ~200 transcripts that exhibit robust (FDR ≤ 5% and  $P \leq 0.001$ ) dysregulated expression at baseline in MDE patients compared with controls (Supplementary Tables 3 and 4). These transcripts encode proteins involved in a broad range of functions, including not only cell adhesion, cell cycle, cell junction, cellular ion homeostasis, cytoskeleton, transcription regulation, but also neuron differentiation and synapse (*BAIAP2*, *CLIC5*, *DENND1A*, *FIG4*, *GNAQ*, *MACF1*, *MYH9*, *NPTN*, *PPT1*, *SNAP23* and *STAT3*). This confirms that molecules important for the function of the nervous system may also be identified in blood. Indeed, MDE candidate genes previously identified using brain tissues were also identified as dysregulated in our MDE PBMC screen, including *RGS7BP*, *EDN1*, *PAQR6*,<sup>46</sup> *PPM1K*,<sup>55</sup> and *ELK3*.<sup>56</sup> Of the biological processes overrepresented within our lists of dysregulated transcripts, cell death, vacuole/lysosome organization, nucleotide binding, nucleosome/chromatin assembly were significantly affected in MDE at baseline and also after treatment response (Table 2). Importantly, all these processes were also found associated to MDE in previous investigations relying on both brain<sup>54–56</sup> and blood materials.<sup>19,62</sup>

Despite a growing interest within the field, miRNAs role in pathophysiology of psychiatric disorders is still not widely documented. Previous studies explored miRNA dysregulation in bipolar disorder and schizophrenia, both with peripheral tissues (PBMCs, serum)<sup>64–66</sup> or with post-mortem brain tissues.<sup>65,67–71</sup> Overall, these studies reported conflicting data on the identities of altered miRNAs, as well as the magnitude of change in expression. These discrepancies are likely due to tissue-specific variations in expression levels, and heterogeneity in quantification and normalization procedures. Probably for the same reasons, our results did not overlap significantly with what was reported in Smalheiser *et al.*,<sup>36</sup> a study conducted with brain cortical samples, which identified only two conserved dysregulated miRNA. Although miR-376a-5p was underexpressed in both our study and the Smalheiser *et al.*'s study, miR-494 was overexpressed in our PBMC samples while underexpressed in the cortex.<sup>36</sup> In addition, out of the several studies conducted with schizophrenia samples, only one relied on PBMCs.<sup>66</sup> Again, we shared only one dysregulated miRNA (miR-200c), while three miRNAs (miR-107, miR-342-5p, and let-7b) were dysregulated in opposing degrees. Nevertheless, as the present study reports the first MDE gene expression analysis with PBMC samples, the data are preliminary and open to further validation from other investigators.

miRNA dysregulation in MDE highlights the importance of gene expression and epigenetic regulation in mood disorders. As shown on Supplementary Table 16, putative interactions between dysregulated mRNAs and miRNAs can be identified without an *a priori* hypothesis. However, these types of interactions remain to be demonstrated in cellular or animal models. In addition, the nature and sequence of the miRNA determines its mRNA target. It was recently demonstrated that common human polymorphisms in the miRNA target element may regulate gene expression with a

concomitant change in phenotype. For example, recent studies suggested that polymorphisms in miR-30e and pre-miR-182 might have a role in major depressive disorder susceptibility.<sup>34</sup> Of note, as described in Supplementary Tables 5–10, other non-coding RNAs, such as long intergenic non-coding RNAs represent a significant portion of dysregulated RNAs in our MDE patients (21 out of 195 at baseline). Although, to our knowledge, this is the first study reporting the mis-expression of long intergenic non-coding RNAs in an affective disorder, functions for each of these molecules remain elusive and are likely additional mechanisms to regulate gene expression.<sup>72</sup> As long intergenic non-coding RNAs are numerous and not yet fully described, their implications in biological pathways are still unclear, underscoring the importance of performing conservative ontology analysis. These complexities remind us that multiple layers of regulation are at play to fine-tune gene expression in both normal physiology and MDE.

After microarray profiling both mRNAs and miRNAs from PBMCs, a set of 14 mRNAs that discriminate responder patients from control subjects were selected for RT-qPCR validation. Of these, 10 candidates (*ACTBL2*, *ATP2A2*, *CELF2*, *HIST1H1A*, *HIST1H1E*, *HIST1H4E*, *IRF2*, *POTEKP*, *PPT1* and *PPP1*) met the stringent statistical significance (FDR < 1%) and/or the membership to a significantly dysregulated biological process. We also decided to test other genes for significance with lower stringency (FDR < 5%), and found candidate genes (*IL1B*, *NRG1*, *SORT1* and *TNF*) that had been identified for affective disorders in previous studies.<sup>13,43,44</sup> We expanded the cohort of patient and healthy subjects for analysis and repeated measurements in three different time points (that is, 0, 2 and 8 weeks). A limitation in trying to reproduce microarray results with a different technology is the difficulty in generating amplicons covering the same portion of a specific transcript as the the microarray oligonucleotide probe. Despite this limitation, we obtained an almost complete overlap for *ATP2A2*, *IL1B*, *NRG1*, *SORT1* and *TNF* in expression levels between the microarray and qPCR data for responder patients, while similar variations were also observed for *HIST1H1A*, *HIST1H4E* and *IRF2* (Table 4). Only two genes (*ACTBL2* and *POTEKP*) out of 14 exhibited conflicting results between the microarray and the qPCR data. Therefore, considering the limitation expressed above, these results indicate that our microarray procedures, which are exclusively based on responder patients, detected reliable variations in gene expression.

To further examine our hypothesis that responder and nonresponder MDE patients express different transcriptional signatures, the candidate genes were tested in a statistical model evaluating the contribution of potential confounding factors (Supplementary Table 18). It allowed us to define a smaller core of six markers, which resist the test of all co-variables and distinguish responders from nonresponders: *HIST1H1A*, *HIST1H4E*, *IL1B*, *POTEKP*, *PPT1* and *TNF*.

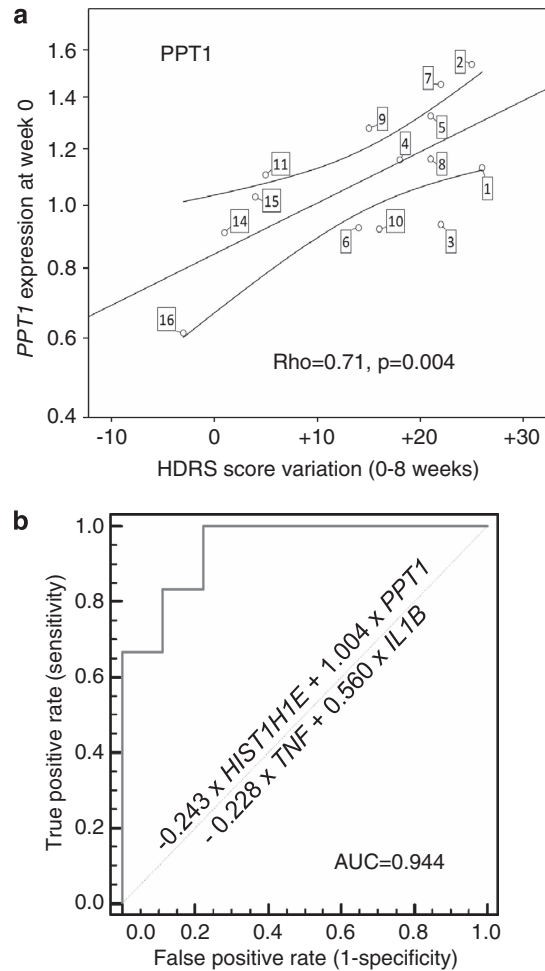
Next, we sought to determine a candidate gene that is predictive of clinical evolution. Our analysis showed that *PPT1* expression was correlated with the change in depression severity (Figure 1a). In addition, in a preliminary attempt to identify a transcriptional biomarker for MDE treatment

response, we submitted the results obtained by RT-qPCR to ROC analysis. This led us to identify four genes that are individually predictive of clinical evolution in our sample: *HIST1H1E*, *IL1B*, *PPT1* and *TNF*. Of note, the combination of these markers provided even better predictive value for treatment response. Although this is not the first attempt to build a classifier gene set for major depressive disorder based on blood gene expression, the seven diagnostic genes previously defined by Spijker *et al.*<sup>22</sup> were identified after *ex vivo* stimulation of the blood cells with lipopolysaccharide.

Looking more specifically at our best predictive gene candidates, we are not surprised to find *TNF* and *IL1B*. These genes encode cytokines that are major players of disease behavior associated with pro-inflammatory responses.<sup>73</sup> Inflammatory response is consistently cited as a key process in dysregulated pathway analysis from affective disorders.<sup>19,21,43,44,51,54,62,74–77</sup> Many studies have validated dysregulation of *IL1B* and *TNF* mRNA/protein in major depression.<sup>78</sup> Genetic studies have also underscored potential polymorphisms of these cytokines linked to MDE and/or treatment response.<sup>79</sup> In this study, we observed that *TNF* and *IL1B* upregulation correlates to responder status, suggesting that a pro-inflammatory response may be associated with a better prognosis. However, this conclusion should be considered with caution as mRNAs are not necessarily correlated with protein expression and serum/plasma levels. Nevertheless, our results suggest that *TNF* and *IL1β* cytokines could be associated with treatment response in a naturalistic point of view. Although potential predictive values of *TNF* or *IL1β* expression in MDE patients for treatment response have been controversial, some recent studies have shown evidence in support of this hypothesis in animal and human studies.<sup>80</sup>

In addition to cytokines, we also observed dysregulated expression of several histone genes that contribute to chromatin organization. Several pangenomic studies have already implied a role of epigenetics in affective disorders,<sup>54,62,75</sup> and chromatin remodeling is described as an important process in pathophysiology of major depression and its treatment.<sup>12</sup> Moreover, key players of epigenetic control of chromatin structure such as histone deacetylases and DNA methyltransferases have been described to be dysregulated in MDE.<sup>13,30,81</sup> In this study, we described a decreased expression of histone H1 family members (Table 4 and Supplementary Table 18) in a responder MDE patient, and a potential predictive value of treatment response for *HIST1H1E* (Supplementary Figure 2). Because linker histones H1 contribute to the so-called ‘histone code’,<sup>82</sup> we believe that our findings adds to the growing evidence of complex epigenetics regulating MDE pathophysiology and its treatment.

Finally, we discovered *PPT1* expression was as one of the most consistently dysregulated genes associated with MDE response, making it an excellent predictive marker for clinical evolution (Table 4, Figure 1 and Supplementary Figure 2). *PPT1* encodes a small glycoprotein involved in the catabolism of lipid-modified proteins during lysosomal degradation and synaptic vesicle endocytosis/recycling in brain.<sup>83</sup> Mutations in *PPT1* lead to infantile-onset neuronal ceroid lipofuscinosis, a recessive neurodegenerative disorder affecting the retina,



**Figure 1** Genes correlating to clinical evolution and predictive of outcome. (a) Spearman's correlation between HDPRS score evolution and mRNA expression level for *PPT1* (Spearman's correlation factor = 0.67,  $P = 0.009$ ). Each circle indicates, for a specified MDE patient (the label attached to the circle refers to the case number in Table 1), the difference of HDPRS scores at inclusion and 8 weeks after inclusion as a function of the *PPT1* FC for MDE<sub>ow</sub> sample, calculated with the  $2^{-\Delta\Delta Ct}$  method. The calibrator was a mathematical pool of control sample Ct. Normalization was performed using *GAPDH* and *PFAFH1B1*. (b) ROC curve for the combined expression of four transcripts (that is, *HIST1H1E*, *IL1B*, *PPT1* and *TNF*) to predict treatment response after 8 weeks. With the highest Youden index, the combination score has a sensitivity value of 100% (54.1–100), specificity value of 77.78% (40–97.2), a positive predictive value of 75% and a negative predictive value of 100%.

cortex and cerebellum. To our knowledge, no previous genetic or proteomic study had implicated *PPT1* in a psychiatric disorder, but its function could be relevant in the context of a neuroplasticity/neurodegeneration hypothesis. Future work will be required to explore the implication of *PPT1* in MDE and its treatment.

Despite our exciting results, we acknowledge several general limitations to our experimental design. We chose to focus our study on the 8-week treatment response period, which is the first stage of recovery. However, a more extended follow-up is required to explore whether the same response and remission biomarker can be used in the long term. Second, although our sample size was determined to ensure

enough statistical power for microarray screening and we performed repeated measures in both the MDE and control groups, our results are limited by the small size of the cohort and would require validation in larger cohort of MDE patients to better evaluate suitability and specificity of the proposed biomarkers. Third, as a consequence of the limited size of our cohort, we could not explore biomarkers for remission that are as important, if not more warranted than biomarkers for treatment response. Therefore a possible design for a more ambitious, future study, is the enrollment, still in naturalistic conditions, of a much larger cohort of patients. It will then be possible to stratify patients according to treatment regimen and to implement an additional time point (6 months) to the protocol during the follow-up of each patient, which would hopefully lead to the identification of remission markers in addition to the validation of treatment-response markers. Fourth, there is an open debate on whether the validation of a biomarker would require treatment-naïve patients at baseline and whether it should be limited to a single treatment with a single targeted phenotype. Therefore, heterogeneities in MDE history and in the naturalistic treatments for all the patients in this study may present difficulties for drawing clear conclusions. Indeed, previous promising results for treatment response or clinical evolution in MDE obtained with biomarker analysis, and relying on biological, electrophysiological or brain imaging, have highlighted the limitation of MDE heterogeneity.<sup>84</sup> Ideally, a biomarker assay for MDE needs to be easy to produce, cost effective and robust, regardless of all potential confounding factors as well as previous treatments received by the patients. Lastly, our study identified a short list of 14 gene candidates based on mRNA profiling that were validated by RT-qPCR according to arbitrary rules. Therefore, we cannot rule out whether among our list of ~200 RNA candidates dysregulated in MDE at baseline, several other candidates would also show robust predictive value for clinical evolution. With this in mind, our next aim will be to test the biomarker predictive value of the most promising mRNA candidates that we validated by RT-qPCR, as well as other mRNA and miRNA candidates significantly dysregulated in our study, in a larger cohort.

In conclusion, we have profiled mRNA and miRNA expression in PBMCs from MDE patients compared with healthy controls in a prospective and naturalistic design. We report new insights into the biological description of MDE and identified *PPT1* as a possible biomarker of MDE clinical evolution. Taken together, we propose that using RNAs from PBMCs as biomarkers may constitute an exciting new method to improve MDE prognosis and to develop personalized medicine in psychiatry.

### Conflict of interest

The authors declare no conflict of interest.

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