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**Clinical variations modulate patterns of gene expression and define blood biomarkers in major depression**

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## **ABSTRACT**

The aim of the study is to compare the expression level of candidate genes between patients suffering from a severe major depressive episode (MDE) and controls, and also among patients during MDE evolution. After a comprehensive review of the biological data related to mood disorders, we initiated a hypothesis-driven exploration of candidate mRNAs. Using RT-qPCR, we analyzed peripheral blood mononuclear cells (PBMCs) mRNA obtained from a homogeneous population of 11 patients who suffered from severe melancholic MDE. To assess the evolution of MDE, we analyzed PBMC mRNAs that were collected on Day 1 and 8 weeks after. Data from these patient samples were analyzed in comparison to age- and sex-matched healthy controls. Among 40 candidate genes consistently transcribed in PBMCs, 10 were differentially expressed in at least one comparison. We found that variations of mRNA levels for *NRG1*, *SORT1* and *TPH1* were interesting state-dependent biological markers of the disease. We also observed that variations in other mRNA expression were associated with treatment efficacy or clinical improvement (*CREB1*, *HDAC5*, *HSPA2*, *HTR1B*, *HTR2A*, and *SLC6A4/5HTT*). Significantly, *5HTT* exhibited a strong correlation with clinical score evolution. We also found a state-independent marker, *IL10*. Moreover, the analysis of 2 separate MDEs concerning a same patient revealed comparable results for the expression of *CREB1*, *HSPA2*, *HTR1B*, *NRG1* and *TPH1*. Overall, our results indicate that PBMCs obtained at different time points during MDE progression represent a promising avenue to discover biological markers for depression.

**KEY WORDS:** depression; PBMC; candidate gene; qPCR; *5HTT*

## INTRODUCTION

Depression is a leading cause of disability worldwide, affecting nearly 16% of the general population (Kessler et al., 2003). This extreme prevalence causes a financial burden estimated to 1% of the Gross Domestic Product (GDP) in developed countries.

Depression is a heterogeneous disorder. Major depressive episode (MDE) is considered a syndromic entity, with multiple distinct causes and pathophysiologies leading to a common final outcome (Nestler et al., 2002). Responses to current treatments are often inconsistent. It is estimated that a third of the patients treated for 8 weeks with an antidepressant will not show any clinical improvement and only 50% of treated patients demonstrate complete remission (Nestler et al., 2002). Accordingly, depressive symptoms evolve in a highly unstable and unpredictable fashion. It is therefore challenging to make a valid prognosis for MDE. The identification of biomarkers could be an important way of improving patient's care and contribute to developing new treatment options for MDE patients.

Although a large amount of research has been focused on understanding the underlying mechanisms of major depressive disorder (MDD), its pathophysiology remains unclear. Based on gene-environment studies and epigenetic studies, a main hypothesis that has emerged proposes that MDD is caused by the convergence of multiple factors including (i) biological factors such as multi-genic vulnerability, hormonal and immunological abnormalities, and (ii) socio-psychological environmental factors as diverse as adverse life events and childhood mistreatment (Bet et al., 2009; Caspi et al., 2003; Glassman & Miller, 2007; Kendler et al., 2006; Kendler et al., 2005; Mill & Petronis, 2007; Ohayon, 2007). Therefore, a multi-genic study of gene expression in MDE is of great interest. mRNA levels are a direct reflection of gene-environment interactions (ie. genetic vulnerability or genetic

polymorphism) and environmental events that regulate transcription, both of which can have socio-psychological influences. Unfortunately, comparative analyses of biological data in MDD remain problematic due to conflicting results. Discrepancies can be explained by the high degree of heterogeneity in i) patient genetic background, ii) severity of MDE, and iii) MDE features (ie melancholic, atypical, catatonic), which collectively may involve different biological pathways (Kessing, 2007; Nestler et al., 2002; Schlatter et al., 2004).

Molecular abnormalities in psychiatric disorders are usually studied using brain tissue samples collected *post-mortem*. However, analyses of *post-mortem* tissues are hampered by many limitations. In particular, death induces rapid cellular and molecular changes, introducing significant heterogeneity and biological biases, due to the lag between death and sample collection (Tomita et al., 2004). *Post-mortem* samples are often clinically heterogeneous and allow analysis of only single time-point, resulting in unreliable correlations between clinical state and biological observations (Tochigi et al., 2008). To circumvent this problem, easily accessible peripheral tissues, such as peripheral blood mononuclear cells (PBMCs) collected from living patients, have been used successfully (Iga et al., 2008). These tissues share numerous biological pathways with the central nervous system (Gladkevich et al., 2004; Sullivan et al., 2006) and significant gene expression similarities were found between blood and brain, including numerous candidate genes in mood disorders (Le-Niculescu et al., 2008; Segman et al., 2005). Moreover, compared to cell lines maintained in culture for a long time, freshly dissociated cells from tissues offer the possibility to more precisely correlate clinical stages with biological observations (Le-Niculescu et al., 2007). Interestingly, variations in mRNA levels of a few genes observed in PBMCs have been linked to clinical stages or the remission of MDD patients (Fujimoto et al., 2008; Hobara et al., 2009; Iga et al., 2005; Iga et al., 2007a; Iga et al., 2006; Iga et al., 2007b; Matuzany-Ruban et al., 2005; Otsuki et al., 2008; Rocca et al., 2002; Su et al., 2009; Tsao et

al., 2006; Wakabayashi et al., 2008; Watanuki et al., 2008). It is therefore not surprising that PBMCs are now considered as sentinel cells in psychiatric disorders (Gladkevich et al., 2004; Iga et al., 2008; Le-Niculescu et al., 2008; Liew et al., 2006; Sullivan et al., 2006) and, as such, represents a promising tool to characterize biomarkers for depression.

Recent technical improvements in molecular biology have increased sensitivity and specificity for gene expression analysis. To date, quantitative real-time RT-PCR (RT-qPCR) remains the best way to validate candidate transcripts identified *via* whole genome exploration with pan-genomic microarrays (Iga et al., 2008).

The aim of our work was to compare the expression level of selected candidate genes in PBMCs, between a homogeneous group of MDD patients characterized by a severe MDE with melancholic features and age- and sex-matched healthy controls. Moreover, our goal was to describe the mRNA variation of expression among patients at two evaluating times: during a severe MDE and 8 weeks later, after delivery of an appropriate treatment.

We predicted that i) gene expression profiles will differ between patients and matched-controls, ii) disparities will be indicative of the efficacy of the treatment as well as the clinical stage, and iii) the set of differentiating mRNAs could be established as biomarkers for MDE.

## MATERIALS AND METHODS

### *Design setting and subjects*

The study design was a dynamic case/control study. A minimum sample size ( $n=10$ ) was determined by using sizepower tool within the R package (Lee & Whitmore, 2002) with the following parameters: False discovery Rate (FDR) = 20 %, number of genes = 40, power of the study = 80 %, expected difference between groups = 0.9, and standard deviation = 1.3. Accordingly, our population study included 11 MDE patients (MDE group) recruited at the Assistance Publique - Hôpitaux de Marseille, and 11 healthy sex- and age-matched controls (control group). Patients of the MDE group were assessed once during study inclusion and a second time, 8 weeks later, in order to investigate clinical evolution and gene altered expression over time. Controls were evaluated only once.

For the MDE group, the inclusion criteria, defined according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) (Association, 1994) were: (i) severe MDE with melancholic features with 17-item Hamilton Rating Scale for Depression (HDRS) score  $\geq 25$ , (ii) recurrent MDD patients, and (iii) patients with a family history of major psychiatric disorders. Patients with substance use disorder, medical problems, abnormal laboratory tests (especially blood cell count) and neurological exploration were excluded. Patients were clinically evaluated by two psychiatrists using an adapted standardized procedure (SCID-P, (Spitzer et al., 1990)). The procedure included the use of all available medical records as well as repeated undirected examinations. The severity of MDE was evaluated with a French version of 17-item HDRS at each evaluation (Guelfi, 1997).

For the control group, subjects were evaluated to exclude any psychiatric disorder history or any somatic disease using the French version of standardized interview validated for health control subjects (SCID-NP, (Spitzer et al., 1990)). Moreover, all participants were

carefully interviewed and had clinical examination to eliminate any psychiatric or medical condition.

All experiments on human subjects were conducted in accordance with the latest version of the Declaration of Helsinki. The project was approved by the local ethics committee and written informed consent was obtained after a complete description of the study to the subjects.

### ***Selection of candidate and reference genes***

After an extensive review of the available biological data relevant to MDD and mood disorders, we focused our study on transcripts of genes (i) with reported mRNA or protein level variations associated to MDD or mood disorders, (ii) with reported DNA polymorphism(s) associated to MDD or mood disorders, (iii) belonging to molecular families involved in MDD or mood disorders and (iv) likely expressed in PBMCs. A final set of 40 genes were studied for the final analysis of all collected samples. Selected genes are listed in Supplementary Tables S1-S4. For each candidate gene, primer sets and probes were selected using the web portal of the manufacturer (Applied Biosystems).

Five reference genes, namely *GAPDH*, *COX6A1*, *ABL1*, *RPL38* and *RPLP0*, were included in the analysis for normalization procedures.

### ***mRNA extraction***

Venous blood was collected from patients and matched-controls in EDTA tubes between 7:00 a.m. and 8:30 a.m. and processed within two hours. PMBCs were isolated from the blood by Ficoll density centrifugation using the same protocol. Total RNAs were extracted from the PBMCs with the RNeasy kit (Qiagen, Hilden, Germany) and DNase-treated according to the manufacturer's protocol. The purity and integrity of the RNA was



determined by OD measurements and by the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). To ensure a robust analysis for the following procedures, samples with an RNA integrity number (RIN) inferior to 9 were excluded.

### ***Real-time RT-PCR***

1 µg of RNA was reverse transcribed with the High Capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). 100 ng of the resulting cDNA was combined with a TaqMan® universal PCR Master Mix (Applied Biosystems) and 48 PCR reactions were simultaneously performed on an ABI PRISM 7900HT thermocycler using tLDA technology according to manufacturer recommendations (Applied Biosystems). All PCR reactions were performed in quadruplicate.

### ***Data analysis***

The mean of the cycle threshold values ( $C_T$ ) was used for subsequent analysis. Data were analyzed according to fold changes and relative quantification (RQ) approaches. Reference genes used for analysis were selected among the ones tested, based on their stability and their proximity in expression compared to each candidate gene. The stability was assessed using the GeNorm software, validated reference genes exhibit average expression stability values (M) under 1.5 (<http://medgen.ugent.be/~jvdesomp/genorm/>) (Vandesompele et al., 2002).  $C_T$  values of candidate mRNAs were then normalized by subtracting the geometric mean of a selected combination of reference genes. The resulting  $\Delta C_{T\text{samples}}$  values provided relative gene expression levels for each sample. A calibrator sample was defined by calculating a mathematical pool of  $C_T$  (i.e. arithmetic mean of control group  $C_T$ ) and was normalized by the selected combination of control genes to obtain the  $\Delta C_{T\text{calibrator}}$ . Finally,  $\Delta\Delta C_T$  values were calculated and converted into fold changes or Relative Quantification (RQ)

between control group and MDE group using the  $2^{-\Delta\Delta C_T}$  method. Using the same method, the RQ between evaluations on the first day and at 8 weeks were calculated using the arithmetic mean of the first sample set,  $C_T$ , as a calibrator.

All statistical tests were made with the SAS 9.1.3 software. Demographic variables were compared between patients and matched-controls using the Mann Whitney Test for metric variables and the Fisher's exact test for nominal variables. Non-parametric unpaired tests were used to compare the RQ between patients and matched-controls at the first evaluation and at 8 weeks. Non-parametric paired tests were used to compare the RQ between the two evaluations in the MDE group. We calculated Spearman's correlations between HDRS evolution and RQ MDE 8 weeks sample for each transcript exhibiting a significant variation of expression between the study groups or at the 8 week follow-up. For all tests, a  $p$ -value  $\leq 0.05$  was considered significant.

For multiple testings, adjusted  $p$ -values were calculated using the false discovery rate (FDR) procedure with the PROC MULTTEST statement (Benjamini & Hochberg, 1995). We chose a FDR threshold of 0.20 in accordance with the candidate genes design of our hypotheses-driven study (Smith et al., 2007).

## RESULTS

### *Demographic and Clinical Variables*

We recruited a homogeneous group of 11 treated inpatients treated for MDE, eligible for study inclusion, and also a sex- and age-matched group of 11 control individuals. A summary of clinical and therapeutic data for each patient is reported in Table 1. The median value of HDRS was 32 for the patients at the first clinical evaluation (interquartile range=13). Eight weeks later, the 8 patients who continued throughout the entire study exhibited a significant clinical improvement (median value=12, interquartile range=14,  $p=0.012$ ). Some patients showed almost complete remission (case numbers 3, 4 and 8, Table 1) while others exhibited partial remission (cases number. 1, 2, 5-7, Table 1). Three patients were lost during the follow-up due to transfer to other clinical wards (cases number 9-11, Table 1). Patients and controls were all of Caucasian origin and the analysis of socio-demographic and clinical parameters (sex, age, and body mass index) showed no significant differences among study groups (Supplementary Table S5).

### *Differentially Expressed Genes*

After an extensive review of the biological data related to mood disorders, we chose to examine the expression of 40 genes using the RT-qPCR technology. The reduction of patient and control samples from the initial number allowed us to verify that the candidate gene mRNAs were reproducibly detected above background levels in PBMCs (data not shown). In particular, we measured the intra-assay variation of our replicate and found that it was very low ( $< 1\%$ ) for more than half of the candidate genes analyzed, and the coefficient of variation was still below 3% for the other genes (Supplementary Table S6). These data indicate the high reproducibility of the microfluidics technology. In addition, the levels of expression for each candidate mRNA were normalized using different reference genes. Among the 5 reference genes included in our study, we found that *COX6A1* and *RPLP0*

exhibited the highest stability, with an average expression stability value (M) comprised between 0.60 and 0.66. Nevertheless, the other reference genes (*RPL38*, *ABL1* and *GAPDH*) provided also satisfactory level of stability (M between 0.68 and 0.70). Because the level of expression varies among the candidate genes (Supplementary Table S6) and because our initial reference genes are either very highly (*RPL38*, *RPLP0*, *GAPDH*) or highly/moderately expressed (*COX6A1*, *ABL1*), we searched among our moderately to weakly expressed candidate genes, genes with a good level of stability. We found that *SYNJ2* and *SYP* were quite stable (M between 0.73 and 0.78) and were moderately and weakly expressed in PBMCs, respectively. We included those genes in our list of reference genes. Thus, we decided to separate the candidate genes into 4 groups: (i) genes with Ct below 25 were normalized with a combination of *RPLP0* and *COX6A1*, (ii) genes with Ct comprised between 25 and 28 were normalized with a combination of *COX6A1* and *ABL1*, (iii) genes with Ct comprised between 28 and 30 were normalized with a combination of *ABL1* and *SYNJ2*, (iv) genes with Ct above 30 were normalized with a combination of *SYNJ2* and *SYP*. By adopting that normalization procedure, we made sure that all candidate genes were normalized by reference genes whose level of expression was in the same range of values.

All quantitative results and statistical analyses are reported in Tables 2 and 3. While about a third of our candidate genes, such as *COMT* (Fig. 1A) were expressed at similar levels among control and patient PBMCs, we found 13 genes differentially expressed ( $p \leq 0.05$ ), in at least one comparison. These genes showed modest fold-change (min=1.20, max=3.05) in accordance with numerous previous studies on psychiatric disorders (Aston et al., 2005; Choudary et al., 2005; Iwamoto et al., 2004; Kalman et al., 2005; Kang et al., 2007; Klempan et al., 2009; Matigian et al., 2007; Ryan et al., 2006; Sequeira et al., 2006; Sequeira et al., 2007; Shao & Vawter, 2008; Tochigi et al., 2008). To circumvent the multi-genic design of

the study, we calculated the FDR for each statistical test and found that 10 genes remained statistically differentially expressed (FDR<0.2, Tables 2 and 3).

Dysregulated genes associated to MDE can be clustered into 3 categories. The first category includes genes exhibiting altered mRNA expression levels during MDE but either reverts back to near normal levels (observed in controls) after clinical improvement (ie. *NRG1* represented on Fig. 1B) or vary in the opposite direction after eight weeks of MDE progression (ie. *SORT1* and *TPHI* represented on Fig. 1C). These genes can be considered state-dependent markers. The second category includes genes with mRNA expression levels that significantly deviate from normal levels during the follow-up (*CREB1* represented on Fig. 1D, *HDAC5* represented on Fig. 1E, *HSPA2*, *HTR1B*, *HTR2A* and *SLC6A4/5HTT*). The expression of these genes is indicative of treatment efficacy or clinical improvement. The third category includes genes with mRNA expression levels that are completely distinct between the control group and the patients. The unique gene we identified in this category, *IL10*, is overexpressed (>2-fold change) in patients, at both inclusion and 8 weeks after inclusion compared to controls (Table 2). No significant difference could be found between inclusion and 8 weeks after inclusion in patient samples with a calculated statistical power over 95%. Indeed, we could propose that *IL10* may represent a state-independent marker (Fig. 1F).

Most genes express various transcripts due to alternative initiation of transcription, polyadenylation, or splicing of the pre-mRNA. To explore the possibility that variations of expression may affect only subsets of transcript isoforms, we studied two genes (*ARRB1* and *NRG1*) using two different sets of primers and probes for each gene. While one set of primers for *ARRB1* only exhibited a trend towards significance (*ARRB1-2*, Tables 2 and 3, Supplementary Table S4) as a marker of treatment efficacy, the gene expression results for *NRG1* clearly differed depending on the set of primers/probe selected (Tables 2 and 3). In fact, one of the two *NRG1* amplicons (*NRG1-2*, Table 2, Supplementary Table S4, Fig. 1B)

was preferentially overexpressed in patients, suggesting that only specific isoforms might be markers of the disease and its treatment.

Finally, the 10 genes, whose expression levels were significantly altered, were examined for potential correlations between mRNA expression levels and HDRS variations. Interestingly, *5HTT* mRNA overexpression was strongly correlated to clinical variations (Spearman's correlation factor =0.95, p=0.0003) as shown in Figure 2.

#### ***A recurrent patient with a yo-yo pattern of expression for some genes***

One of the patients was enrolled twice in the study due to two different MDEs (case number 3, Table 1). During the first episode, his HDRS-17 items scored was 38. Eight weeks later, his score collapsed to 2. Further clinical interviews determined that remission was prolonged for at least the next six months. Nevertheless, two years after his first participation in our study, the same patient suffered another severe MDE with a score of 34. However, 8 weeks later, his clinical score returned to 9 on the HDRS scale (Table 4 with treatments indicated). Remarkably, the pattern of transcriptional changes of all the 9 candidate genes dysregulated during the follow-up (bold genes in Table 3) was reproduced during both MDEs. *CREB1*, *HSPA2*, *HTR1B*, *NRG1-2* and *TPHI* levels of dysregulation were reproduced with a high degree of similarity (Table 4). In addition, the changes observed for *HDAC5*, *HTR2A*, *SLC6A4* and *SORT1* were not of identical intensities during both MDEs but were in the same orientation.

## **DISCUSSION**

The current study describes a quantitative expression analysis of 10 selected genes in a homogeneous group of MDD patients. We found that candidate genes exhibited differential patterns of mRNA levels during MDE when compared to control subject and/or over time. We also obtained comparable results for two different MDEs from the same patient. Altogether, these results suggest that some mRNAs could be promising biological markers for MDE and underscore the influence of clinical state on gene expression.

We focused on patients with severe melancholic MDE suffering from recurrent MDD with family histories of psychiatric disorders. Taken together, these clinical features are likely associated with a more homogeneous and genetically determined phenotype (Sullivan et al., 2000). We also used quantitative phenotypic state measures as criteria for inclusion (HDRS  $\geq$  25) and follow-up. As expected, the 8-week treatment resulted in a global clinical improvement in our MDE patient population with either full remission or partial therapeutic response as shown in Table 1. Thus, we were able to test whether differentially expressed genes are associated with clinical evolution in this patient population. As a result, we strongly reduced clinical heterogeneity and obtained more significant, reproducible and specific results despite our modest sample size compared to previous studies.

We used PBMCs, a fresh and easily accessible tissue to study gene expression in MDE. PBMCs are a heterogeneous population of cells whose variations, in both density and composition, could affect gene expression. However, studies have demonstrated that the levels of only a few mRNA are dependent on cell counts (Eady et al., 2005). In our investigations, standard cell count was made for all participants and numbers were all within normal range of variation. Beyond these potential limitations, PBMCs collection is easily reproducible within and between patients and could be a valuable material for obtaining standardized biological approaches of psychiatric disorders. Importantly, PMBCs offer the

possibility to correlate the clinical status of the patient to the biological activities of the cells at the time of collection (Begemann et al., 2008; Iga et al., 2007a; Iga et al., 2006; Iga et al., 2007b; Matuzany-Ruban et al., 2005; Rocca et al., 2002; Segman et al., 2005; Tsao et al., 2006; Wakabayashi et al., 2008). Many candidate genes, reported to sustain function(s) specific to the nervous system, are detected in PBMCs. In accordance with findings in previous studies based on non-neural cells (Iga et al., 2008), we were able to study selected candidate genes in PBMCs and correlate gene expression to the clinical status of patients.

Results in gene expression studies is dependent on normalization procedure (de Jonge et al., 2007). To prevent the erroneous use of a control gene with a variable expression levels, we tested the steadiness of several internal control genes using GeNorm (Vandesompele et al., 2002). We ended using 5 different reference genes with different level of expression. Thus we were able to normalize the level of expression of candidate genes by using stably and closely expressed reference genes.

The transcriptional variations reported herein could be suggestive of different biological signatures that are still difficult to discriminate. Future studies will require larger sample sizes in order to replicate, consolidate and clarify the observed trends. Within our pilot study, we propose that some *NRG1* transcripts whose expression level was altered during MDE and returned close to normal levels, eight weeks after treatment (Tables 2-3 and Fig. 1B), may be interesting biological markers of MDE. In addition, the observation that *SORT1* and *TPH1*, whose mRNA expression level is altered during MDE and vary in the opposite direction eight weeks after treatment (Tables 2-3 and Fig. 1C), may reveal both disease and drug-response signatures. By contrast, the mRNA expression levels of *CREB1*, *HDAC5*, *HSPA2*, *HTR1B*, *HTR2A* and *SLC6A4* varied significantly only after clinical improvement (Tables 2-3 and Fig. 1D-E), suggestive of a drug-response and/or clinical remission signature. Finally, the distinct levels of *IL10* expression between patients and matched-controls, during



the MDD acute phase and after 8 weeks of treatment (Tables 2-3 and Fig. 1F), likely reflects a non-specific treatment intake signature without any link to clinical stage. In fact, it is well established that depression is associated to cytokine dysregulation, and antidepressants have an effect on cytokine secretion (Capuron et al., 2009; Miller et al., 2009; Schiepers et al., 2005).

However, we cannot exclude the possibility that heterogeneity of treatment (electroconvulsive therapy, ECT, versus antidepressant molecule), the dose and the duration of each treatment, the addition in some case of an antipsychotic may impact mRNA expression. Numerous studies have revealed the importance of the location-dependent (i.e. tissue specificity) and time-dependent regulation of gene expression following antidepressant treatment (Conti et al., 2007; Donnici et al., 2008; Surget et al., 2009). While some of our candidate genes, like *CREBI*, are known to be regulated by antidepressants (Blendy, 2006), the effects of these treatments on our other candidate genes expression remain to be tested. It is also known that antipsychotics modify the expression of many genes (Choi et al., 2009; Girgenti et al., ; Ikeda et al., ; Molteni et al., 2009; Schmidt et al., 2009). Therefore, as an exploration for a potential bias due to the non-uniform use of antipsychotics, we compared the level of expression of the candidate genes according to the treatment by an antipsychotic drug at inclusion (n=7 for MDE receiving antipsychotics at inclusion, n=4 for MDE without antipsychotics at inclusion) and found a significant effect of antipsychotic drug for *HSPA2* (p=0.008) and *COMT* (p=0.046) without correction for multiple testing (data not shown). Even if these results must be interpreted with caution because of the sample size and *post hoc* design of analysis, it suggests that the use of antipsychotics may represent a confounding factor for some candidate genes. Ideally, to circumvent most of the biases linked to treatments, a wash-out should be performed before inclusion. In practice, however, we had to reject this procedure for ethical reasons, due to the severity of MDE. In addition, it is not known exactly how long the effects of a treatment can persist after being administered.

As a strong support for a biological link between gene expression variation and clinical improvement, we found that variation in HDRS score was correlated to *5HTT* mRNA levels of expression, as shown on Figure 2. We are aware that an important bias of our study concern the variability of treatment during the follow-up, and of course, that important observation should be reproduced in patients receiving identical medications. Moreover, analysis of two different MDE episodes for a same patient revealed reproducible results, especially for *CREB1*, *HSPA2*, *HTR1B*, *NRG1-2* and *TPHI* (Table 4). This individual was treated with a combination of antidepressant/antipsychotic drugs during the first MDE while he received the same antidepressant with valproic acid during the second MDE. Thus, we observed similar molecular signatures despite the use of 2 different treatment protocols. Altogether, these observations strengthen the significance of these genes as biomarkers despite potential confounding factors. As suggested by others, it will be of interest to conduct a longitudinal follow-up for these specific genes (Iga et al., 2005). Although the 8-week interval of observation was commonly used in previous studies, a recent study suggested that early improvement observed in the first 2 weeks of MDE treatment is indicative of treatment outcome (Szegedi et al., 2009). Thus, in addition to a long term follow-up, we plan to test the correlation between clinical responses and mRNA expression levels in PBMCs, at time points earlier than 8 weeks.

Our observations underscore the necessity to clearly define and evaluate clinical phenotypes when assessing mRNA expression variations. Psychiatric clinical phenotypes, particularly in mood disorders, are highly variable for each patient over time and are highly dependent upon clinical evolution and treatment responses. Indeed, repetitive prospective biological studies may be more informative than isolated evaluations. Moreover, our study highlights the importance of normalization procedures. In addition, we tested for selected genes (*ARRB1*, *NRG1*, and others, data not shown) using different sets of primers/probes that

are specific to the same gene but not to the same transcripts, revealing that gene expression data can vary depending on the primers used for analysis (*NRG1*, Tables 2-3). This result indicates that mRNA biogenesis processes, including alternative transcription initiation/splicing/polyadenylation, should be taken into account for in the interpretation of such results. In the future, a more precise investigation of specific transcripts, not just genes, is warranted. Finally, our study has demonstrated the utility of PBMC mRNAs as easily measurable and accessible markers of MDD evolution. In the near future, this technique can be developed into a valuable diagnostic and prognostic tool for MDD.

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## FIGURE LEGENDS

**FIG. 1.** Gene expression variations during MDE delineate state-dependent and treatment efficacy markers. Asterisks indicate significant difference between the groups (\*,  $p < 0.05$  and \*\*,  $p < 0.01$ ). Horizontal lines represent the median value for each group.

**FIG. 2.** Spearman correlation between HDRS score evolution and mRNA expression level for *5HTT* (Spearman's correlation factor = 0.95,  $p = 0.0003$ ). 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 HDRS scores at inclusion and 8 weeks after inclusion as a function of the *SLC6A4/5HTT* RQ for MDE 8 weeks sample, calculated with the  $2^{-\Delta\Delta Ct}$  method. The calibrator was a mathematical pool of CONTROL sample Ct. Normalization was performed using *SYNJ2* and *SYP*.